☞ 11hr_AC-NR_CRule_10-123_pt03b

☞

(FORM UPDATED: 08/11/2010)

WISCONSIN STATE LEGISLATURE ...
PUBLIC HEARING - COMMITTEE RECORDS

2011-12
(session year)

Assembly
(Assembly, Senate or Joint)

Committee on Natural Resources...

COMMITTEE NOTICES ...

- Committee Reports ... **CR**
- Executive Sessions ... **ES**
- Public Hearings ... **PH**

INFORMATION COLLECTED BY COMMITTEE FOR AND AGAINST PROPOSAL

- Appointments ... **Appt** (w/Record of Comm. Proceedings)
- Clearinghouse Rules ... **CRule** (w/Record of Comm. Proceedings)
- Hearing Records ... bills and resolutions (w/Record of Comm. Proceedings)
  (**ab** = Assembly Bill) (**ar** = Assembly Resolution) (**ajr** = Assembly Joint Resolution)
  (**sb** = Senate Bill) (**sr** = Senate Resolution) (**sjr** = Senate Joint Resolution)
- Miscellaneous ... **Misc**

* Contents organized for archiving by: Stefanie Rose (LRB) (August 2013)

critical to test the efficacy of specific disease prevention strategies applied not only within donor and recipient communities, but also in the realm where they intersect.

References and Notes

1. J. O. Lloyd-Smith *et al.*, *Science* **326**, 1362 (2009).
2. C. E. Rupprecht, C. A. Hanlon, T. Hemachudha, *Lancet Infect. Dis.* **2**, 327 (2002).
3. V. P. Hsu *et al.*, *Emerg. Infect. Dis.* **10**, 2082 (2004).
4. S. Riley *et al.*, *Science* **300**, 1961 (2003).
5. A. Moya, E. C. Holmes, F. González-Candelas, *Nat. Rev. Microbiol.* **2**, 279 (2004).
6. M. Anishchenko *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 4994 (2006).
7. H. D. Song *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 2430 (2005).
8. C. R. Parrish *et al.*, *Microbiol. Mol. Biol. Rev.* **72**, 457 (2008).
9. T. Kuiken *et al.*, *Science* **312**, 394 (2006).
10. J. D. Blanton, K. Robertson, D. Palmer, C. E. Rupprecht, *J. Am. Vet. Med. Assoc.* **235**, 676 (2009).
11. D. A. Brass, in *Rabies in Bats: Natural History and Public Health Implications*, D. A. Brass, Ed. (Livia Press, Ridgefield, CT, 1994), pp. 151–162.
12. J. S. Smith, L. A. Orciari, P. A. Yager, *Semin. Virol.* **6**, 387 (1995).
13. G. J. Hughes, L. A. Orciari, C. E. Rupprecht, *J. Gen. Virol.* **86**, 1467 (2005).
14. O. R. P. Bininda-Emonds *et al.*, *Nature* **446**, 507 (2007).
15. Materials and methods are available as supporting material on *Science* Online.
16. D. M. de Vienne, M. E. Hood, T. Giraud, *J. Evol. Biol.* **22**, 2532 (2009).
17. G. S. Gilbert, C. O. Webb, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 4979 (2007).
18. T. J. Davies, A. B. Pedersen, *Proc. Biol. Sci.* **275**, 1695 (2008).
19. G. M. Baer, J. H. Shaddock, R. Quirion, T. V. Dam, T. L. Lentz, *Lancet* **335**, 664 (1990).
20. S. Finke, K. K. Conzelmann, *Virus Res.* **111**, 120 (2005).

21. For helpful discussion and comments, we thank P. Beerli, J. Davies, S. Altizer, A. Park, P. Rohani, J. Allgeier, B. Han, P. Stephens, and three anonymous reviewers. For contributing rabid bats, we thank the Arizona State Public Health Laboratory, the California Department of Public Health, the Georgia Department of Community Health, the Florida Department of Health, the Idaho Department of Health and Welfare, the Indiana State Department of Health, the University of Iowa's University Hygienic Laboratory, the Mississippi State Department of Health, the New Jersey Department of Health and Senior Services, the Tennessee Department of Health, the Texas Department of State Health Services, the Virginia Consolidated Laboratory, and the Washington State Department of Health. For providing museum-vouchered bat tissues, we thank the Angelo State Natural History Collection, the Carnegie Museum of Natural History, the Centro de Investigaciones Biológicas del Noroeste, the Louisiana State University Museum of Natural Science, the Museum of Vertebrate Zoology, the U.S. National Museum of Natural History, the Royal Ontario Museum, and the University of Alaska Museum. The sequences generated in this study can be found at GenBank under accession numbers GU644641 to GU645012 and GU722925 to GU723257 (table S1). This work was supported by Association of Public Health Laboratories/Centers for Disease Control Emerging Infectious Diseases and NSF Graduate Research Fellowships to D.G.S., NSF-NIH Ecology of Infectious Disease grant 0430418 to G.F.M., and funding from the U.S. Army Engineer Research Development Center–Construction Engineering Research Laboratory and Western Michigan University to M.J.V.

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5992/676/DC1
Materials and Methods
SOM Text
Fig. S1
Tables S1 to S9
References

26 February 2010; accepted 10 June 2010
10.1126/science.1188836

An Emerging Disease Causes Regional Population Collapse of a Common North American Bat Species

Winifred F. Frick,[1,2]* Jacob F. Pollock,[3] Alan C. Hicks,[4] Kate E. Langwig,[4,1] D. Scott Reynolds,[5,1] Gregory G. Turner,[6] Calvin M. Butchkoski,[6] Thomas H. Kunz[1]

White-nose syndrome (WNS) is an emerging disease affecting hibernating bats in eastern North America that causes mass mortality and precipitous population declines in winter hibernacula. First discovered in 2006 in New York State, WNS is spreading rapidly across eastern North America and currently affects seven species. Mortality associated with WNS is causing a regional population collapse and is predicted to lead to regional extinction of the little brown myotis (*Myotis lucifugus*), previously one of the most common bat species in North America. Novel diseases can have serious impacts on naïve wildlife populations, which in turn can have substantial impacts on ecosystem integrity.

Emerging infectious diseases are increasingly recognized as direct and indirect agents of extinction of free-ranging wildlife (*1*–*4*). Introductions of disease into naïve wildlife populations have led to serious declines or local extinctions of different species in the past few decades, including amphibians from chytridiomycosis (*5*, *6*), rabbits from myxomatosis in the United Kingdom (*7*), Tasmanian devils from infectious cancer (*3*), and birds in North America from West Nile virus (*8*). Here we demonstrate that white-nose syndrome (WNS), an emerging infectious disease, is causing unprecedented mortality among hibernating bats in eastern North America and has caused a population collapse that is threatening regional extinction of the little brown myotis (*Myotis lucifugus*), a once widespread and common bat species.

WNS is associated with a newly described psychrophilic fungus (*Geomyces destructans*) that grows on exposed tissues of hibernating bats, apparently causing premature arousals, aberrant behavior, and premature loss of critical fat reserves (*9*, *10*) (Fig. 1). The origin of WNS and its putative pathogen, *G. destructans*, is uncertain (*9*). A plausible hypothesis for the origin of this disease in North America is introduction via human trade or travel from Europe, based on recent evidence that *G. destructans* has been observed on at least one hibernating bat species in Europe (*11*). Anthropogenic spread of invasive pathogens in wildlife and domestic animal populations, so-called pathogen pollution, poses substantial threats to biodiversity and ecosystem integrity and is of major concern in conservation efforts (*1*, *2*).

WNS has spread rapidly and now occurs throughout the northeastern and mid-Atlantic regions in the United States and in Ontario and Québec provinces in Canada and currently affects at least seven species of hibernating bats (Fig. 2). Many species of bats in temperate North America hibernate in caves and mines (*12*) in aggregations of up to half a million individuals in a single cave (*13*). In late spring, these winter aggregations typically disperse into smaller sex-segregated groups of conspecifics, when adult females form maternity colonies and adult males mostly roost alone (*14*, *15*). From August to October, females and males assemble at hibernacula or swarming sites to mate before hibernating (*16*, *17*). The mechanisms for the persistence and transmission of *G. destructans* during summer and fall months are unknown, but spread of the fungus to new geographic regions and to other species may result from social and spatial mixing of individuals across space and time.

During the past 4 years, WNS has been confirmed in at least 115 bat hibernacula in the United States and Canada and has spread over 1200 km from Howe Cave near Albany, New York, where it was first observed in February

[1]Center for Ecology and Conservation Biology (CECB), Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215, USA. [2]Department of Environmental Studies, University of California Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA. [3]Department of Ecology and Evolutionary Biology, University of California Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA. [4]Endangered Species Unit, New York State Department of Environmental Conservation, 625 Broadway, Albany, NY 12233, USA. [5]St. Paul's School, Concord, NH 03301, USA. [6]Wildlife Diversity Division, Pennsylvania Game Commission, 2001 Emerton Avenue, Harrisburg, PA 16669, USA.

*To whom correspondence should be addressed. E-mail: wfrick@batresearch.org

2006 (*9*) (Fig. 2). Decreases in bats at infected hibernacula range from 30 to 99% annually, with a regional mean of 73%, and all surveyed sites have become infected within 2 years of the disease arriving in their region (Fig. 3, A to C). Such sharp declines and rapid spread raise serious concerns about the impact of WNS on the population viability of affected bat species.

We investigated the impacts of disease-associated mortality on the regional population of little brown myotis in the northeastern United States by comparing trends in pre- and post-WNS populations and simulating 100 years of post-WNS population dynamics to assess the consequences of the introduction of the disease for bat population viability (*18*). We used a population matrix model parameterized with survival and breeding probabilities estimated from 16 years (1993–2008) of mark and recapture data at a maternity site of little brown myotis (*19*) to estimate population growth before WNS (table S1). We also calculated geometric mean growth rates from winter count surveys of this species conducted over the past 30 years at 22 hibernacula ranging across five states in the northeastern United States to determine regional population trends before the emergence of WNS (table S2).

Deterministic population growth calculated from the population matrix model of mean vital rates was positive [yearly population growth rate (λ) = 1.008], demonstrating that population growth was stable or increasing before the emergence of WNS. Estimates of long-term growth rates over the past 30 years indicate that 86% of hibernacula (n = 19 out of 22) had stable or increasing populations (λ = >1). Regional mean growth equaled 1.07 (range: 0.98 to 1.2) (table S2), suggesting that the regional population was growing before WNS and that vital rates estimated from the maternity site represent regional patterns. The growth of hibernating populations over the past 30 years may be in response to conservation measures, such as protective gating of mines and caves (*20*), the installation of bat houses (*21*), and the potential amelioration of impacts from pesticides banned in the 1970s (*22*).

To assess the impact of disease-related mortality on population viability, we simulated population dynamics using a stochastic population model that included demographic data from both infected and susceptible (uninfected) populations (*18*). We performed 1000 simulations of 100 years of growth from a starting population of 6.5 million bats, using means, variances, and correlations from vital rates (*19*) that incorporated environmental variability (*23*). The probability of extinction for each year was defined as the proportion of 1000 runs for which the simulated population dropped below a quasi-extinction threshold during that year. Quasi-extinction was specified as 0.01% of the starting population (that is, 650 bats). Defining extinction thresholds at low population sizes accounts for processes such as demographic stochasticity and potential Allee effects (*23*–*26*).

In the simulation model, the susceptible population retained pre-WNS vital rates estimated from the 16-year mark and recapture data (*19*), and infected populations were given vital rates associated with annual declines calculated from infected hibernacula where consecutive yearly counts were available (n = 22) (*18*). The increase of prevalence of WNS was estimated as the percentage of uninfected hibernacula that became infected each year (2007, 5%; 2008, 49%; 2009, 59%) and was incorporated into the simulation as the proportion of the susceptible population that becomes infected each year.

Because of the inherent uncertainty in predicting the dynamics of a recently emergent disease, we evaluated the potential for disease fadeout and its influence on population viability. We estimated annual declines for each of 3 years after infection and constructed nine a priori models to test hypotheses regarding the influence of density and time since infection on population growth rates at infected hibernacula (table S3). From these estimates, there is little evidence of density-dependent declines, although model results suggest that the rate of decline ameliorates with the time since infection (Fig. 3D and table S3). To incorporate this time amelioration effect into the simulation model, we used predicted values of population growth from a nonlinear model [$\lambda = 1 - 1.16 \times \exp(-0.31 \times t)$, where t = years since infection] for each of 16 years after infection, when predicted population growth stabilized (λ = 1) (Fig. 3D).

We simulated population growth for five scenarios related to this time amelioration effect, including declines ameliorated according to predicted values (Fig. 3D) at each yearly time step and that persisted at 45% (3rd-year actual mean), 20% (6th-year predicted mean), 10% (8th-year predicted mean), 5% (10th-year predicted mean), and 2% (13th-year predicted mean) per year (Fig. 4). By comparing the probabilities of extinction over 100 years for these five scenarios, we evaluated the vulnerability of the regional population to extinction,

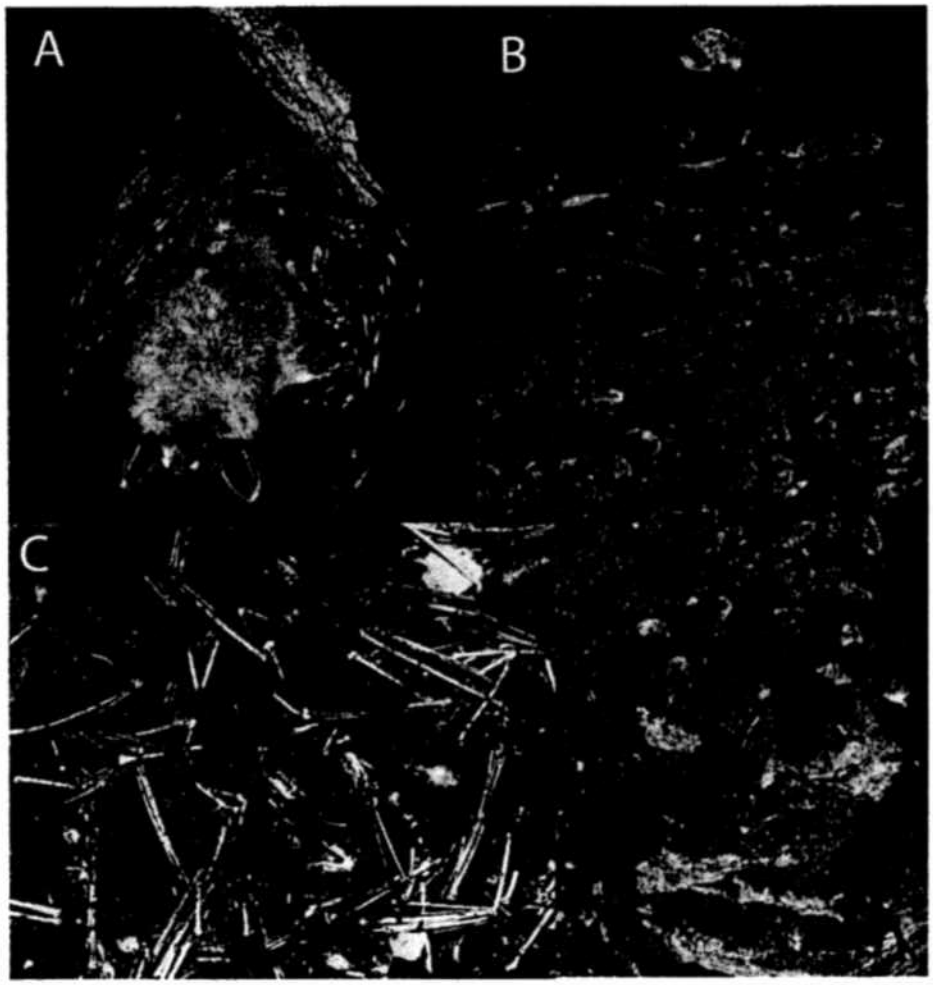


Fig. 1. (**A**) Photograph of hibernating little brown myotis infected with WNS. White fungus is visible on wings, ears, muzzle, and other exposed skin tissues. [Photo: Ryan Von Linden] (**B**) Bat carcasses piled on a cave floor, illustrating mass mortality at hibernacula infected with WNS. [Photo: Alan Hicks] (**C**) Skulls, bones, and decomposed carcasses covering the cave floor after multiple years of infection. [Photo: Marianne Moore]

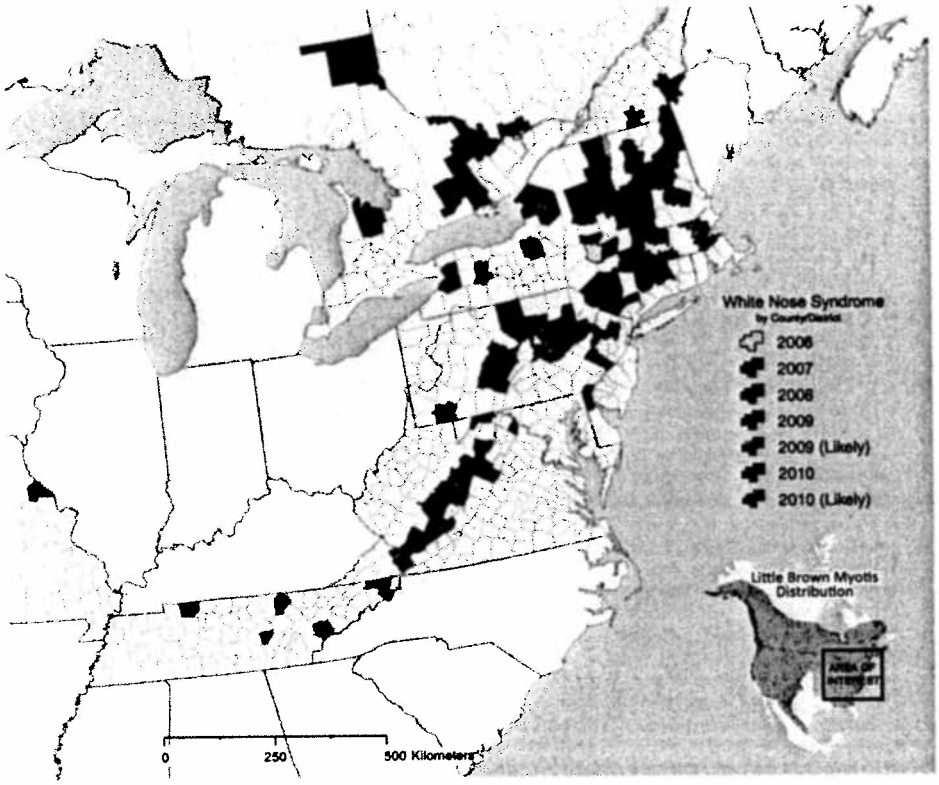


Fig. 2. Map of current distribution and spread of WNS across eastern North America.

given the uncertainty in how declines from disease mortality may persist in the future.

Using vital rates derived from mean declines in the first 3 years of infection and persisting at the observed third-year mean decline of 45% per year thereafter (Fig. 3D), we expect a 99% chance of regional extinction of little brown myotis within the next 16 years (Fig. 4A). If declines continue to ameliorate with time since infection, timelines to probable extinction lengthen but remain greater than 90% by 65 years, even if declines ameliorate and stabilize at 10% per year (Fig. 4A). Model results indicate that annual declines from WNS would have to ameliorate to less than 5% per year to significantly reduce the chance of extinction over 100 years (Fig. 4A). Even if disease mortality lessens over time, the regional population is expected to collapse from an estimated starting population of 6.5 million bats to fewer than 65,000 (1% of the pre-WNS population) in less than 20 years (Fig. 4B).

Our results paint a grim picture of a once-healthy population of an abundant and widely distributed species now experiencing unprecedented losses from WNS and facing a serious threat of regional extinction within the next 16 years (Fig. 4). Such a severe population decline, especially if the disease spreads farther south and west of its current distribution in eastern North America, may result in unpredictable changes in ecosystem

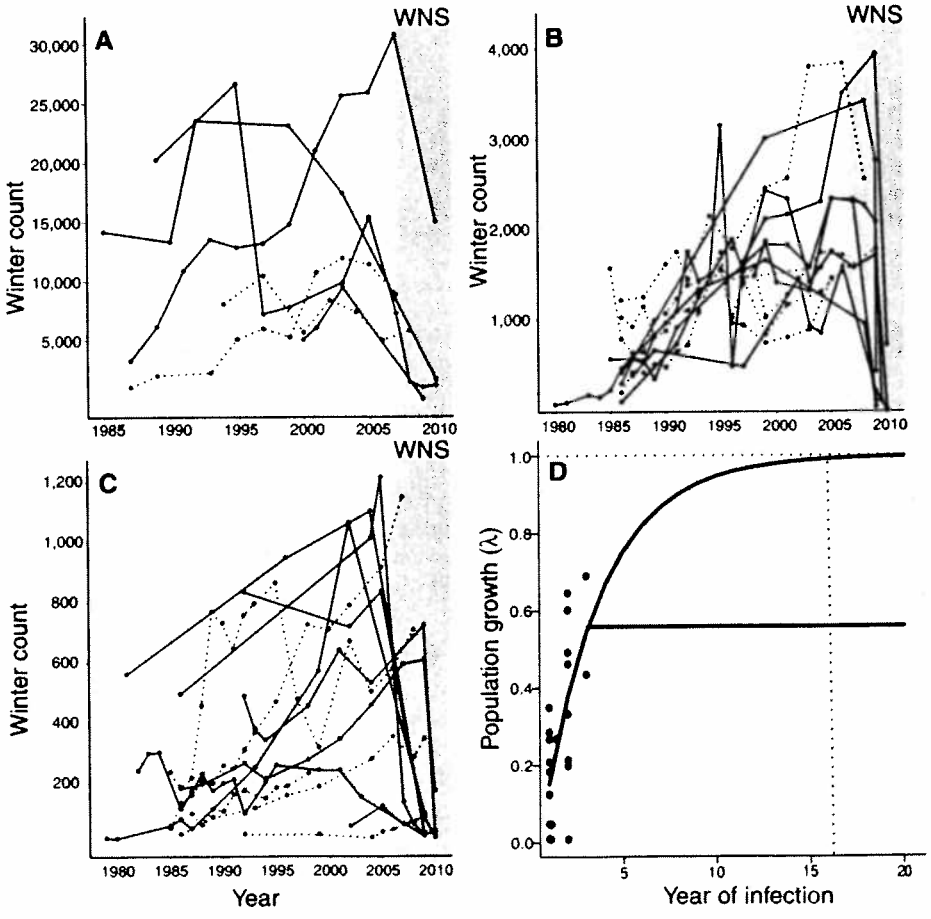


Fig. 3. (**A** to **C**) Population trends of little brown myotis over the past 30 years at (**A**) small (<1500 bats), (**B**) medium (<5000 bats), and (**C**) large (>5000 bats) hibernating colonies in the northeastern United States. Solid lines represent sites with bats infected with WNS; dotted lines represent uninfected sites. Hibernacula infected with WNS experienced a significant reduction in numbers as compared to the lowest available count from the past 30 years (Wilcoxon test = 190; $P < 0.002$). Large decreases in winter counts at a few hibernacula in the mid-1990s were related to winter flood events. (**D**) Population growth (λ) at hibernacula (black circles) by year since infection. The curved fitted line represents the nonlinear time-dependent model, showing amelioration of mortality from WNS until population growth reaches equilibrium at $\lambda = 1$ in 16 years since the first year of infection (vertical dotted line). The hockey-stick line represents declines from WNS persisting at the third-year mean of 45% per year, after a first-year decline of 85% and a second-year decline of 62%.

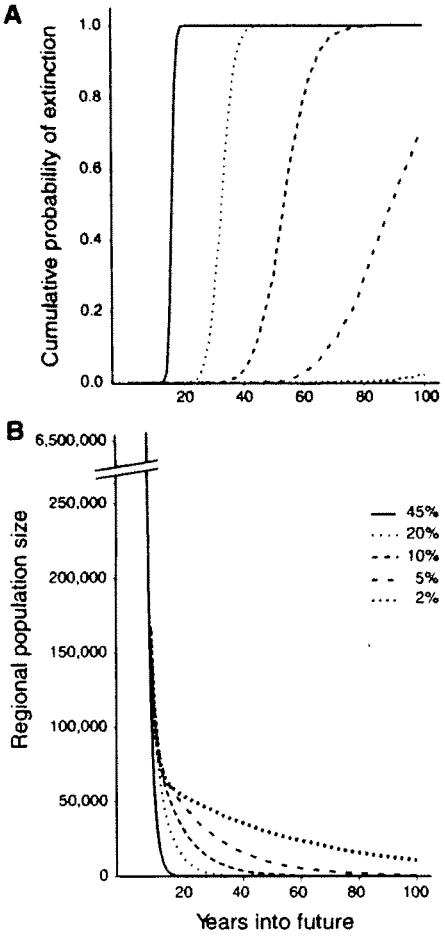


Fig. 4. **(A)** Cumulative probability of regional extinction of little brown myotis for five scenarios of time-dependent amelioration of disease mortality from WNS, based on matrix model simulation results. Each scenario represents predicted time-dependent declines for a specified number of years after infection and then holds the decline rate constant at either 45, 20, 10, 5, or 2% to demonstrate the impact of amelioration on the probability of extinction over the next 100 years. **(B)** Population size in each year averaged across 1000 simulations for each of the five scenarios of time-dependent amelioration of mortality from WNS.

structure and function (*27*, *28*). The rapid geographic spread of WNS since 2006, coupled with the severity and rapidity of population declines, support the hypothesis of introduction of a novel pathogen into a naïve population and demonstrate the seriousness of pathogen pollution as a conservation issue (*1*). Our analysis focused on little brown myotis in the northeastern United States, but several other bat species are experiencing similar mortality from WNS and may also be at significant risk of population collapse or extinction. This rapid decline of a common bat species from WNS draws attention to the need for increased research, monitoring, and management to better understand and combat this invasive wildlife disease (*1*).

References and Notes

1. P. Daszak, A. A. Cunningham, A. D. Hyatt, *Science* **287**, 443 (2000).
2. H. McCallum, A. Dobson, *Trends Ecol. Evol.* **10**, 190 (1995).
3. H. McCallum, *Trends Ecol. Evol.* **23**, 631 (2008).
4. A. M. Kilpatrick, C. J. Briggs, P. Daszak, *Trends Ecol. Evol.* **25**, 109 (2010).
5. L. Berger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9031 (1998).
6. K. R. Lips *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 3165 (2006).
7. F. Fenner, *FEMS Microbiol. Rev.* **24**, 123 (2000).
8. S. L. LaDeau, A. M. Kilpatrick, P. P. Marra, *Nature* **447**, 710 (2007).
9. D. S. Blehert *et al.*, *Science* **323**, 227 (2009).
10. A. Gargas, M. T. Trest, M. Christensen, T. J. Volk, D. S. Blehert, *Mycotaxon* **108**, 147 (2009).
11. S. J. Puechmaille *et al.*, *Emerg. Infect. Dis.* **16**, 290 (2010).
12. T. J. O'Shea, M. A. Bogan, *Monitoring Trends in Bat Populations of the United States and Territories: Problems and Prospects* (Biological Resources Discipline, Information and Technology Report USGS/BRD/ITR-2003-003, U.S. Geological Survey, Washington, DC, 2003).
13. R. Barbour, W. Davis, *Bats of America* (Univ. Press of Kentucky, Lexington, KY, USA, 1969).
14. T. H. Kunz, L. F. Lumsden, in *Bat Ecology*, T. H. Kunz, M. B. Fenton, Eds. (Univ. of Chicago Press, Chicago, IL, 2003), pp. 3–89.
15. T. H. Kunz, D. S. Reynolds, in (*12*), pp. 9–20.
16. W. H. Davis, H. B. Hitchcock, *J. Mammal.* **46**, 296 (1965).
17. D. W. Thomas, M. B. Fenton, R. M. R. Barclay, *Behav. Ecol. Sociobiol.* **6**, 129 (1979).
18. Information on materials and methods is available on *Science* Online.
19. W. F. Frick, D. S. Reynolds, T. H. Kunz, *J. Anim. Ecol.* **79**, 128 (2010).
20. E. T. Posluszny, C. Butchkoski, in *Proceedings of Bat Conservation and Mining: A Technical Interactive Forum* (Bat Conservation International and U.S. Department of the Interior, Office of Surface Mining, St. Louis, MO, 2000), pp. 159–168.
21. M. D. Tuttle, D. Hensley, *Bats* **11**, 3 (1993).
22. K. N. Geluso, J. S. Altenbach, D. E. Wilson, *Science* **194**, 184 (1976).
23. W. F. Morris, D. F. Doak, *Quantitative Conservation Biology: Theory and Practice of Population Viability Analysis* (Sinauer, Sunderland, MA, 2002).
24. F. Courchamp, T. Clutton-Brock, B. Grenfell, *Trends Ecol. Evol.* **14**, 405 (1999).
25. P. A. Stephens, W. J. Sutherland, *Trends Ecol. Evol.* **14**, 401 (1999).
26. F. Courchamp, B. Grenfell, T. Clutton-Brock, *Proc. Biol. Sci.* **266**, 557 (1999).
27. K. J. Gaston, *Science* **327**, 154 (2010).
28. G. W. Luck, G. C. Daily, P. R. Ehrlich, *Trends Ecol. Evol.* **18**, 331 (2003).

29. Funding was provided by grants from the U.S. Fish and Wildlife Service (USFWS) to W.F.F., J.F.P., D.S.R., T.H.K., and G.G.T. We thank three anonymous reviewers, J. P. Hayes, and D. F. Doak for helpful reviews and A. M. Kilpatrick for fruitful discussion. Funding for winter counts of bats at hibernacula was provided by USFWS Section 6 and State Wildlife Grants issued to the Pennsylvania Game Commission, and by Federal Aid in Wildlife Restoration Grant WE-173-G issued to the New York State Department of Environmental Conservation. Count data from hibernating colonies were kindly provided by the Connecticut Department of Environmental Protection; the Pennsylvania Game Commission; the New York Department of Environmental Conservation; Vermont Fish and Game; the Massachusetts Division of Fisheries and Wildlife; and K. Berner, State University of New York at Cobleskill. We are grateful to the many individuals who were involved in conducting annual counts of bats at hibernacula over the past 30 years. Data are available upon request from the authors.

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5992/679/DC1
Materials and Methods
Figs. S1 and S2
Tables S1 to S3
References

22 February 2010; accepted 24 May 2010
10.1126/science.1188594

Sex-Specific Parent-of-Origin Allelic Expression in the Mouse Brain

Christopher Gregg,[1,2] Jiangwen Zhang,[3] James E. Butler,[1,2] David Haig,[4] Catherine Dulac[1,2]*

Genomic imprinting results in preferential gene expression from paternally versus maternally inherited chromosomes. We used a genome-wide approach to uncover sex-specific parent-of-origin allelic effects in the adult mouse brain. Our study identified preferential selection of the maternally inherited X chromosome in glutamatergic neurons of the female cortex. Moreover, analysis of the cortex and hypothalamus identified 347 autosomal genes with sex-specific imprinting features. In the hypothalamus, sex-specific imprinted genes were mostly found in females, which suggests parental influence over the hypothalamic function of daughters. We show that *interleukin-18*, a gene linked to diseases with sex-specific prevalence, is subject to complex, regional, and sex-specific parental effects in the brain. Parent-of-origin effects thus provide new avenues for investigation of sexual dimorphism in brain function and disease.

Genomic imprinting is an epigenetic mode of gene regulation involving preferential expression of the paternally or maternally inherited allele (*1*). Sexual dimorphism is a central characteristic of mammalian brain function and behavior that influences major neurological diseases in humans (*2*). Here we address the potential existence of differential genomic imprinting in the brain according to the sex of individuals. Imprinting refers to gene expression differences between maternal and paternal chromosomes (*3*) and is also used more strictly to define complete allele-specific silencing (*4*). Our analysis encompasses sex differences in parent-

[1]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA. [2]Howard Hughes Medical Institute, Harvard University, Cambridge, MA 02138, USA. [3]FAS Research Computing, Harvard University, Cambridge, MA 02138, USA. [4]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA.

*To whom correspondence should be addressed. E-mail: dulac@fas.harvard.edu


FORWARD

Science
AAAS

www.sciencemag.org/cgi/content/full/329/5992/679/DC1

Supporting Online Material for

An Emerging Disease Causes Regional Population Collapse of a Common North American Bat Species

Winifred F. Frick,* Jacob F. Pollock, Alan C. Hicks, Kate E. Langwig, D. Scott Reynolds, Gregory G. Turner, Calvin M. Butchkoski, Thomas H. Kunz

*To whom correspondence should be addressed. E-mail: wfrick@batresearch.org

Published 6 August 2010, *Science* **329**, 679 (2010)
DOI: 10.1126/science.1188594

This PDF file includes:

SUPPORTING ONLINE MATERIAL:

Materials and Methods

Data sources

30-year winter hibernacula count data

Systematic surveys to count the number of hibernating bats were conducted at winter hibernacula throughout the northeastern U.S.A. over a 30-year period (1979-2009) by different individuals representing state departments of natural resources. Winter survey protocols consisted of trained researchers searching all sections of a hibernaculum to identify to species and count all bats observed. Multiple species are often present in hibernacula and many surveys were focused on the endangered Indiana myotis (*Myotis sodalis*). For this analysis, only complete counts of our focal species, little brown myotis (*Myotis lucifugus*), are included and we constrained the sample to counts conducted between 1 December and 31 March to reduce variability due to seasonal movements. Surveys were limited to only one per year at each hibernaculum, because repeated or prolonged disruption of bats during torpor can cause increased energy expenditure from arousals and premature depletion of fat stores (*1-3*). Duration of surveys depended on size of the site and number of bats present, although efforts were made to limit time spent inside a hibernaculum to reduce disturbance. The majority of counts were conducted *in situ*, although in some instances counts were verified or enhanced by subsequent analysis of photographic images.

Vital rates

We used published vital rates estimated from 16 years of mark-recapture data (1993-2008) at a maternity colony of little brown myotis located near Peterborough, New Hampshire (*4*) (Table S1).

Detection of WNS

The first known WNS infection in little brown myotis occurred in February 2006, but was not confirmed until a later review of photographs taken at this site. The photographs show white fungal growth present on the noses, ears, and forearms of bats, characteristic of WNS infection. This was likely the early stage of infection at this site, because mortality of bats was not yet apparent. Because WNS has rapidly expanded geographically, monitoring efforts have focused on searching for visible presence of *Geomyces destructans*, often making it possible to detect presence of the putative pathogen before symptomatic mortality associated with the disease has occurred. Surveys in the winter of 2006-2007 relied primarily on symptomatic evidence of infection, such as presence of white fungus on bats in conjunction with aberrant behavior or unusually high numbers of dead or moribund bats, whereas winter site surveys in 2007-2008, 2008-2009 and 2009-2010 relied more on visible presence of white fungus on one or more bats, and in some cases a laboratory confirmation of presence of *G. destructans* (*5*). All sites that had presence of *G. destructans* confirmed without symptomatic mortality in their first year had symptomatic mortality the following year. For calculation of annual declines from WNS, we defined sites with symptomatic mortality as infected with WNS.

Analyses

Calculation of pre-WNS population growth from vital rates

We constructed a 2-stage Lefkovitch matrix using estimated annual survival, breeding and fecundity probabilities for adult and juvenile stage classes of little brown myotis (Fig. S1) from 16-years of mark-recapture data (*4*), yielding:

$$\begin{bmatrix} S_j * B_j * F & S_a * B_a * F \\ S_j & S_a \end{bmatrix}$$

where, S_j represents the annual survival probability of a female little brown myotis in its first year (juvenile stage class); S_a represents the annual survival probability of a female adult bat (adult stage class); B_j represents the probability that a female returns to the maternity colony to breed in the year following her birth, B_a represents the probability that an adult female returns to the maternity colony ($B_a = 1$ in our model); and F represents the fecundity or probability that a female will reproduce each year at a maternity colony. Because data were collected during a post-breeding census, fecundity estimates apply to both juvenile and adult stages because juvenile females enter the adult stage class by the time they breed the following year (Fig. S1). Deterministic growth (λ) was calculated as the greatest eigenvalue of this matrix and equaled 1.008 prior to WNS.

Calculation of pre-WNS annual growth rates from winter count surveys at hibernacula

We calculated geometric mean growth rates for each of 22 hibernacula in the northeastern U.S.A. that had more than seven years of winter counts collected between 1979 and 2009 to characterize regional growth trends prior to WNS (Table S2).

We used the log of the geometric mean of population growth (μ) to represent average population growth:

$$\mu = \log \lambda_G \approx \frac{\log \lambda_t + \log \lambda_{t-1} + \log \lambda_{t-2} \ldots \log \lambda_0}{t}$$

Where, λ_t represents population growth between each year (*e.g.*, $\lambda_t = N_{t+1}/N_t$) and λ_G is geometric growth over all years (*6*). Values $\lambda_G > 1$ represent positive growth and $\lambda_G < 1$ correspond to negative population growth (*i.e.*, decline). Because counts were conducted at different times, we estimated μ using a linear regression technique with y_i as the dependent variable and x_i as the independent variable, where:

$$y_i = \frac{\log\left(\frac{N_{i+1}}{N_i}\right)}{\sqrt{t_{i+1} - t_i}} \text{ and } x_i = \sqrt{t_{i+1} - t_i}$$

The slope of the regression estimates μ and the mean square error estimates its variance (σ^2) (*6*).

Population Viability Analyses (PVA)

Pre-WNS population viability analysis

We simulated probabilities of quasi-extinction and calculated the stochastic

growth rate for pre-WNS conditions using a stochastic demographic population model based on the population matrix model and estimated vital rates described above. We ran 1,000 simulations of 100 years of population growth, drawing random vital rates for each year of each run from beta distributions with means, variances and correlations of the vital rates estimated from the mark-recapture data (*4*, *6*). We designated a starting population size of 6.5 millions bats (see below) and defined a quasi-extinction threshold at 0.01% of the starting population (e.g. 650 bats). The probability of extinction for a given year is the proportion of the 1,000 runs for which the population drops below the quasi-extinction threshold on or before that year. The mean stochastic yearly growth (λ) for the simulation before incorporating WNS mortality was 1.006 (95% C.I.: 0.94-1.07).

No robust estimates exist for the total regional population of little brown myotis in the northeastern U.S.A. prior to the occurrence of WNS. Informed researchers have estimated that over 1 million bats have died since WNS was first observed in 2006. To provide the model with a reasonable starting regional population size, we estimated minimum regional population size by calculating a grand average of the number of little brown myotis counted in surveyed hibernacula each year since 1985 (for NY, CT, VT, and MA) and since 1999 (for PA). This generated a rough estimate of 650,000 bats for all five states. Because the proportion of known, surveyed hibernacula to total hibernacula in the region is unknown, we increased our estimate by an order of magnitude to 6.5 million bats to provide an upper estimate. The consequence of over-estimating the starting population size is to lengthen the time to extinction, whereas under-estimating the regional population size will shorten time to extinction. By defining an extinction threshold as a percentage of the starting population size (we conservatively used 0.01% of the starting population), we ameliorate this potential bias.

Post-WNS population viability analysis

To determine the impact of WNS on regional population viability, we modified our stochastic demographic model to incorporate two types of sub-populations: infected and susceptible. The susceptible subpopulation experienced pre-WNS vital rates and variances as described above. For the infected populations, we incorporated mortality from WNS into vital rates matrices by first using estimated annual declines at 22 hibernacula where consecutive yearly counts were available. Annual decline was defined as:

$$1 - \frac{N_{t+1}}{N_t}$$

where, N_t = number of hibernating little brown myotis counted in year t; N_{t+1} = number of hibernating little brown myotis counted the next year, and N_{t+1}/N_t is the 1-year growth rate (λ). To back-calculate survival rates from these decline rates, we created a look-up table that contained 10,000 incremental values of adult survival (S_a) from 0 to 1 and the corresponding values for lambda (λ). Lambdas were calculated as the greatest eigenvalue of the 2-stage Lefkovitch matrix with fecundity (F) and age-specific breeding probabilities (B_a and B_j) held constant at their 16-year means (Fig. S2). Juvenile survival was fixed as a constant proportion of adult survival (S_j= .47 * S_a), based on the mean proportion of juvenile to adult survival estimated from the 16 year mark-recapture data.

We investigated the influence of density at hibernacula (N_t) and time since infection in years on population growth at 22 sites where we had consecutive post-WNS yearly counts. We built nine models of post-WNS annual growth that tested for main, additive, and interactive effects among density [$\ln(N_t)$] and time since infection [year and ln(year)], as well as a null model of constant growth (Table S3). We used Aikaike Information Criteria (AIC) model selection methods to compare relative support among these *a priori* candidate models. Model selection results showed strong support for an influence of year since infection on population growth ($\Delta AIC_c = 0$; AIC_c weight = 0.19) (Table S3). The coefficient estimate for the influence of year since infection equaled 0.20 (95% C.I.: 0.11-0.31), suggesting that declines have ameliorated by a mean 20% for each of three years since infection (Fig. 3D). To predict how declines may ameliorate with time, we fit a non-linear model [λ = 1-b*exp(-c*year) + error, where b and c are fitted constants] that forced population growth to asymptote at the equilibrium value of λ = 1. The non-linear model had equal support from the data ($\Delta AIC_c = 0.03$; AIC_c weight = 0.19) and was used to generate predicted values of population growth for each year of 16 years since infection until population growth stabilized at 1.

The simulation model used between 3 and 16 classes of infected subpopulations, each one denoted by the number of years since infection, starting with year 1. In each year of the simulation, a specified number of bats become infected and move from the susceptible population to the 1st year infected class. The next year, the remaining individuals in the 1st year infected class transition to 2nd year infected class et cetera for up to 16 years. Individuals then remain in the last specified infected class for the rest of the current run of the simulation. To test how different amelioration scenarios would affect probability of extinction we altered the number of classes such that individuals stayed in classes associated with either 45% declines (class 3), 20% declines (class 6), 10% declines (class 8), 5% declines (class 10) and 2% declines (class 13). Adult and juvenile survival rates for each of the infected year classes were derived by using the look-up table for predicted values from the non-linear time-dependent growth model for each of post-infection years 1-16.

Transition from the susceptible class to the 1st year infected class was based on observed rates of the number of surveyed uninfected hibernacula that became infected from 2007-2009 and starts in year 1 of the simulation at 5%, increasing in year 2 to 49% and persisting at 59% thereafter. We ran 1,000 simulations of 100 years of population growth using starting population size of 6.5 million bats and a quasi-extinction threshold of 0.01% of the starting population (e.g. 650 bats).

References

1. D. W. Thomas, M. Dorais, J.-M. Bergeron, *J. Mammal.* **71**, 475 (1990).
2. J. R. Speakman, P. I. Webb, P. A. Racey, *J. Appl. Ecol.* **28**, 1087 (1991).
3. D. W. Thomas, *J. Mammal.* **76**, 940 (1995).
4. W. F. Frick, D. S. Reynolds, T. H. Kunz, *J. Anim. Ecol.* **79**, 128 (2010).
5. A. Gargas, M. T. Trest, M. Christensen, T. J. Volk, D. S. Bleher, *Mycotaxon* **108**, 147 (2009).
6. W. F. Morris, D. F. Doak, *Quantitative Conservation Biology: Theory and Practice of Population Viability Analysis.* (Sinauer Associates, Inc., Sunderland, MA, 2002), pp. 480.

Table S1. Estimates of annual vital rates for little brown myotis used in population viability analysis for pre-WNS conditions. Annual estimates of B_j (probability of returning to maternity colony to breed for juveniles) and S_j (probability of annual survival of juveniles) were calculated based on median date of birth (DOB) for pups for each year and annual survival of adult females (S_a) was dependent on cumulative summer precipitation (*4*). Estimates of fecundity apply to both adult and juvenile stage classes as juveniles enter adult stage class by the following year. Probability that adult bats return to the maternity colony to breed (B_a) was fixed at 1. Numbers in parentheses represent 95% confidence limits for each estimate.

Year (t)	Med. Julian DOB	Breeding of Juveniles (B_j)	Survival of Juveniles (S_j)	Survival of Adults (S_a)	Fecundity (t+1)
1993	171	0.41 (0.27, 0.56)	0.37 (0.28, 0.46)	0.64 (0.59, 0.69)	0.95
1994	173	0.40 (0.26, 0.55)	0.36 (0.30, 0.42)	0.68 (0.64, 0.71)	0.93
1995	172	0.40 (0.27, 0.56)	0.36 (0.30, 0.43)	0.69 (0.66, 0.72)	0.95
1996	179	0.36 (0.23, 0.52)	0.33 (0.27, 0.40)	0.64 (0.59, 0.69)	0.95
1997	181	0.35 (0.22, 0.51)	0.33 (0.26, 0.40)	0.63 (0.56, 0.68)	0.97
1998	176	0.38 (0.25, 0.52)	0.35 (0.28, 0.41)	0.72 (0.69, 0.76)	0.99
1999	170	0.41 (0.27, 0.57)	0.37 (0.31, 0.44)	0.75 (0.70, 0.79)	0.96
2000	180	0.36 (0.23, 0.52)	0.33 (0.27, 0.40)	0.73 (0.69, 0.76)	0.99
2001	169	0.42 (0.28, 0.58)	0.37 (0.31, 0.44)	0.63 (0.58, 0.69)	0.94
2002	178	0.37 (0.24,0.53)	0.34 (0.28, 0.40)	0.71 (0.67, 0.74)	0.96
2003	179	0.36 (0.23,0.52)	0.33 (0.27, 0.40)	0.80 (0.73, 0.85)	0.95
2004	177	0.38 (0.24,0.53)	0.34 (0.28, 0.41)	0.81 (0.73, 0.87)	0.95
2005	178	0.37 (0.24, 0.53)	0.34 (0.28, 0.40)	0.88 (0.77, 0.94)	0.96
2006	180	0.36 (0.23, 0.52)	0.33 (0.27, 0.40)	0.90 (0.78, 0.95)	0.97
2007	174	0.39 (0.26, 0.54)	0.35 (0.29, 0.42)	0.79 (0.72, 0.84)	0.87

Table S2. Results from count-based estimates of population growth for little brown myotis at 22 hibernacula from 5 states in the northeastern U.S.A. Values of $\lambda_G > 1$ indicate positive population growth and $\lambda_G < 1$ indicate negative population growth (*i.e.* decline).

Hibernacula Site Code	No. of annual counts	Range of years	Geometric mean of population growth (λ_G and 95% CL)
CT-1	10	1986-2007	1.084 (0.927, 1.268)
MA-1	8	1979-2002	1.200 (1.009, 1.427)
MA-3	8	1980-1999	1.177 (0.981, 1.411)
NY-2	6	1989-2005	0.983 (0.697, 1.385)
NY-5	15	1982-2003	0.978 (0.816, 1.173)
PA-1	7	1987-2006	1.077 (0.865, 1.341)
PA-2	17	1986-2009	1.118 (0.840, 1.488)
PA-3	9	1986-2009	1.116 (1.004, 1.239)
PA-4	11	1987-2007	1.119 (1.030, 1.215)
PA-5	9	1985-2004	1.000 (0.839, 1.192)
PA-6	7	1994-2008	0.977 (0.823, 1.159)
PA-7	10	1987-1998	1.082 (0.862, 1.359)
PA-8	15	1986-2009	1.024 (0.939, 1.117)
PA-9	15	1985-2007	1.155 (1.021, 1.307)
PA-10	15	1986-2005	1.109 (0.950, 1.294)
PA-11	11	1986-2009	1.131 (0.971, 1.317)
PA-12	16	1985-2008	1.049 (0.909, 1.211)
PA-13	8	1995-2006	1.008 (0.809, 1.256)
PA-14	10	1987-2007	1.114 (0.962, 1.290)
PA-15	12	1986-2008	1.034 (0.947, 1.129)
VT-1	7	1992-2009	1.071 (0.840, 1.365)
VT-2	9	1986-2009	1.054 (1.004, 1.108)

Table S3. Model selection results for 9 models of population growth (λ) as a function of density dependence (N_t) or time since infection (year). A non-linear model forcing population growth to asymptote at 1 (indicated in bold) had equal support from the data based on AIC_c and was used for generating predicted values of population decline ($1-\lambda$) for stochastic simulation models.

Model	AIC_c	ΔAIC_c	AIC_c weights	-log(*L*)	No. Parameters
$\lambda \sim$ year	-12.52	0.00	0.19	9.57	2
$\lambda \sim 1-b*e^{(-c*year)}$	**-12.49**	**0.03**	**0.19**	**9.56**	**2**
$\lambda \sim$ ln(year)	-12.33	0.19	0.18	9.48	2
$\lambda \sim \ln(N_t)$ + year	-12.33	0.19	0.18	10.83	3
$\lambda \sim \ln(N_t)$ + ln(year)	-11.80	0.72	0.13	10.57	3
$\lambda \sim \ln(N_t)$ * year	-10.73	1.78	0.08	11.54	4
$\lambda \sim \ln(N_t)$ * ln(year)	-9.56	2.96	0.04	10.95	4
$\lambda \sim \ln(N_t)$	-5.44	7.08	0.01	6.04	2
$\lambda \sim 1$ (null)	-2.39	10.13	0.00	3.30	1

Figure S1. Life-cycle representing a stage-structured model for little brown myotis and transition probabilities used in projection population matrix. Juvenile stage includes first year of life for bats captured as young-of-the-year. Reproduction is represented by two components: 1) Breeding probability (B) = probability that an individual returns to the maternity colony to reproduce in a given year; and 2) Fecundity (F) = proportion of reproducing females at the colony. S_a = adult annual survival; S_j = juvenile annual survival; B_a = adult annual breeding probability; B_j = juvenile annual breeding probability; F = fecundity.

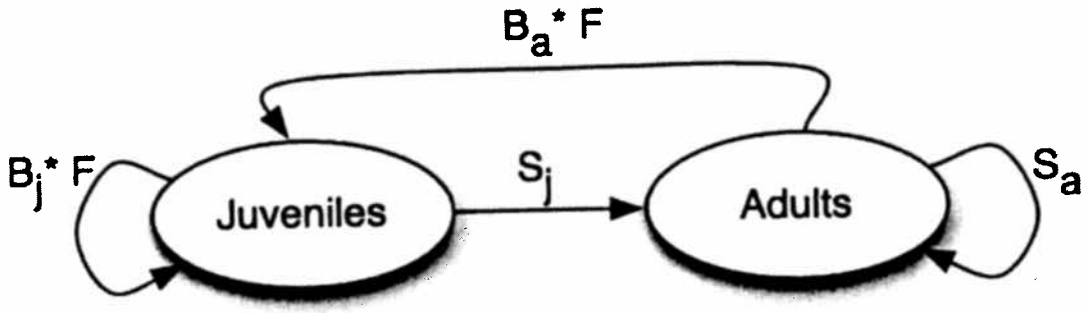
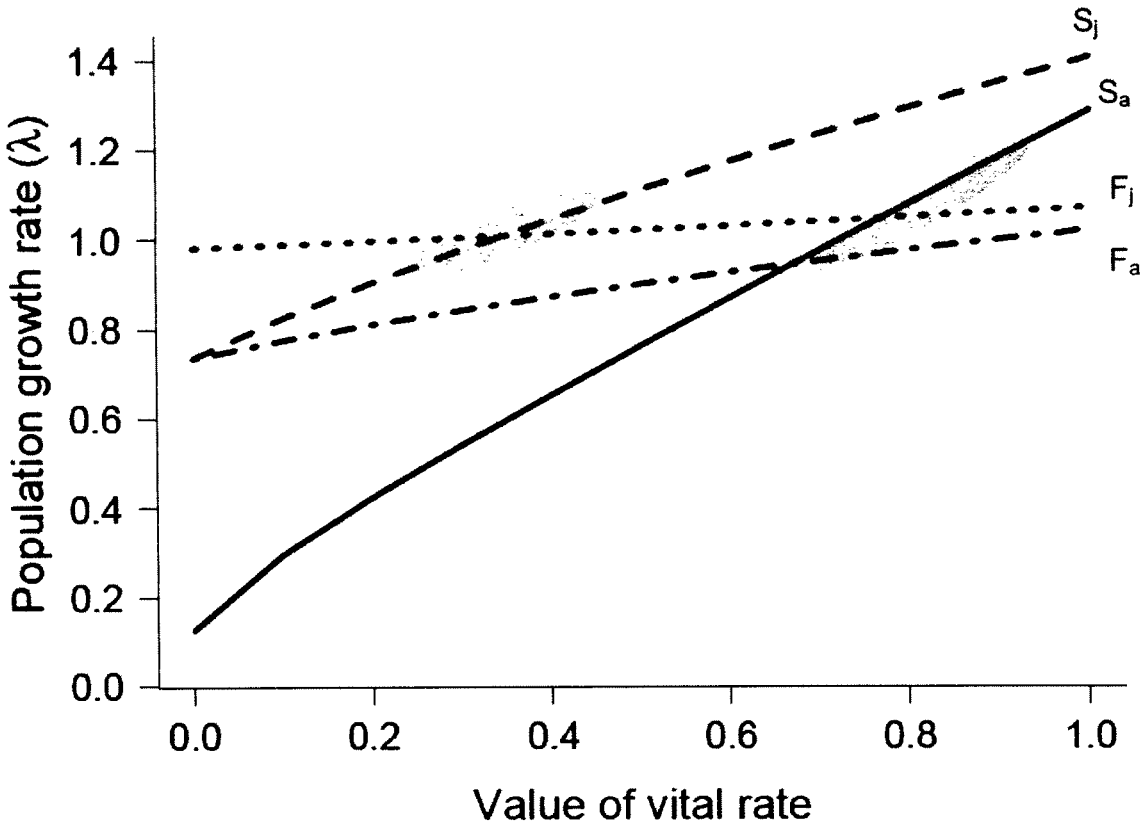


Figure S2. Relationship of population growth rate and vital rates for a 2-stage population model in little brown myotis. Shaded ellipses indicate the range of estimated values for adult survival (S_a; solid line) and juvenile survival (S_j; dashed line) under normal pre-WNS conditions. S_a = adult survival; S_j = juvenile survival; F_j = juvenile fecundity; F_a = adult fecundity.




FORWARD

BAT RESEARCH NEWS

Volume 50: Number 3

Fall 2009

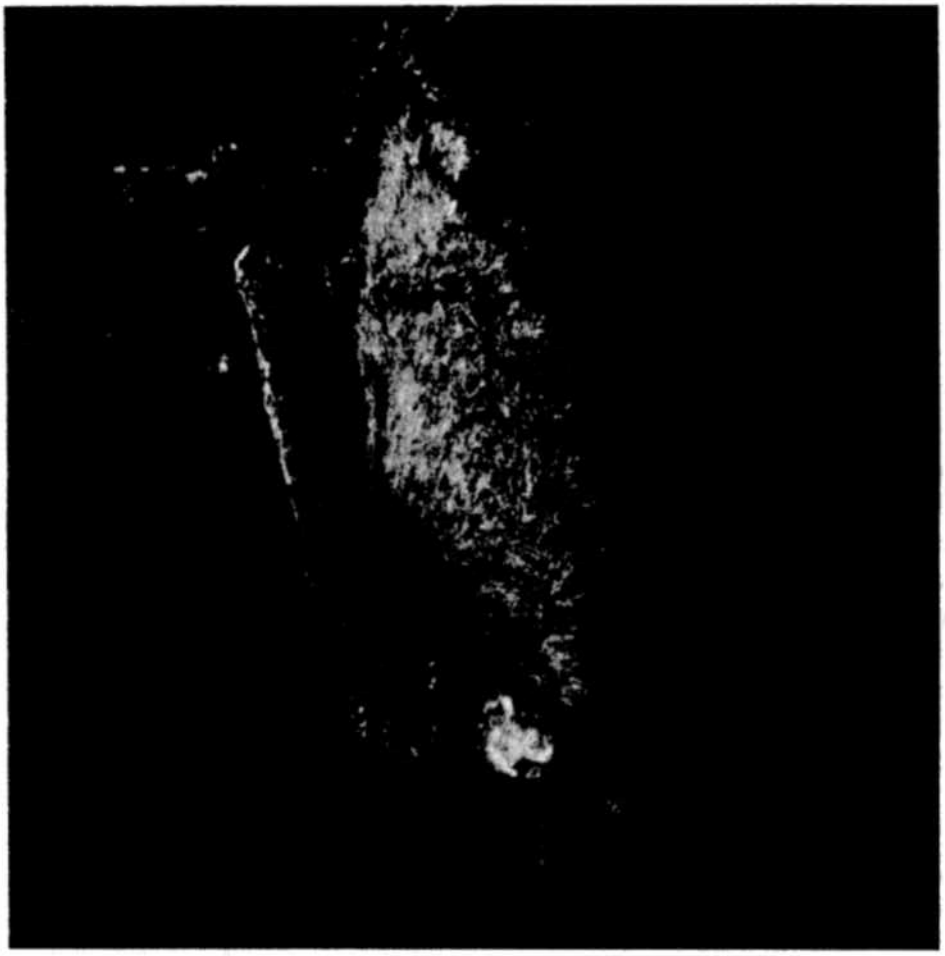


Figure 1. Little brown bat (*Myotis lucifugus*) afflicted with white nose syndrome. The bat was found in an abandoned mine in Lackawanna County, Pennsylvania. Photograph courtesy of Greg Turner, Pennsylvania Game Commission.

Pennsylvania, West Virginia, and Virginia (Figure 2).

Anthropogenic transmission?—Concern over anthropogenic transmission was raised initially in 2008, after discovery that all but two or three new WNS sites found that year had been visited by either biologists or recreational users who had been in at least one of the original four sites noted in 2007. This concern highlighted the need for establishing decontamination protocols, as well as the need to verify that they were effective. Large-scale movements (jumps) of WNS occurred in 2009 into areas where WNS was not thought to exist, leaving sites in-between unaffected. These jumps occurred in central Pennsylvania, West Virginia, and Virginia, and several factors pointed toward a human connection. First, most sites had small hibernating populations. Although the possibility existed for spread by an infected bat that had migrated a long distance to a small hibernating population in the new area, it was unlikely that this would have occurred multiple times, particularly when hibernacula with much larger populations existed nearby, as was the case at several of these newly infected sites. Second, most new sites had

very high recreational use, and third, several sites were confirmed to have visitation by people or mud-covered gear that had been in affected sites in New York, prior to establishment of decontamination protocols. Due to this suspicion that anthropogenic movements were likely, that no causal agent had been identified, and that decontamination protocols were not thoroughly tested, some states reduced or halted population surveys during hibernation and the U.S. Fish and Wildlife Service requested a voluntary moratorium on caving until more was learned (http://www.fws.gov/northeast/wnscaveadvisory.html).

Bat-to-bat transmission?—In addition to anthropogenic spread, bat-to-bat transmission surely also occurred and may have been the primary mechanism of spread. This mode of transmission was supported by a wave-like pattern of spread away from the epicenter (in addition to long-distance jumps) and the fact that in both 2008 and 2009 some newly affected sites had not been visited by

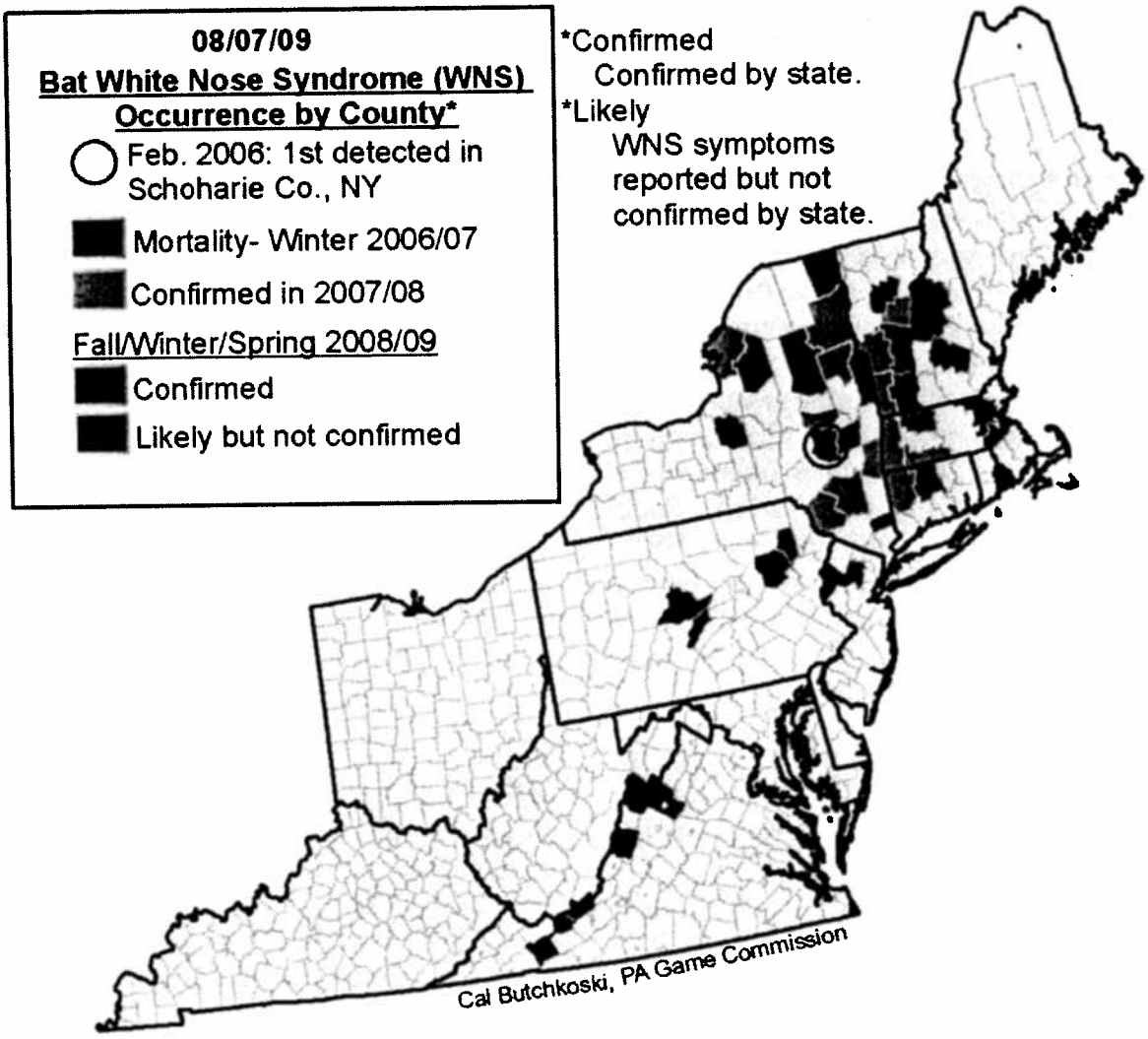


Figure 2. Map depicting the spread of white nose syndrome by county across hibernating seasons. Courtesy of Cal Butchkoski, Pennsylvania Game Commission.

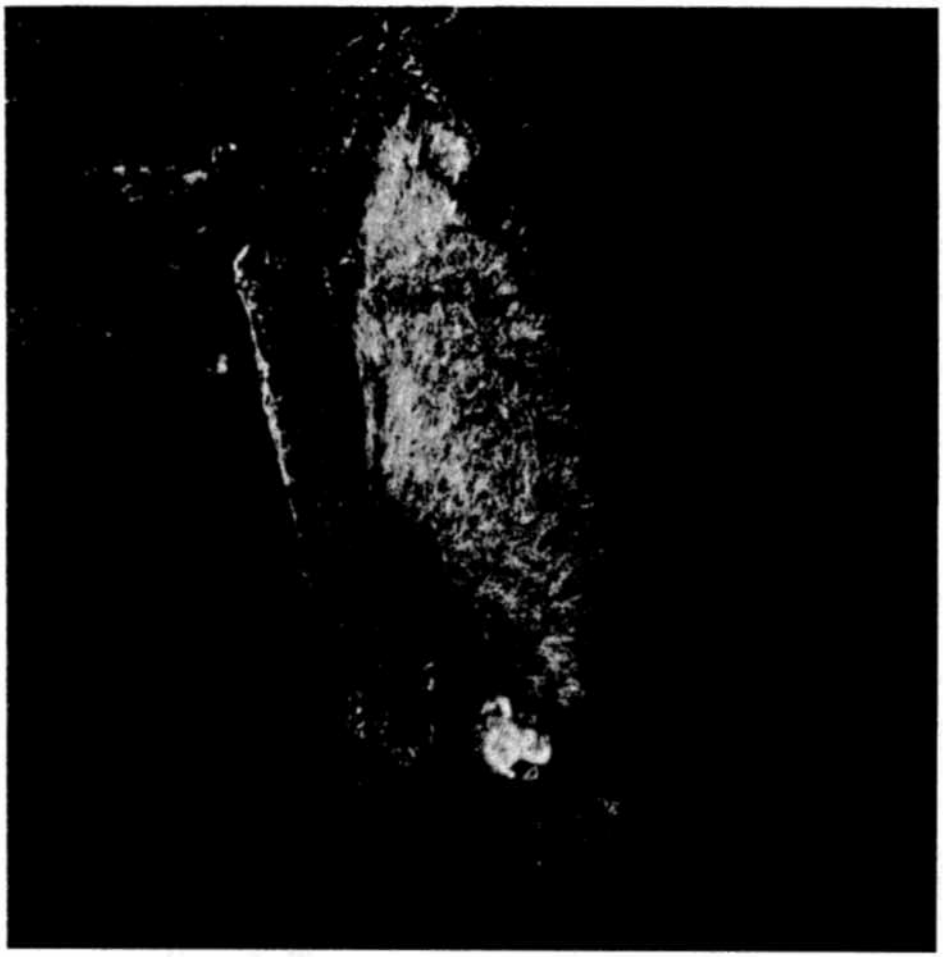


Figure 1. Little brown bat (*Myotis lucifugus*) afflicted with white nose syndrome. The bat was found in an abandoned mine in Lackawanna County, Pennsylvania. Photograph courtesy of Greg Turner, Pennsylvania Game Commission.

Pennsylvania, West Virginia, and Virginia (Figure 2).

Anthropogenic transmission?—Concern over anthropogenic transmission was raised initially in 2008, after discovery that all but two or three new WNS sites found that year had been visited by either biologists or recreational users who had been in at least one of the original four sites noted in 2007. This concern highlighted the need for establishing decontamination protocols, as well as the need to verify that they were effective. Large-scale movements (jumps) of WNS occurred in 2009 into areas where WNS was not thought to exist, leaving sites in-between unaffected. These jumps occurred in central Pennsylvania, West Virginia, and Virginia, and several factors pointed toward a human connection. First, most sites had small hibernating populations. Although the possibility existed for spread by an infected bat that had migrated a long distance to a small hibernating population in the new area, it was unlikely that this would have occurred multiple times, particularly when hibernacula with much larger populations existed nearby, as was the case at several of these newly infected sites. Second, most new sites had

of the Mississippi River. To examine this question, sediment samples were collected from hibernacula within and outside the WNS-affected region in winter 2009, and preliminary analyses indicated a diversity of fungi related to, but distinct from *G. destructans* (D. Blehert, pers. comm.). The presence of these closely related species made analyzing samples for *G. destructans* labor intensive. Nonetheless, Blehert noted that *G. destructans* was found in sediments from a number of hibernacula within the WNS-infected region.

Scientific studies supporting the fungus as the sole causative agent of mortality are on-going but not conclusive at this time. Many researchers within the WNS community currently believe that the fungus is the most likely culprit. Additionally, anecdotal evidence of the likely role of *G. destructans* in causing WNS continues to grow. Consider first, that if humans are capable of spreading this fungus from site to site via caving equipment, the odds of transmission would appear greater with a resistant fungal spore than with other pathogens. Although fungi are common inhabitants of caves and mines and are occasionally documented on live bats, the unique morphological characteristics of this fungus had never been seen and/or reported in the United States until 2006, and the fungus continues to be found only in sites confirmed to be affected and displaying high mortality. In one central Pennsylvania site, the fungus was noted and confirmed prior to the observation of the clinical signs of roost shifting, distortions of typical arousal patterns, lethargy, and early emergence or death. Further, these clinical signs increased as growth of the fungus on individual bats progressed and as a greater number of bats became affected. Finally, evidence that the *G. destructans* can be found in sediments in affected sites supports the hypothesis that humans may represent a potential vector.

Research investigating the efficacy of fungicides for decontaminating affected gear and compounds for potential use as treatment for affected bats is also underway. Preliminary results indicate that the vegetative structures of a similar, but non-pathogenic fungus are rather easy to kill but the spores are quite resistant (H. Barton, pers. comm.). Thus far, over 80 compounds have been tested by Barton and the efficacies of these treatments are being analyzed. The combining of different compounds to achieve synergistic decontamination and/or treatment, while causing minimal damage to either the unique cave biota or the performance of technical gear is a major challenge but is showing promise. Barton preliminarily notes that washing caving equipment in Woolite (Reckitt Benckiser, Inc., Parsippany, New Jersey) prior to decontamination is critical, because it removes mud, clay, and other sediments that contain charged surfaces that attract disinfectants, decreasing their efficacy. These studies and their widespread application hinge on confirmation of the fungus as a causative agent of mortality.

If the fungus is eventually documented as the causative agent of WNS, the immediate question that follows is: where did this fungus come from? It is possible that this pathogenic fungus evolved from one of the naturally occurring and closely related species found in nearly all hibernacula investigated so far, but the fungus could also be an introduced species to which North American bats have no resistance. On this front, several European scientists, upon hearing about WNS, have noted that a fungus with similar morphological traits can be found on their hibernating bats but with no signs of mass mortality at this time. The arrival of an exotic cold-loving fungus is a "perfect storm" for killing hibernating bats, because bats have extremely high rates of contact, the fungus attacks them at a time when their capability

for mounting any immune response is minimized to save energy, and this period of inactivity and immune suppression is lengthy. Regardless of the causative agent(s), the levels of mortality are unprecedented in the known history of bats, and the potential loss of millions of bats across this region gives everyone reason to be greatly concerned.

Hope for the Future?

Despite all this, there may be hope for North American bats. If the fungus is actually the causative agent, then what we know suggests that non-hibernating bats should largely not be affected as the fungi will not have the ability to grow for prolonged periods. Evidence from Pennsylvania suggests a single year's natural spread may be only around 15–20 miles per year without anthropogenic transmission. Therefore, if we can determine the mechanisms and timing by which natural transmission is occurring, we may be able to slow the spread and allow for containment or treatments to be developed. Progress is also being made on testing procedures to decontaminate all gear used underground. Compounds that have anti-fungal capabilities are now being tested to determine whether there are impacts to bats or the many unique and globally rare creatures that live among them and will hopefully lead to some management options. These compounds may help delay or break the cycle of transmission, or even better, they may increase the survival rate at affected sites until even more can be learned.

There are also things people can do in both affected and not-yet-affected areas. In those parts of the continent not currently affected, intense surveillance can provide estimates of pre-WNS population size and allow for better tracking and potential mitigation of WNS. One excellent example of this type of activity is the Appalachian bat count (http://www.pgc.state.pa.us/pgc/cwp/view.asp?a=458&Q=176676&PM=1).

People can also install bat boxes to provide alternate roosts for bats. Although this would not affect the fungus directly, installing bat boxes could provide fungus-free environments over the summer months and also reduce migratory distances between winter hibernacula and summer sites by providing suitable roosts, both of which could enhance the survival of as many bats as possible. Examples of boxes that work well in the eastern United States can be found by visiting the websites of the Pennsylvania State Game Commission (http://www.pgc.state.pa.us/pgc/lib/pgc/wildlife/woodcrafting/plan11.pdf) or Bat Conservation International (http://www.batcon.org). As the disease moves through new areas, locating resistant individuals and those few remaining summer and winter colonies will be of critical importance to the future recovery efforts of our night-flying friends. We hope and expect that some bats will survive, but even survivors will face tremendous challenges, because they can be expected to have limited fat reserves for migration and winter survival. Minimal winter disturbance will be critical for these bats to give them a fighting chance. Even so, with their low reproductive rates, it will be decades before bat populations in WNS-affected areas are restored. Finally, people can inform their state and federal representatives that significant governmental funding is desperately needed, as WNS is clearly an issue for all of North America—not just the Northeast. Lastly, several mechanisms for collecting personal donations to assist WNS-related research have been established (http://www.indstate.edu/ecology/centers/bat.htm, http://www.batcon.org/, or http://www.caves.org/WNS/).

Literature Cited

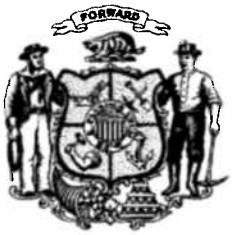
Blehert, D. S., A. C. Hicks, M. Behr, C. U. Meteyer, B. M. Berlowski-Zier, E. L. Buckles, J. T. H. Coleman, S. C. Darling, A. Gargas, R. Niver, J. C. Okoniewski, R. J. Rudd, and W. B. Stone. 2009. Bat white-nose syndrome: an emerging fungal pathogen? Science, 323:227.

Gargas, A., M. T. Trest, M. Christensen, T. J. Volk, and D. S. Blehert. 2009. *Geomyces destructans* sp. nov. associated with bat white-nose syndrome. Mycotaxon, 108: 147–154.

Reeder, D. M., and G. R. [G. G.] Turner. 2008. Working together to combat white-nose syndrome: a report of a meeting on 9–11 June 2008, in Albany, New York. Bat Research News, 49:75–78.

Reichard, J. D., and T. H. Kunz. In press. White-nose syndrome inflicts lasting injuries to the wings of little brown bats (*Myotis lucifugus*). Acta Chiropterologica.


WISCONSIN STATE LEGISLATURE


FORWARD

In Press at Mycologia, published on October 17, 2010 as doi:10.3852/10-262

Short title: *Geomyces destructans*

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

Daniel L. Lindner

US Forest Service, Northern Research Station, Center for Forest Mycology Research, One Gifford Pinchot Drive, Madison, Wisconsin 53726

Andrea Gargas

Symbiology LLC, Middleton, Wisconsin 53562

Jeffrey M. Lorch

Molecular and Environmental Toxicology Center, University of Wisconsin at Madison, Medical Sciences Center, 1300 University Avenue, Madison, Wisconsin 53706 and US Geological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711

Mark T. Banik

Jessie Glaeser

US Forest Service, Northern Research Station, Center for Forest Mycology Research, One Gifford Pinchot Drive, Madison, Wisconsin 53726

Thomas H. Kunz

Center for Ecology and Conservation Biology, Department of Biology, Boston University, 5 Cummington Street, Boston, Massachusetts 02215

David S. Blehert[1]

US Geological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711

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

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.

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 1, 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 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*.

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 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 environments 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*.

ACKNOWLEDGMENTS

The authors thank Peter Youngbaer (NSS), Mike Warner (Speleobooks Inc.) and Alan Hicks (NY DEC) for their assistance in coordinating collection of samples and the many individuals who volunteered to collect samples for this project. We thank Kyah Norton (CFMR) for her assistance with PCR, cloning and sequencing of DNA and are indebted to Paul Cryan (USGS-FORT) for helpful suggestions during the preparation of this manuscript and for his assistance in creating FIG. 1. We also thank LeAnn White (USGS-NWHC) and two anonymous reviewers for their thoughtful comments on this manuscript. Financial support for this project was provided to DSB and THK by the National Speleological Society and to DSB by US Fish and Wildlife Service intergovernmental agreement 501819H057. Use of trade, product or firm names is for descriptive purposes only and does not imply endorsement by the US government.

LITERATURE CITED

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402.

Blehert DS, Hicks AC, Behr M, Meteyer CU, Berlowski-Zier BM, Buckles EL, Coleman JTH, Darling SR, Gargas A, Niver R, Okoniewski JC, Rudd RJ, Stone WB. 2009. Bat white-nose syndrome: an emerging fungal pathogen? Science 323:227.

Casadevall A. 2005. Fungal virulence, vertebrate endothermy and dinosaur extinction: Is there a connection? Fungal Genet Biol 42:98–106.

Chaturvedi V, Springer DJ, Behr MJ, Ramani R, Li X, Peck MK, Ren P, Bopp DJ, Wood B, Samsonoff WA, Butchkoski CM, Hicks AC, Stone WB, Rudd RJ, Chaturvedi S. 2010. Morphological and molecular characterizations of psychrophilic fungus *Geomyces destructans* from New York bats with white-nose syndrome (WNS). PLOSone 5:e10783.

Courtin F, Stone WB, Risatti G, Gilbert K, van Kruiningen HJ. 2010. Pathologic findings and liver elements in hibernating bats with white-nose syndrome. Vet Pathol 47:214–219.

Frick WF, Pollock JF, Hicks AC, Langwig KE, Reynolds DS, Turner GG, Butchkoski CM, Kunz TH. 2010. An emerging disease causes regional population collapse of a common North American bat species. Science 329:679–682.

Gargas A, DePriest PT, Taylor JW. 1995. Positions of multiple insertions in SSU rDNA of lichen-forming fungi. Mol Biol Evol 12:208–218.

———, Trest MT, Christensen M, Volk TJ, Blehert DS. 2009. *Geomyces destructans* sp. nov. associated with bat white-nose syndrome. Mycotaxon 108:147–154.

Jumpponen A. 2007. Soil fungal communities underneath willow canopies on a primary successional glacier forefront: rDNA sequence results can be affected by primer selection and chimeric data. Microb Ecol 53:233–246.

Lindner DL, Banik MT. 2009. Effects of cloning and root-tip size on observations of fungal ITS sequences from *Picea glauca* roots. Mycologia 101:157–165.

Lorch JM, Gargas A, Meteyer CU, Berlowski-Zier BM, Green DE, Shearn-Bochsler V, Thomas NJ, Blehert DS. 2010. A rapid diagnostic test for bat white-nose syndrome using PCR. J Vet Diagn Invest 22:224–230.

Meteyer CU, Buckles EL, Blehert DS, Hicks AC, Green DE, Shearn-Bochsler V, Thomas NJ, Gargas A, Behr MJ. 2009. Pathology criteria for confirming white-nose syndrome in bats. J Vet Diagn Invest 21:411–414.

Puechmaille SJ, Verdeyroux P, Fuller H, Ar Gouilh MM, Bekaert M, Teeling EC. 2010. White-nose syndrome fungus (*Geomyces destructans*) in bat, France. Emerg Infect Dis 16:290–293.

Simon UK, Weiss M. 2008. Intragenomic variation of fungal ribosomal genes is higher than previously thought. Mol Biol Evol 25:2251–2254.

Turner GG, Reeder DM. 2009. Update of white-nose syndrome in bats, September 2009. Bat Res News 50:47–53.

White TJ, Bruns TD, Lee SB, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, White T, eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press. p 315–321.

Wibbelt G, Kurth A, Hellmann D, Weishaar M, Barlow A, Veith M, Prüger J, Görföl T, Grosche T, Bontadina F, Zöphel U, Seidl H-P, Cryan PM, Blehert DS. 2010. White-nose syndrome fungus (*Geomyces destructans*) in bats, Europe. Emerg Infect Dis 16:1237–1242.

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion [doctoral thesis]. Austin: Univ. Texas Press.

LEGENDS

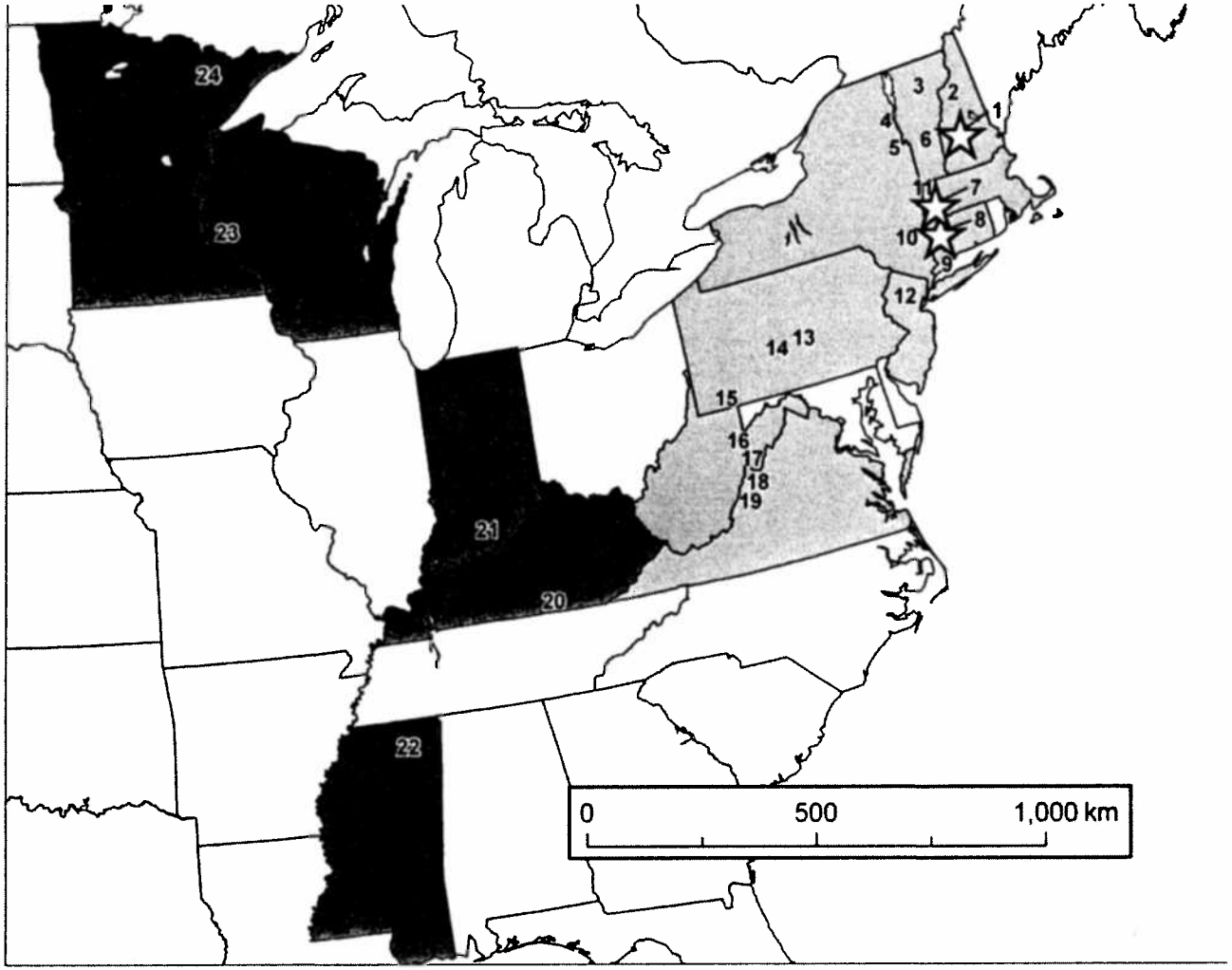
FIG. 1. White-nose syndrome (WNS) occurrence and environmental sample collection locations. States where WNS was documented when the samples were collected (winter 2008–2009) are light gray; states where the disease had not yet been identified are dark gray. Sample collection locations are designated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

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.

FOOTNOTES

Submitted 29 Aug 2010; accepted for publication 26 Sep 2010.

[1]Corresponding author. E-mail: dblehert@usgs.gov


24
23
21
20
22
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
0
500
1,000 km

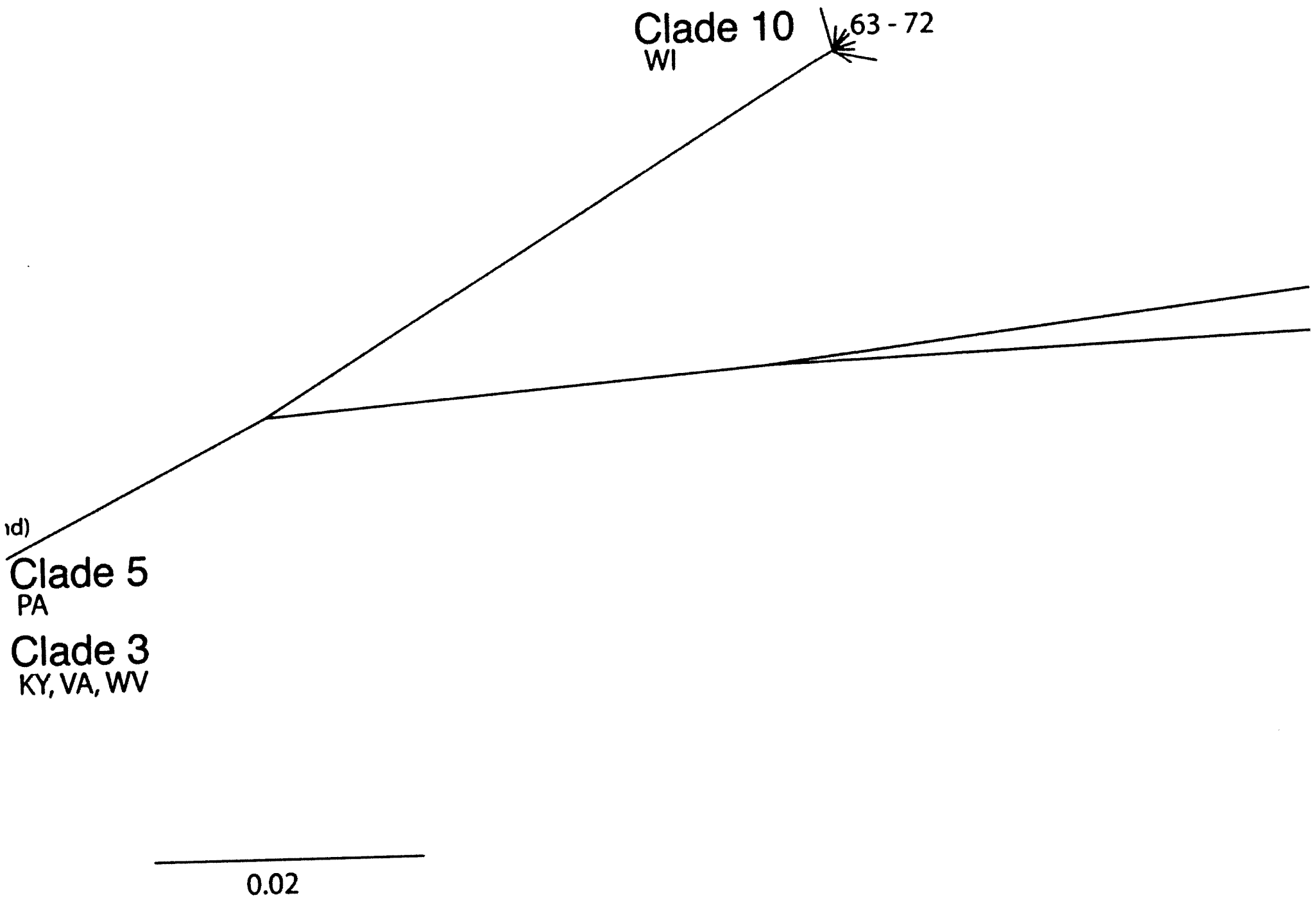

Clade 10
WI
63 - 72
id)
Clade 5
PA
Clade 3
KY, VA, WV
0.02

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.

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