In Press at Mycologia, preliminary version published on September 6, 2012 as doi:10.3852/12-242

Short title: Real-time PCR for *Geomyces destructans*

Bat white-nose syndrome: a real-time TaqMan polymerase chain reaction test targeting the intergenic spacer region of *Geomyces destructans*

Laura K. Muller

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

Jeffrey M. Lorch

Molecular and Environmental Toxicology Center, University of Wisconsin at Madison, Medical Sciences Center, 1300 University Avenue, Madison, Wisconsin 53706

Daniel L. Lindner[1]

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

Michael O'Connor[1]

Wisconsin Veterinary Diagnostic Laboratory, 445 Easterday Lane, Madison, Wisconsin 53706

Andrea Gargas

Symbiology LLC, Middleton, Wisconsin 53562 USA

David S. Blehert[2]

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

Copyright 2012 by The Mycological Society of America.

Abstract: The fungus *Geomyces destructans* is the causative agent of white-nose syndrome (WNS), a disease that has killed millions of North American hibernating bats. We describe a real-time TaqMan PCR test that detects DNA from *G. destructans* by targeting a portion of the multicopy intergenic spacer region of the rRNA gene complex. The test is highly sensitive, consistently detecting as little as 3.3 fg genomic DNA from *G. destructans*. The real-time PCR test specifically amplified genomic DNA from *G. destructans* but did not amplify target sequence from 54 closely related fungal isolates (including 43 *Geomyces* spp. isolates) associated with bats. The test was qualified further by analyzing DNA extracted from 91 bat wing skin samples, and PCR results matched histopathology findings. These data indicate the real-time TaqMan PCR method described herein is a sensitive, specific and rapid test to detect DNA from *G. destructans* and provides a valuable tool for WNS diagnostics and research.

Key words: diagnostic test, IGS, pathogen, PCR, wildlife disease

INTRODUCTION

White-nose syndrome (WNS) is an emergent disease causing unprecedented mortality in several species of North American hibernating bats (Turner et al. 2011). First photo-documented in 2006 at a cave in east central New York (Blehert et al. 2009), the disease has spread to 19 US States and four Canadian provinces (see http://www.fws.gov/whitenosesyndrome/maps/WNSMAP04-27-12_300dpi.jpg). Bat mortality has approached 100% at some affected hibernacula (Turner and Reeder 2009). Although insectivorous bats are often inconspicuous to humans, they provide natural pest-control services (Kunz et al. 2011) valued at approximately 22.9 billion dollars per year to the United States agricultural industry (Boyles et al. 2011).

The causative agent of WNS is the recently described psychrophilic fungus *Geomyces destructans* (Gargas et al. 2009, Lorch et al. 2011, Warnecke et al. 2012). The fungus grows on bats while they hibernate, initially colonizing the skin, then penetrating the epidermis of the wings, ears and muzzle (Meteyer et al. 2009). At present, the gold-standard for diagnosing WNS is identification of characteristic skin lesions by histopathology. However, this method is time-consuming, requires specialized training and for a thorough analysis a large amount of wing tissue (approximately 1.5 cm × 3.0 cm; Lorch et al. 2010) must be collected from each bat, either restricting analyses to dead animals or necessitating euthanasia.

PCR provides an alternative diagnostic tool to rapidly detect DNA from *G. destructans* in association with small amounts of bat skin (approximately 3 mm × 3 mm). Two PCR methods have been described (Lorch et al. 2010, Chaturvedi et al. 2011). The method developed by Lorch et al. (2010) targets the internal transcribed spacer (ITS) region of the rRNA gene complex using conventional PCR technology. However, because of the recently discovered diversity of other fungi closely related to *G. destructans* in bat hibernacula (Lindner et al. 2011, Lorch et al. unpubl), this method is best conducted in conjunction with sequencing of PCR product when analyzing diagnostic samples from bats, and it cannot be used to specifically detect DNA from *G. destructans* in environmental samples without cloning and sequencing. A real-time TaqMan PCR that targets the alpha-L-rhamnosidase gene (hereafter referred to as the ALR test) was developed by Chaturvedi et al. (2011). Although the ALR test has been screened against 16 isolates of *Geomyces* and related teleomorphs, the relationships of these fungal isolates to *G. destructans* has not been phylogenetically defined. Thus, specificity of the ALR test for use with environmental samples containing *Geomyces* spp. closely related to *G. destructans* (Lindner et al. 2011, Lorch et al. unpubl) remains unknown. In addition, the ALR test targets a gene that

likely exists only as a single copy, which may reduce sensitivity compared to a PCR test targeting a multicopy gene region.

The goal of this study was to develop a highly specific real-time TaqMan PCR test with greater sensitivity than existing tests to detect DNA from *G. destructans*. The intergenic spacer (IGS) region of the rRNA gene complex was chosen as the target for this test due to its high copy number and its interspecific sequence variability compared to other portions of this gene region (Jackson et al. 1999).

MATERIALS AND METHODS

Fungal culture and DNA extraction.—Fungal isolates were grown as pure cultures on Sabouraud dextrose medium (BD Diagnostic Systems, Sparks, Maryland) incubated at 7 C. Genomic DNA (gDNA) was extracted from fungal isolates with three methods. To produce high purity gDNA from the type isolate of *G. destructans* (American Type Culture Collection number ATCC MYA-4855) for generation of standard curves, fungal biomass (approximately 800 mg) grown in liquid Sabouraud dextrose medium (BD Diagnostic Systems) was washed twice with 1 mL sterile deionized water, then ground approximately 10 min in liquid nitrogen to yield a fine powder. Genomic DNA extraction was initiated with a commercial kit (Gentra® Puregene® Genomic DNA Purification Kit, QIAGEN Inc., Valencia, California) according to the manufacturer's instructions for cell lysis, proteinase K digestion, and RNase treatment steps except the amount of proteinase K was increased to 20 mg/mL (final concentration). Genomic DNA from the resulting lysate was further purified with a second kit (OmniPrep™ for Fungi, G-Biosciences, Maryland Height, Missouri) following the manufacturer's instructions beginning at the chloroform extraction step. Concentration of type isolate gDNA was measured with the Quant-iT™ High-Sensitivity DNA Assay Kit (Invitrogen, Carlsbad, California). Genomic DNA was extracted from nine additional isolates of *G. destructans* (for PCR and DNA sequencing applications) using the OmniPrep™ for Fungi kit (G-Biosciences) according to the manufacturer's instructions. Genomic DNA also was extracted from a group of near-neighbor fungi consisting of 54 isolates (TABLE I), including 40 identified as congeneric with *G. destructans* based on analysis of ITS sequences by Lorch et al. (unpubl). An additional three isolates, cultured according to the methods of Lorch et al. (2010) and identified as *Geomyces* spp. based on ITS sequence analysis, also were included. These three isolates were cultured from animals collected in 2010, including a tricolored bat (*Perimyotis subflavus*) from Tennessee, a silver-haired bat

(*Lasionycteris noctivagans*) from Tennessee and a tricolored bat from Wisconsin. Genomic DNA extractions for these 54 isolates were conducted with the protocol of Lindner and Banik (2009) with reagent volumes modified proportionally for use with eight-well 0.2 mL PCR strip tubes as described by Lorch et al. (unpubl).

IGS analysis, primers and probe.—Complete IGS region was amplified by PCR of gDNA extracted from the type isolate of *G*. *destructans*. Amplification primers (synthesized by the University of Wisconsin Biotechnology Center, Madison, Wisconsin) were complementary to the 3′– end of the large subunit (LSU) rRNA gene (CNL12: 5′– CTG AAC GCC TCT AAG TCA G–3′; Anderson and Stasovski 1992) and the 5′– end of the small subunit (SSU) rRNA gene (CNS1: 5′– GAG ACA AGC ATA TGA CTA CTG –′; White et al. 1990). Polymerase chain reaction was conducted per the manufacturer's instructions with TaKaRa ExTaq proofreading DNA polymerase (Clontech Laboratories Inc., Madison, Wisconsin). Reactions included 1 μL gDNA in a volume of 50 μL. Cycling conditions consisted of an initial denaturation at 98 C for 2 min, followed by 35 cycles of 98 C for 10 s, 50 C for 30 s, and 72 C for 4 min, with a final extension at 72 C for 7 min. Full-length amplification product (2793 nt) was directly sequenced in both directions with a primer-walking strategy. Sequencing reactions were run by the University of Wisconsin Biotechnology Center with the BigDye Terminator® 3.1 system (Applied Biosystems, Foster City, California), and reaction products were analyzed with a 3730xl DNA Analyzer (Applied Biosystems). Complementary strand sequencing reaction results were assembled and edited with SeqMan Pro 9.0.5 (DNASTAR, Madison, Wisconsin). The IGS region sequence with flanking nucleotides from the type isolate of *G*. *destructans* is deposited in GenBank (accession No. JX415267).

The IGS region of the type isolate of *G*. *destructans* was aligned with IGS region sequences from 34 fungal isolates previously identified as close relatives of *G*. *destructans* based on analysis of their ITS sequences (TABLE I; Lorch et al. unpubl). All IGS nucleotide positions were numbered starting with position 1 following the 3′– terminal nucleotide of the LSU. A variable region spanning nucleotides 114–310 was identified within the IGS, and specific primers and probe were developed with the sequence generated from the type isolate of *G*. *destructans* (FIG. 1). This region was analyzed with commercial software (Primer Express 3.0, Applied Biosystems), and the following primers and probe were synthesized by commercial laboratories (University of Wisconsin Biotechnology Center and Biosearch Technologies Inc., Novato, California, respectively). Forward primer nu-IGS-0169-5′–Gd: 5′– TGC CTC TCC GCC ATT AGT G –3′; reverse primer nu-IGS-0235-3′–Gd: 5′– ACC ACC GGC TCG CTA GGT A –3′; and probe nu-IGS-0182/0204-Gd: 5′– (FAM) CGT TAC AGC TTG CTC GGG CTG CC (BHQ-1) –3′. To ensure

the nucleotide region targeted for primer and probe development did not exhibit intraspecies variability, sequence of the 5′– end of the IGS region (622 nt) was determined for nine additional isolates of *G*. *destructans* cultured from the skin of bats collected in Connecticut, Massachusetts, New Jersey, New York, Pennsylvania, Vermont, Virginia and West Virginia with primers nu-IGS-0033-5′–Gd: 5′– TCT CCC ATT AAC TTG CAG GCT AG –3′; and nu-IGS-0613-3′–Gd: 5′– AGT GCC TCT CGC CCT AGA AC –3′. The portion of the IGS regions sequenced for all isolates of *G*. *destructans* were identical and did not match any other sequences in GenBank, including those of near-neighbor isolates described by Lorch et al. (unpubl).

Development of the real-time IGS PCR test.—Real-time IGS PCR (hereafter referred to as the IGS test) was conducted using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems) and commercial master mix (QuantiFast™ Probe PCR+ROX Vial Kit, QIAGEN Inc.) according to the manufacturer's instructions. Standard laboratory practices to avoid cross contamination of samples (e.g. unidirectional workflow) were followed. Each 25 µL PCR reaction contained 12.5 µL 2× master mix, 0.5 µL 50× ROX dye solution, 0.5 µL of each 20 µM PCR primer solution, 0.25 µL 20 µM dual-labeled BHQ probe and 5 µL template DNA. A positive control of gDNA from *G*. *destructans* and a no-template control were included in each 96-well assay plate. PCR cycling conditions included an initial Taq polymerase activation step of 95 C for 3 min followed by 40 cycles of 95 C for 3 s and 60 C for 30 s. For the purpose of standardization between assays (i.e. IGS vs. ALR), plate runs and sample types (as background fluorescence levels occasionally differed), the threshold baseline was set to 10% of the maximum fluorescence as determined by positive control samples (King and Guidry 2004). Any reaction that crossed the threshold baseline within 40 cycles was considered positive.

Tenfold serial dilutions 3.3–3.3 ag gDNA from the type isolate of *G*. *destructans* were used as template to generate a standard curve. Each dilution of gDNA was loaded in triplicate into a 96-well assay plate and amplified by the IGS test. The lowest concentration of gDNA template that was detected in three out of three replicates (for which the replicates did not vary by greater than one C_t value) was identified as the lower detection limit.

To determine specificity of the real-time IGS PCR test, a near-neighbor panel consisting of purified gDNA template from fungal isolates cultured from bats or from soil collected within bat hibernacula (TABLE I; Lorch et al. unpubl) was diluted tenfold and assembled in a 96-well assay plate. Triplicate positive and negative controls containing gDNA from *G*. *destructans* and no template respectively also were included. To rule out the possibility of PCR inhibition, duplicate samples of three representative gDNA extracts prepared according to Lindner and

Banik (2009) were spiked with 33 pg gDNA from the type isolate of *G. destructans* and amplified by the IGS test. Spiked samples within one C_t value of the average C_t of identical samples lacking added gDNA were identified as non-inhibitory.

Real-time IGS PCR analysis of bat skin samples were compared to histological analyses conducted previously with the same skin samples from bats submitted to the U.S. Geological Survey National Wildlife Health Center for diagnostic testing 2009–2011. Ninety-one bats of 12 species from 57 collection events in 19 states (Alabama, California, Connecticut, Florida, Indiana, Maryland, Maine, Missouri, New Jersey, New Mexico, New York, Ohio, Pennsylvania, Tennessee, Texas, Virginia, Vermont, Wisconsin, and Virginia), both within and outside the known range of WNS at the time of collection, were analyzed. Of these 91 samples, 42 were previously identified as WNS negative and 49 were WNS positive by histology. All tissues were stored frozen at either −20 C or −80 C until extracted with the Gentra® Puregene® Genomic DNA Purification Kit (QIAGEN Inc.) as described by Lorch et al. (2010), and the extracted gDNA was diluted 100-fold. To rule out the possibility of PCR inhibition, duplicate aliquots of gDNA from WNS negative skin samples each were spiked with 33 pg gDNA from the type isolate of *G. destructans* and amplified by the IGS test. Spiked histology-negative skin samples within one C_t value of the average C_t of the positive controls were identified as non-inhibitory. Triplicate positive control reactions each containing the same amount of gDNA from *G. destructans* also were amplified.

Comparison of IGS and ALR PCR tests.—A direct comparison of the sensitivity and specificity of the ALR (Chaturvedi et al. 2011) and IGS PCR tests was conducted. Primers for the ALR test were as described by Chaturvedi et al. (2011), and the probe was synthesized by a commercial laboratory (Biosearch Technologies Inc.) as follows: 5′– (FAM) TTC GGC GGC CAG CCG CG (BHQ-1) –3′. Reagents, cycling conditions and analyses for the ALR PCR were otherwise as described above. The ALR test sensitivity was compared to that of the IGS test by simultaneously generating two concentration curves in the same 96-well assay plate. The two concentration curves each were prepared in triplicate by serially diluting gDNA from the type isolate, as described above, and each set of serially diluted gDNA templates was amplified with the appropriate primer/probe combination (IGS or ALR) together in the same 96-well assay plate. Because the maximum fluorescence of the ALR assay was lower than that of the IGS assay, the threshold baseline was manually set at 10% of the maximum fluorescence for each respective assay.

The specificity of the ALR test was assessed with DNA templates from the near-neighbor panel as described above. A subset of the diagnostic samples described above also was used to further assess the sensitivity and specificity of the ALR test. These samples included wing skin from 22 bats diagnosed as WNS negative by histopathology and 35 bats diagnosed WNS positive by histopathology. As with all PCR runs, both positive and negative controls were included.

RESULTS

Standard curve.—A standard curve was prepared by PCR amplifying triplicate serial dilutions (3.3 ng–3.3 fg) gDNA from *G. destructans* (FIG. 2). The assay was consistently linear over seven logs with an R^2 value greater than 0.99. Amplification efficiency was calculated as 104.86% (efficiency = $-1 + 10^{(-1/\text{slope})}$). Product also was amplified at the 10^{-7} template dilution (330 ag) but was not included in the standard curve because it was detected only in one of three replicate samples. Because the 10^{-6} template dilution (3.3 fg) was the lowest concentration yielding amplification for all three replicates, 3.3 fg gDNA (per 25 µL reaction) was identified as the lower limit of detection.

Near-neighbor screen.—Target DNA was not amplified with the IGS PCR test from any of the 54 fungal isolates designated as near neighbors to *G. destructans*. Positive and negative controls in this screen performed as expected. Duplicates of three of the samples containing fungal gDNA were spiked with gDNA from *G. destructans* to test for PCR inhibition. Spiked samples varied by < 1 C_t value from identically prepared positive control samples that lacked near-neighbor fungal gDNA, indicating that inhibition did not occur.

Diagnostic screen.—Genomic DNA extracts from 42 wing skin samples previously diagnosed as WNS negative by histopathology all were confirmed negative by the real-time IGS PCR test. Genomic DNA extracts from 49 wing skin samples previously diagnosed WNS positive by histopathology all were positive by the real-time IGS PCR test. To test for inhibition, gDNA

extracts from the 42 negative samples were spiked with gDNA from *G. destructans* and retested by real-time IGS PCR. PCR results for spiked diagnostic samples differed by < 1 C_t value from identically prepared positive control samples that lacked wing skin DNA, indicating that inhibition did not occur.

Test comparison.—Sensitivity and specificity of the IGS and ALR (Chaturvedi et al. 2011) tests were compared with the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Using purified gDNA as template, the ALR test consistently detected 330 fg gDNA in all three replicates at its lower detection limit while the IGS test consistently detected 3.3 fg gDNA in all three replicates at its lower detection limit. When amplifying replicate gDNA samples, C_t values for the ALR test were on average 7.8 cycles higher than those of the IGS test. When amplifying gDNA extracts from diagnostic samples, both tests responded equivalently with samples from bats determined to be WNS negative by histopathology (no amplification). However, compared to the IGS test, C_t values were higher in all instances when using the ALR test when amplifying diagnostic samples from bats determined to be WNS positive; C_t values for the ALR assay were on average 7.1 cycles higher than those resulting for the IGS assay. In addition, the ALR test yielded false-negative results for extractions from seven of 35 (20%) diagnostic samples determined to be positive for WNS by histopathology. Despite lower sensitivity, the ALR test was equally specific as the IGS test based upon analysis of gDNA extracts from near-neighbor fungi; no amplification of gDNA from the near neighbor panel was detected.

DISCUSSION

As WNS continues to spread across North America, development of enhanced diagnostic tests to detect the causative agent, *G. destructans*, improves the ability to conduct disease surveillance and provides researchers with needed laboratory tools. We developed a specific and sensitive

real-time TaqMan PCR test (the IGS test) to detect DNA from *G. destructans* by targeting the multicopy IGS region of the rRNA gene complex. The IGS test proved to be specific, agreeing with histopathologic diagnoses for 91 of 91 previously analyzed bat skin samples and appropriately discriminating target DNA from a panel of 54 closely related fungal isolates. In addition, the IGS test was approximately 100-fold more sensitive than a previously published real-time TaqMan PCR test (the ALR test; Chaturvedi et al. 2011).

The IGS region of the rRNA gene complex has been shown to be a useful target for development of PCR tests suitable for identifying fungal species/subspecies (Radford et al. 1998, Williamson et al. 2000, Suarez et al. 2005, López-Flores et al. 2008). Because the IGS region is not transcribed, there may be less selective pressure for conservation of nucleotide sequence yielding high interspecific variability (Hillis, et al. 1991, Jackson et al. 1999). In addition, as a high-copy DNA target (approximately 100–400 copies per fungal genome [Boyle et al. 2004]), IGS region offers greater PCR sensitivity compared to single or low copy-number genes. Furthermore, should spontaneous mutation or intraspecific sequence variation occur within a gene, multicopy PCR primer/probe binding sites are less likely to lose primer/probe specificity than are single-copy DNA targets.

To verify suitability of the IGS region as a PCR target for the detection of *G. destructans*, IGS regions from 10 isolates of *G. destructans* were PCR amplified. The 5′– end (622 nt) of all resulting amplification products were directly sequenced and determined to be identical. Subsequently equivalent partial IGS sequences of two fungal isolates cultured from bats and 32 isolates cultured from soil collected in bat hibernacula and identified as close relatives of *G. destructans* based on analysis of their ITS sequences (TABLE I; Lorch et al. unpubl) were compared. This comparison revealed that, although these isolates exhibited low ITS region

variability, there was high variability among analyzed portions of their IGS regions. Based upon this analysis, a variable region within the IGS, downstream of the LSU of the rRNA gene region, was identified for development of primers and probe (FIG. 1).

The lower limit of detection of the IGS test was 3.3 fg of gDNA per 25 μL reaction, or approximately 0.1 genome equivalents (based on an estimated size of 30.65 Mb for the genome of *G. destructans* [see http://www.broadinstitute.org/annotation/genome/Geomyces_destructans/GenomeStats.html] and the assumption that an average base pair is 650 Daltons). In our laboratory the lower limit of detection of the ALR test was 330 fg gDNA per 25 μL reaction, or approximately 10 genome equivalents. This was higher than the detection limit of two conidia (presumably two genome equivalents) per real-time PCR reaction reported for the ALR test by Chaturvedi et al. (2011). This disparity could result from differences in the platforms used to conduct the real-time PCR or from discrepancies between using viable conidia as compared to purified gDNA to determine the lower limit of detection. Nonetheless, when compared with standardized methods the IGS test was approximately 100 times more sensitive than the ALR test. This difference in sensitivity most likely results from differences in copy numbers of the DNA targets for each PCR test. Specifically, the IGS test targets the multicopy IGS region, while the ALR test targets the alpha-L-rhamnosidase gene, which presumably exists only as a single copy per genome.

The ALR test (Chaturvedi et al. 2011) was specific when assessed against gDNA from the near-neighbor panel. However, the ALR test failed to detect DNA from *G. destructans* in 20 percent of diagnostic samples previously determined to be positive for WNS by histopathology. In contrast, results of the IGS test for the same samples agreed with the previous histopathology findings. In addition, for the ALR test samples consistently crossed the threshold baseline

approximately seven cycles (approximately two orders of magnitude) behind those of the IGS test. The observed reduced sensitivity of the ALR test compared to the IGS test when analyzing diagnostic samples was consistent with measured differences in lower limits of detection for both tests determined through concentration curve analyses.

Our analyses demonstrate that the real-time TaqMan PCR method described herein (the IGS test) provides a sensitive, specific and rapid tool for detecting DNA from *G. destructans*. This test thus provides an enhanced tool for both WNS diagnostic investigations and research. Furthermore, by demonstrating the IGS test did not amplify target DNA from closely related fungal isolates cultured from soil collected in bat hibernacula, we show this test likely has specificity sufficient for the analysis of environmental samples.

ACKNOWLEDGMENTS

We thank A. Minnis (U.S. Forest Service) for assistance with phylogenetic analyses. We acknowledge technical assistance provided by B. Berlowski-Zier (U.S. Geological Survey) and M. Banik (U.S. Forest Service). D.S.B. was financially supported by the U.S. Geological Survey and D.L.L. by the U.S. Forest Service. Use of trade, product or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

LITERATURE CITED

Anderson JB, Stasovski E. 1992. Molecular phylogeny of northern hemisphere species of *Armillaria*. Mycologia 84:505–516.

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.

Boyle DG, Boyle DB, Olsen V, Morgan JA, Hyatt AD. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time TaqMan PCR assay. Dis Aquat Organ 60:141-148.

Boyles JG, Cryan PM, McCracken GF, Kunz TH. 2011. Economic importance of bats in agriculture. Science 332:41-42.

Chaturvedi S, Rudd RJ, Davis A, Victor TR, Li X, Appler KA, Rajkumar SS, Chaturvedi V. 2011. Rapid real-time PCR assay for culture and tissue identification of *Geomyces destructans* : the etiologic agent of bat geomycosis (white-nose syndrome). Mycopathologia 172:247–256.

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

Hillis DM, Dixon MT. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q Rev Biol 66:411-453.

Jackson CJ, Barton RC, Evans EGV. 1999. Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J Clin Microbiol 37:931–936.

King JL, Guidry C. 2004. Müller cell production of insulin-like growth factor-binding proteins in vitro: modulation with phenotype and growth factor stimulation. Invest Ophthalmol Vis Sci 45:4535–4542.

Kunz TH, Braun de Torrez EC, Bauer DM, Lobova TA, Fleming TH. 2011. Ecosystem services provided by bats. In: Ostfeld RS, Schlesinger WH, eds. The year in ecology and conservation (Annals of the New York Academy of Sciences). New York: Wiley-Blackwell. p 1–38.

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.

———, Gargas A, Lorch JM, Banik MT, Glaeser J, Kunz TH, Blehert DS. 2011. DNA-based detection of the fungal pathogen *Geomyces destructans* in soils from bat hibernacula. Mycologia 103:241–246.

López-Flores I, Robles F, Vlencia JM, Grau A, Villalba A, de la Herrán R, Garrido-Ramos MA, Ruiz-Rejón C, Ruiz-Rejón M, Navas JI. 2008. Detection of *Marteilia refringens* using nested PCR and in situ hybridization in *Chamelea gallina* from the Balearic Islands (Spain). Dis Aquat Organ 82:79–87.

Lorch JM, Gargas A, Meteyer CU, Berlowski-Zier BM, Green DE, Shearn-Bochsler V, Thomas NJ, Blehert DS. 2010. Rapid polymerase chain reaction diagnosis of white-nose syndrome in bats. J Vet Diagn Invest 22:224–230.

———, Meteyer CU, Behr MJ, Boyles JG, Cryan PM, Hicks AC, Ballmann AE, Coleman JTH, Redell D, Reeder DM, Blehert DS. 2011. Experimental infection of bats with *Geomyces destructans* cause white-nose syndrome. Nature 480:376–378.

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

Radford SA, Johnson EM, Leeming JP, Millar MR, Cornish JM, Foot ABM, Warnock DW. 1998. Molecular epidemiological study of *Aspergillus fumigatus* in a bone marrow transplantation unit by PCR amplification of ribosomal intergenic spacer sequences. J Clin Microbiol 36:1294–1299.

Suarez MB, Walsh K, Boonham N, O'Neill T, Pearson S, Barker I. 2005. Development of real-time PCR (TaqMan) assays for the detection and quantification of *Botrytis cinerea* in planta. Plant Physiol Biochem 43:890–899.

Turner GG, Reeder DM, Coleman JTH. 2011. A five-year assessment of mortality and geographic spread of white-nose syndrome in North American bats and a look to the future. Bat Res News 52:13–27.

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

Warnecke L, Turner JM, Bollinger TK, Lorch JM, Misra V, Cryan PM, Wibbelt G, Blehert DS, Willis CKR. 2012. Inoculation of bats with European *Geomyces destructans* supports the novel pathogen hypothesis for the origin of white-nose syndrome. Proc Natl Acad Sci USA. 109:6999–7003.

White TJ, Bruns T, Lee S, Taylor J. 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 315–322.

Williamson EC, Speers D, Arthur IH, Harnett G, Ryan G, Inglis TJ. 2001. Molecular epidemiology of *Scedosporium apiospermum* infection determined by PCR amplification of ribosomal intergenic spacer sequences in patients with chronic lung disease. J Clin Microbiol 39:47–50.

LEGENDS

FIG. 1. Schematic representation of a portion of the rRNA gene complex of *Geomyces destructans*, illustrating the location of the intergenic spacer (IGS) region that was targeted for development of the real-time TaqMan polymerase chain reaction test. The expanded section shows the nucleotide sequence of the 103 base pair portion of the IGS region amplified by the assay, including locations of the *G. destructans*-specific primers and probe.

FIG. 2. Standard curve for amplification and detection of the rRNA gene intergenic spacer region from genomic DNA purified from *Geomyces destructans*. All points represent the mean C_t value of amplification reactions conducted in triplicate with error bars denoting standard deviation. The assay was consistently linear over seven logs 3.3 fg–3.3 ng genomic DNA ($R^2 = 0.9995$).

FOOTNOTES

Submitted 27 Jun 2012; accepted for publication 30 Jul 2012.

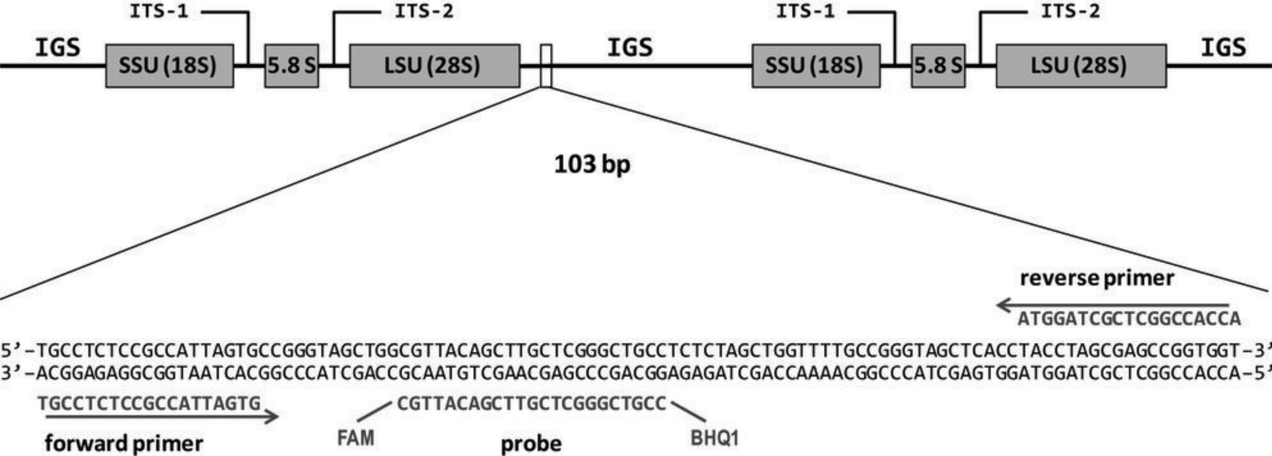
[1]D.L.L. and M.O. contributed equally to this work.

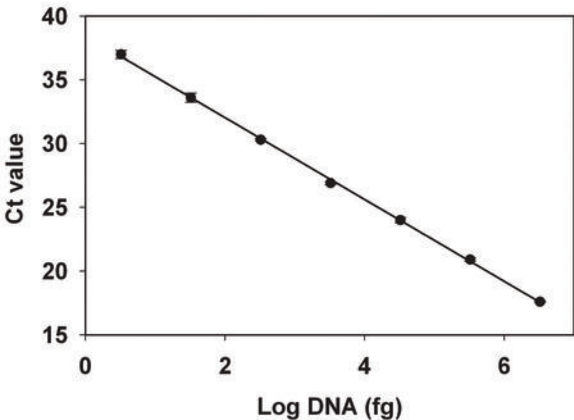
[2]Corresponding author. E-mail: dblehert@usgs.gov

TABLE I. GenBank accession numbers of the rRNA gene internal transcribed spacer (ITS) region and if applicable intergenic spacer (IGS) region of close relatives of *Geomyces* isolated from caves and mines (Lorch et al. unpubl) or from bats (fungal isolates 22984-1-I1, 23014-1-I2, 23342-1-I1) that were used for development and specificity testing of the real-time TaqMan polymerase chain reaction assay. All isolates, including those for which the IGS region was not sequenced, were screened with the IGS test to assess its specificity.

		GenBank accession numbers	
Fungal isolate	Genus	ITS	IGS
01NH06	*Geomyces*	JX270341	–
01NH08	*Geomyces*	JX270343	–
02NH08	*Geomyces*	JX270353	JX270196
02NH11	*Geomyces*	JX270356	–
02NH14	*Geomyces*	JX270359	JX270198
04NY11	*Geomyces*	JX270375	–
05NY05	*Geomyces*	JX270384	–
06VT05	*Geomyces*	JX270393	JX270206
06VT12	*Geomyces*	JX270400	JX270210
07MA07	*Geomyces*	JX270407	JX270215
07MA08	*Geomyces*	JX270408	JX270216
10NY10	*Geomyces*	JX270434	–
11MA03	*Geomyces*	JX270438	JX270233
11MA07	*Geomyces*	JX270442	JX270236
12NJ08	*Geomyces*	JX270454	JX270243
12NJ10	*Geomyces*	JX270456	–
12NJ13	*Geomyces*	JX270459	JX270246
13PA01	*Geomyces*	JX270462	–
14PA05	*Geomyces*	JX270468	–
15PA02	*Geomyces*	JX270477	JX270252
15PA05	*Geomyces*	JX270480	–
15PA10A	*Geomyces*	JX270485	JX270256
17WV03	*Geomyces*	JX270510	JX270269
17WV05	*Geomyces*	JX270512	JX270271
18VA08	*Geomyces*	JX270528	–
18VA13	*Geomyces*	JX270533	JX270279
18VA15	*Geomyces*	JX270535	–
18VA16	*Geomyces*	JX270536	JX270281
20KY12	*Geomyces*	JX270565	JX270296
21IN06	*Geomyces*	JX270573	JX270301
21IN10	*Geomyces*	JX270577	JX270303
23WI05	*Geomyces*	JX270595	–
23WI06	*Geomyces*	JX270596	JX270315

23WI08	*Geomyces*	JX270598	JX270316
24MN04	*Geomyces*	JX270612	JX270322
24MN06	*Geomyces*	JX270614	JX270323
24MN09	*Geomyces*	JX270617	JX270325
24MN13	*Geomyces*	JX270621	JX270328
24MN18	*Geomyces*	JX270626	JX270331
24MN28	*Geomyces*	JX270628	JX270333
11MA09	*Mycoarthris*	JX270444	–
07MA11	*Oidiodendron*	JX270411	JX270219
15PA18	*Oidiodendron*	JX270494	–
18VA18	*Oidiodendron*	JX270538	–
18VA21	*Oidiodendron*	JX270541	JX270283
23WI01A	*Oidiodendron*	JX270591	JX270312
23WI17	*Oidiodendron*	JX270607	JX270320
23WI18	*Oidiodendron*	JX270608	JX270321
01NH05	*Pseudeurotium*	JX270340	–
02NH04	*Pseudeurotium*	JX270349	–
15PA01	*Pseudeurotium*	JX270476	–
22984-1-I1	*Geomyces*	JX415262	-
23014-1-I2	*Geomyces*	JX415263	JX415265
23342-1-I1	*Geomyces*	JX415264	JX415266


ITS-1
ITS-2
ITS-1
ITS-2
IGS
SSU (18S)
5.8 S
LSU (28S)
IGS
SSU (18S)
5.8 S
LSU (28S)
IGS
103 bp
reverse primer
ATGGATCGCTCGGCCACCA
5'-TGCCTCTCCGCCATTAGTGCCGGGTAGCTGGCGTTACAGCTTGCTCGGGCTGCCTCTCTAGCTGGTTTTGCCGGGTAGCTCACCTACCTAGCGAGCCGGTGGT-3'
3'-ACGGAGAGGCGGTAATCACGGCCCATCGACCGCAATGTCGAACGAGCCCGACGGAGAGATCGACCAAAACGGCCCATCGAGTGGATGGATCGCTCGGCCACCA-5'
TGCCTCTCCGCCATTAGTG
CGTTACAGCTTGCTCGGGCTGCC
forward primer
FAM
probe
BHQ1


40
35
30
25
20
15
0
2
4
6
Ct value
Log DNA (fg)