
FINAL REPORT

to

U.S. Department of the Interior, National Park Service

**APPALACHIAN HIGHLANDS NETWORK RESEARCH GRANT:
Prevalence and potential threat of an emerging disease to amphibians
in the Appalachian Highlands**

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INTRODUCTION

The 40 species of amphibians inhabiting Great Smoky Mountains National Park comprise a diverse and globally significant biological resource (Dodd 2004). Because of their complex life cycles, limited mobility, and often restricted geographic ranges, amphibians are sensitive to stressors like habitat alteration, climate change, and disease. There are ample reasons to be concerned about the well-being of amphibian populations in this region, given expected changes in environmental conditions with climate change (Dodd 1997, Corser 2001) and reports of population declines (Highton 2005). Furthermore, amphibians in the Mesoamerican highlands are experiencing severe declines following outbreaks of a disease caused by the pathogenic fungus, *Batrachochytrium dendrobatidis* (Lips et al. 2003). This unusual organism in the phylum Chytridiomycota is the only member of that group known to parasitize vertebrates (Berger et al. 1998). *B. dendrobatidis* infects keratinized tissue in the mouthparts of larval amphibians and the skin of adults. Lethality probably results from interference with water and ion exchange through the skin of the amphibian. Chytrid fungi generally occur in moist habitats and *B. dendrobatidis* cannot survive high temperatures ($> 30^{\circ}\text{C}$; Longcore et al. 1999), which supports the idea of chytridiomycosis as a contributor to the decline of amphibian populations in high-elevation sites. The origin of this pathogen is unclear, although molecular genetic evidence suggests a fairly recent, sudden range expansion, perhaps facilitated by human transport or introduction of a carrier organism (Morehouse et al. 2003, Rachowicz et al. 2005).

Information on the relative susceptibility of different amphibian taxa is lacking. In the southeastern U.S., chytrid infection has been documented in the families Ranidae, Hylidae, and Salamandridae (Daszak et al. 2005, Rothermel et al., in review). Despite its prevalence, only one or two severe mortality events due to chytrid have been reported. Bullfrogs (*Rana catesbeiana*)

seem to be resistant to the disease, and could act as carriers (Daszak et al. 2004, Garner et al. 2006). Worldwide, there have been only a handful of reports of chytridiomycosis in plethodontid salamanders in the wild. Cummer et al. (2005) found a single infected individual of the entirely terrestrial Jemez Mountains salamander (*Plethodon neomexicanus*). More recently, Lauer et al. (2007) have detected *B. dendrobatidis* in a population of red-backed salamanders (*Plethodon cinereus*) in Virginia. Other researchers have collected morbid salamanders during mass mortality events in Mexico and Panama (Lips et al. 2003, Parra-Olea et al. 2005).

From a conservation standpoint, it is critical to determine how much of a threat this pathogen may pose to the many endemic species of amphibians in the Southern Appalachians. Ultimately, we need to understand which species are susceptible to infection and under what environmental conditions disease outbreaks might occur. As an initial step toward that end, we tested both aquatic-breeding and terrestrial amphibians for chytrid infection and sampled populations in contrasting habitats within the park. We hypothesized that chytrid is most likely to be found at lower elevations, because the cold temperatures at high elevations may limit fungal growth. In the laboratory, *B. dendrobatidis* grows best at temperatures between 17°C and 25°C (Piotrowski et al. 2004). Furthermore, we expected chytrid infections to be more prevalent in aquatic-breeding species which are known to be susceptible to chytrid infection, such as ranid frogs and newts (*Notophthalmus viridescens*).

METHODS

We conducted field surveys for amphibians in Great Smoky Mountains National Park and used relatively new, sensitive molecular genetic techniques to detect presence/absence of *B. dendrobatidis* (hereafter “*Bd*”) in the samples. Our goal was to maximize the likelihood of detecting chytrid if it is present in amphibian populations in the park. Based on our current knowledge of the fungus, we optimized our chances of finding positive samples by sampling amphibians in late spring and early summer (Kriger & Hero 2006). Because we were interested in the potential susceptibility of all amphibian taxa, we sampled both aquatic-breeding (e.g., *Ambystoma*, *Notophthalmus*, *Bufo*, *Rana*) and terrestrial (e.g., *Plethodon*) species. The sample collection and processing methods differed between these two groups of amphibians and depended on life stage, as described below. All captured amphibians were examined in the field for signs of disease, including other fungal infections or iridoviruses; iridovirus outbreaks have occurred at Gourley Pond in Cades Cove (Dodd 2004). Throughout the surveys, we followed appropriate protocols for preventing the spread of infectious agents, including disinfecting all nets, boots, and minnow traps with bleach solution prior to entering the park and at the conclusion of each sampling visit.

Pond-breeding Amphibians

Sample Collection – Personnel from Austin Peay State University visited Great Smoky Mountains National Park on two occasions in spring 2007 (April 13-15 and May 15-17) to collect samples for this study. We concentrated our collecting efforts in the area around Cades Cove, where most of the park’s pond habitat occurs. We sampled 10 sites and collected more than 500 samples of larval and post-metamorphic pond-breeding amphibians, as well as 15 samples of plethodontid salamanders (Appendix). The plethodontid samples were sent to Matthew Chatfield at the University of Michigan to ensure that all plethodontid salamanders were analyzed using the same genetic assay techniques (see below).

Because of unusually dry conditions in spring 2007, Gourley Pond and Gum Swamp were completely dry and Finley Cane Sinkhole #2 was reduced to a small puddle. At sites that were holding water, we collected larvae using dipnets and minnow traps. The larvae were euthanized by immersion in a concentrated solution (i.e., overdose) of MS-222, which does not destroy *Bd* (Webb et al. 2005). Euthanized larvae were then preserved in 70% ethanol for subsequent genetic assays. Very small tadpoles were preserved in individual 2-ml vials, whereas many of the larger tadpoles were preserved in specimen jars containing multiple individuals of the same species. The latter obviously creates an opportunity for cross-contamination (i.e., zoospores from one infected individual spreading to others in the same container) and necessitates treating these as pooled samples. Thus, some groups of larvae collected from the same pond on the same date were not kept separate and were treated as pooled samples in subsequent analyses.

Post-metamorphic amphibians were captured by searching under cover objects in the terrestrial habitat adjacent to ponds and streams, or by driving the Cades Cove Loop at night and searching for amphibians on the road. Each post-metamorphic individual was placed in an individual plastic container or Ziploc bag with a small amount of water until it could be processed. We collected skin samples from post-metamorphic (juvenile and adult) amphibians using non-lethal techniques and released the animals within 24 hrs at the point of capture. The sampling method entailed swabbing the ventral skin (and hind-toe webbing of anurans) 15-20 times, following the protocol of Livo (2004; <http://wildlife.state.co.us/NR/rdonlyres/710BBC95-2DCF-4CF9-8443-D4561DBC3B69/0/PCRsampling2004.pdf>). Each swab sample was preserved in a 2-ml vial containing 70% ethanol.

Sample Processing – The preserved swab samples from adults and preserved tissue samples from larvae were analyzed using polymerase chain reaction (PCR)-based assays that are highly effective in detecting even low levels of chytrid infection. Prior to running these assays (but after collecting the samples), we learned that larvae of some ambystomatid species, including *A. maculatum* and *A. opacum*, lack keratinized tissues and thus would not be susceptible to *Bd* infection (M. Venesky, University of Memphis, pers. comm.). Given limited funding, we chose not to analyze the larval specimens of these species, which would presumably have tested negative for *Bd*. The analyses of all other samples of pond-breeding amphibians were conducted in the laboratory of Dr. Chad Brooks at Austin Peay State University. His lab maintains cultures of *Bd* for use as positive controls in all tests and employs conventional PCR according to the protocols of Annis et al. (2004; Fig. 1).

DNA isolation techniques varied depending on whether the sample was a whole larval amphibian or a skin swab. In larval anurans, *Bd* localizes in the mouthparts because the jaw sheaths and tooth rows are the only keratinized tissues. Thus, we excised the oral discs of tadpoles and subjected the oral disc tissue to PCR. Each tadpole was placed on a disposable vinyl plastic plate and its oral disc was aseptically excised using a razorblade. Plastic plates and razorblades were later reused after washing for 5 min in 10% sodium hypochlorite (bleach) wash, 5 min in a 70% ethanol wash then rinsed in deionized water. The oral disc specimen of each individual sample was kept isolated and processed separately throughout. However, some pooled samples of larvae were large (as many as 73 tadpoles per jar) and had to be subdivided into tubes containing 3 to 7 oral discs.

Once excised, the oral discs were digested and cells lysed in 200 µl of 0.2-mg/ml proteinase K (Sigma-Aldrich Chemical Company, St. Louis, MO) in 200 mM NaCl-20 mM Tris-HCl (pH 8.0)-50 mM EDTA-1% sodium dodecyl sulfate (Sigma-Aldrich Chemical Company) for 16 hours at 37°C. After incubation, the digested tissue samples were triturated to disrupt cellular matrixes and 400 µl of buffered phenol (pH 8.0) and 400 µl of chloroform (Sigma-Aldrich Chemical Company) were added to the samples. The sample was vortexed thoroughly for 10 seconds and allowed to set at room temperature for approximately 5 minutes. The sample was then centrifuged at 10,000 x g for 5 min and the top, aqueous layer was moved to a new tube. DNA was precipitated by adding two volumes of isopropanol and 100 µl of 3M sodium acetate then placed at -80°C for 5 minutes followed by centrifugation at 10,000 x g for 10 min. The resulting DNA pellet was washed once in 80% ethanol and subsequently centrifuged at 10,000 x g for 5 min. The DNA pellet was air-dried and re-suspended in 50 µl of molecular grade water then frozen until further DNA analysis.

In addition to testing the oral discs, we also retained and tested the ethanol from each pooled sample of larvae. The ethanol was passed through a 0.22 µm filter to collect possible free-floating *Bd* zoospores. The cellular debris was reverse-osmosed to collect the debris in a new tube. The sample was then boiled in a hot water bath for 5 min and the resulting cell lysate was frozen until further DNA analysis.

To isolate DNA from skin swabs, we pressed the swab against the sides of its 2-ml vial to remove most of the retained ethanol (and any *Bd*) from the cotton. The tubes containing ethanol were then centrifuged at 10,000 x g to create pellets of swab-debris and possible zoospores. The ethanol was moved to a new tube and the original cotton swab was placed back into the ethanol, then stored for possible re-analysis. The swab-debris pellet was re-suspended in 100 µl of molecular grade water and boiled in a hot water bath for 5 min. The resulting cell lysate was frozen until further DNA analysis.

We used specific DNA primers for the 16s ribosomal subunit of *Bd* and PCR to determine whether *Bd* was present in either the oral disc tissue (larvae) or swab sample (postmetamorphic amphibians). Briefly, 5 to 10 µl of each DNA specimen was added to a total volume of a 20 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 100 µM each of the four dNTP, 1.25 units of Taq DNA polymerase and 25 pmol of each primer. Amplification was carried out for 35 cycles using an annealing temperature of 58°C for the primers employed. The success of the PCR reactions was verified by gel electrophoresis of the DNA sample amplicons and *Bd* control DNA through a 0.8% agarose gel in 1 x TAE buffer.

Determining sensitivity of conventional PCR – Prior to DNA analysis of field-collected specimens, *in vitro* cultivated *Bd* zoospores were enumerated and added to sterile swabs and tadpole oral discs to simulate *Bd* infection. The oral disc tissue came from presumably uninfected treefrog tadpoles (*Hyla chrysoscelis*) that were removed from a swimming pool in Montgomery County, Tennessee. *Bd* zoospores were serially diluted to statistically represent 0.1, 1, 10, 100, 1,000, and 10,000 zoospores and added to water, swabs, and oral discs in triplicate. Swabs and oral discs were treated as described above to isolate DNA and perform PCR analysis. As few as 100 zoospores were detectable in swab extracts and 10 or fewer zoospores in oral discs (Figs. 2a and 2b).

Plethodontid Salamanders

Sample Collection – Plethodontid salamanders (and a single specimen of *Cryptobranchus alleganiensis*) were captured using visual daytime surveys from 25 localities in Great Smoky Mountains National Park in May-July 2006 (n = 139) and April-July 2007 (n = 89; Appendix). All salamanders were post-metamorphic with the exception of one *Eurycea wilderae/E. junaluska* larva captured at Tremont Road #2. Each salamander was swabbed (approximately 20 strokes), with care taken to thoroughly sample the throat, chin, belly, vent, and ventral surfaces of the feet and tail. Swabs were subsequently stored in 70% ethanol. All salamanders were released at the site of capture except the single *Eurycea* larva, which was euthanized and preserved in 70% ethanol.

In addition to sampling 225 healthy animals, three sick, dying or dead animals were also sampled using the same swabbing protocol. These included one emaciated, but still active, *Plethodon metcalfi* from Palmer Creek Trail; one nearly dead *Desmognathus imitator/D. ocoee* (likely the result of a predation attempt) from Gregory Bald Trail; and one dead *Pseudotriton ruber* from the Sinks region of the park. These sick, dying and dead animals were not collected.

Sample Processing – Genomic DNA was extracted from swabs using the Gene Releaser general protocol (BioVentures, Inc.). Swab samples were tested for the presence of *Bd* using real-time polymerase chain reaction (RT-PCR) Taqman assays and species-specific primers developed by Boyle et al. (2004). An internal control (VICTM dye, Applied Biosystems) was used to help determine the presence of PCR inhibitors, which may result in false negatives (Hyatt et al. 2007). The internal control was added to every sample well of the assay. Additionally, a sample known to contain *Bd* was run concurrently as a positive control. In order to avoid the high costs associated with the highly accurate RT-PCR methodology, samples from identical species and locations were pooled, with up to three samples per reaction well (Hyatt et al. 2007). To further avoid high costs, all assays were performed in singlicate (following Kriger et al. 2006) rather than the widespread method of performing assays in triplicate (following Boyle et al. 2004). Actual detection probability is not expected to decline significantly with these cost-cutting measures (Kriger et al. 2006, Hyatt et al. 2007). All DNA extractions and RT-PCR reactions were carried out at the University of Michigan, Museum of Zoology and Medical School, respectively.

RESULTS

Pond-breeding Amphibians

We did not find any dead or obviously sick amphibians during pond surveys. However, we detected *Bd* in samples from four sites, all in or near Cades Cove: Sparks Lane/Cooper Branch, Stupka's Sinkhole, Finley Cane Sinkholes, and Tremont Road #3 (Table 1). All of the positive samples were larval anurans. We found infected wood frog (*R. sylvatica*) larvae at Stupka's Sinkhole (in ethanol of a pooled sample, and in 1 of 27 individual oral disc samples), Finley Cane Sinkhole #1 (in ethanol of a pooled sample and in 1 of 24 subsamples of oral discs), and Tremont Road #3 (in 2 of 16 subsamples of oral discs). We also found one infected toad (*Bufo* sp.) larva at Finley Cane Sinkhole #2. Finally, *Bd* was detected in ethanol (but not in oral discs) from a pooled sample of chorus frog (*Pseudacris* sp.) larvae from the Sparks Lane/Cooper Branch site.

At sites where *Bd* was detected, prevalence (i.e., percent of individuals infected) was apparently very low. We can infer prevalence from samples in which large numbers of larvae were collected from a given site on the same date, and individual larvae were preserved and analyzed separately. At Finley Cane Sinkhole #2, only 1.6% of toad larvae were infected. At Stupka's Sinkhole, 3.7% of wood frog larvae were infected. Likewise, the one positive subsample of wood frog larvae from Finley Cane Sinkhole #1 contained 3 larvae, which suggests prevalence was $\leq 4\%$.

Plethodontid Salamanders

According to the RT-PCR assays, every plethodontid swab sample was negative for the presence of *Bd*. By determining the presence of *Bd* in the positive control, we confirmed that the reaction worked. However, the standard dilution of 1/10 of the swab extract yielded a mere 20% success rate in internal control reactions, indicating PCR inhibition was a significant problem. To overcome the presence of PCR inhibitors, all samples were rerun with a 1/100 dilution. The success rate of internal control reactions was 100% following the increased dilution factor. In the singlicate method proposed by Kriger et al. (2006), any positive sample would be rerun in triplicate to confirm its positive status. This second step proved unnecessary in this analysis because every sample was negative.

DISCUSSION

Chytridiomycosis has contributed to recent amphibian population declines in western North America, Central America, and Australia (Collins and Storfer 2003). The primary purpose of this study was to determine if *B. dendrobatidis*, the pathogen that causes chytridiomycosis, is present in Great Smoky Mountains National Park. Indeed, our sampling revealed the presence of this pathogen in a limited number of sites and species within the Park. The detection of *Bd* in Great Smoky Mountains National Park is one of a growing list of occurrences in the southeastern U.S. For example, recent surveys have also found chytrid-infected amphibians at Congaree National Park, Chattahoochee River National Recreation Area and sites in the Blue Ridge Mountains of North Carolina and Georgia (Rothermel et al., in review).

Another goal of this study was to provide preliminary information regarding *Bd* prevalence and which species are infected. Consistent with our predictions, we detected *Bd* only in the larval stages of pond-breeding anurans in the genera *Bufo*, *Rana*, and possibly *Pseudacris*. The detection of *Bd* only in ethanol and not in oral discs of chorus frog larvae is not strong diagnostic evidence of infection, although we presume there was shedding of infected tissue from the mouthparts during transport and storage in ethanol. Additional sampling of chorus frogs and toads would be warranted to gain more information on prevalence in these taxa. We could not assess the influence of elevation on *Bd* occurrence because elevation and taxonomic associations were confounded; that is, samples (including positives) of pond-breeding species tended to be from lower-elevation sites and samples (all negative) of plethodontid salamanders tended to be from higher-elevation sites (Appendix).

Infections have been reported in these genera in other locations throughout the southeastern U.S. In particular, infections of bullfrogs (*R. catesbeiana*) and other ranids seem to be fairly common in our region. For example, *Bd* infection was detected in 4 of 10 post-

metamorphic *R. catesbeiana*, 1 of 4 post-metamorphic *R. clamitans*, and 2 of 38 larval *R. clamitans* collected from Chattahoochee River National Recreation Area in 2005 (Rothermel, unpubl. data). At Congaree National Park, 22 of 29 post-metamorphic pickerel frogs (*R. palustris*) were infected (Rothermel, unpubl. data). Green and Dodd (2007) sampled larval amphibians at Warm Springs National Fish Hatchery in Georgia and detected oral chytridiomycosis in 4 of 5 *R. catesbeiana*, whereas larvae of *R. clamitans*, *B. fowleri*, and *Hyla cinerea* were not infected.

In the absence of data from historical specimens, it is impossible to estimate how long *Bd* has been present in the park. Analyses of preserved frog specimens from sites in South Carolina and Virginia have confirmed that *Bd* has been present in the southeastern U.S. for at least three decades (Daszak et al. 2005, Ouellet et al. 2005). Specimens collected by Dodd (2004) in the Cades Cove area (mostly from Gourley Pond) in 1999-2001 were not infected with *Bd* based on histological examination. However, given the large numbers of visitors to the park and natural vectors such as wading birds, *Bd* could easily have been introduced long ago.

The non-detection of *Bd* in post-metamorphic life stages of pond-breeding species could be due to relatively low sample sizes. Most (87%) of our samples of pond-breeding amphibians were larvae, resulting in greater probability of detection for this life stage compared to juveniles and adults. However, other factors may have also contributed. In many species, *Bd* causes only sublethal infections in larvae, and it is usually during or after metamorphosis that infected individuals succumb to chytridiomycosis and die (Rachowicz & Vredenburg 2004). In addition, our minimum detection limit for swab samples using conventional PCR was approximately 100 zoospores, which means there was a slightly greater chance that light infections of post-metamorphic amphibians could have gone undetected. An interesting result was the lack of infection in adult newts (*N. viridescens*; n = 37), which in some cases were co-inhabiting ponds with infected larvae of other species. Infected newts have been found in several other sites in the southeastern U.S. (Rothermel et al., in review), but it is not known how susceptible they are to chytridiomycosis.

We used the most sensitive genetic techniques currently available (RT-PCR) to test the samples of plethodontid salamanders, because little is known regarding their potential susceptibility and because their vulnerability to infection is of great concern from a conservation standpoint. Thus, we can say with high confidence that the plethodontid salamanders we tested were not infected. Although some species of *Plethodon* and *Desmognathus* are susceptible to infection, they seem resistant to chytridiomycosis (Vazquez & Rothermel, in prep.). Resistance to *Bd* derives from antimicrobial peptides produced by skin glands (Rollins-Smith et al. 2005) or cutaneous bacteria that inhibit growth of fungi, in some cases *Bd* (Harris et al. 2006, Lauer et al. 2007). Different species and populations possess various combinations of these inhibitory factors, resulting in variation in resistance to chytridiomycosis. Furthermore, woodland salamanders that are direct-developing may have low likelihood of infection simply because they do not inhabit streams and other aquatic habitats where *Bd* occurs.

Implications for Park Management

Any comprehensive monitoring strategy for amphibians should incorporate surveillance for emerging diseases. Although the presence of *Bd* in the park is cause for concern, evidence is accumulating throughout the southeastern U.S. that other pathogens may be equally or more

problematic for amphibians in this region. To date there have been very few instances of amphibian mortality attributed to chytridiomycosis in the southeastern U.S. In contrast, there have been several severe outbreaks and local die-offs of pond-breeding amphibians due to ranavirus or an as-yet-unidentified protozoan pathogen (Dodd 2004, Davis et al. 2007, Petranka et al. 2007). The only way to determine whether *Bd* and other pathogens are negatively affecting amphibians is to conduct intensive, long-term monitoring of populations of vulnerable species.

In the meantime, park personnel and others should take steps to limit the spread of pathogens. It may not be necessary to disinfect when moving between ponds located in close proximity (e.g., within Cades Cove), since other natural vectors - including dispersing amphibians - are capable of spreading pathogens regardless of precautions taken by humans. However, disinfection of sampling gear and boots is warranted when moving among sites in different areas of the park (see Speare et al. 2004 for appropriate disinfection strategies). Researchers visiting aquatic habitats in the park should be encouraged to follow disinfection protocols, especially if they have been visiting other aquatic habitats outside the park.

Ultimately, there are at least two unanswered questions that warrant a precautionary approach. First, it is not clear whether there are different strains of *Bd*. If there are, then it is possible that a more virulent strain could be introduced to the park. Secondly, changing environmental conditions or additional anthropogenic stressors could create more favorable conditions for *Bd* and other pathogens in the future. Researchers in Central America and Europe, for example, have proposed links between rising temperatures and *Bd*-associated amphibian declines (Bosch et al. 2006, Pounds et al. 2006).

Dissemination of Research Findings

A manuscript is in preparation and copies will be forwarded to the National Park Service upon acceptance. Preliminary results of this research were presented at the 2007 Annual Meeting of the Tennessee Academy of Science. An article about one of our collecting trips, written by Jason Love of the Great Smoky Mountains Institute at Tremont, appeared in the Summer 2007 issue of the *Walker Valley Reflections* newsletter.

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Table 1. Results of surveys for *Bd* infection in pond-breeding amphibians in Great Smoky Mountains National Park. Samples were collected in April and May 2007. Conventional PCR was used to determine the presence of *Bd* (pooled samples) or number of individuals infected (individual samples). L = larval, P = post-metamorphic.

Site	Collection Date	Species	Life Stage	Sample Type	Conventional PCR					
					Pooled ethanol		Pooled oral discs		Individual swabs or oral discs	
					n	Bd	n	Bd	n	Bd
Sparks Lane/ Cooper Branch	4/14/2007	<i>Pseudacris</i> spp.	L	Pooled	7	+	2 tubes	-		
	4/14/2007	<i>Pseudacris crucifer</i>	P	Individual					6	0
	4/14/2007	<i>Pseudacris feriarum</i>	P	Individual					1	0
	4/14/2007	<i>Bufo americanus</i>	P	Individual					2	0
	4/14/2007	<i>Notophthalmus viridescens</i>	P	Individual					3	0
	4/14/2007	<i>Ambystoma maculatum</i>	P	Individual					2	0
Methodist Church Pond	5/16/2007	<i>Rana</i> spp.	L	Pooled	4	-	3 tubes	-		
	5/16/2007	<i>Notophthalmus viridescens</i>	P	Individual					10	0
	4/14/2007	<i>Notophthalmus viridescens</i>	P	Individual					13	0
	4/14/2007	<i>Ambystoma maculatum</i>	P	Individual					1	0
Stupka's Sinkhole	4/14/2007	<i>Rana sylvatica</i>	L	Pooled	4	+	1 tube	-		
	4/14/2007	<i>Rana sylvatica</i>	L	Individual					27	1
	4/14/2007	<i>Notophthalmus viridescens</i>	P	Individual					8	0
Gum Swamp	4/14/2007	<i>Notophthalmus viridescens</i>	P	Individual					3	0
Finley Cane Sinkhole #1	5/16/2007	<i>Rana sylvatica</i>	L	Pooled	72	+	24 tubes	+ (1 tube)		
	5/16/2007	<i>Rana catesbeiana</i>	P	Individual					1	0
	5/16/2007	<i>Rana clamitans</i>	P	Individual					1	0
Finley Cane Sinkhole #2	5/16/2007	<i>Bufo</i> sp.	L	Individual					61	1
Tremont Road #2	5/16/2007	<i>Bufo</i> sp.	L	Pooled	61	-	11 tubes	-		
	5/16/2007	<i>Rana clamitans</i>	L	Pooled	4	-	4 tubes	-		
	5/16/2007	<i>Rana clamitans</i>	P	Individual					2	0
Tremont Road #3	5/16/2007	<i>Rana sylvatica</i>	L	Pooled	68	-	16 tubes	+ (2 tubes)		
The Sinks	5/16/2007	<i>Rana sylvatica</i>	L	Pooled	73	-	20 tubes	-		
	5/16/2007	<i>Pseudacris</i> sp.	L	Individual					1	0
Cades Cove Road	5/16/2007	<i>Bufo americanus</i>	P	Individual					1	0
	5/16/2007	<i>Bufo</i> sp.	P	Individual					3	0

Figure 1. Image of a gel resulting from conventional PCR. Lane 1 shows a PCR reaction using DNA isolated from *in vitro* cultivated *Bd* zoospores (+) and lane 2 shows a PCR reaction lacking *Bd* DNA (-).

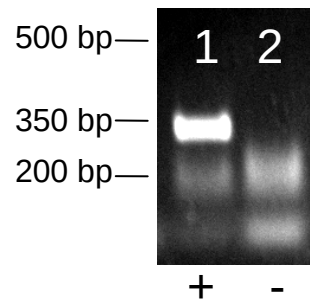


Figure 2a. Zoospores of *B. dendrobatidis* were serial-diluted and added to water to determine the minimum detection limit of conventional PCR used to analyze tissue samples from pond-breeding amphibians in Great Smoky Mountains National Park. Below: (A) Conventional PCR was able to detect as little as 1 zoospore per PCR reaction and could robustly detect 10 zoospores per PCR reaction. (B) A higher resolution image of the 1 zoospore dilution demonstrating that 1 zoospore could often be detected.

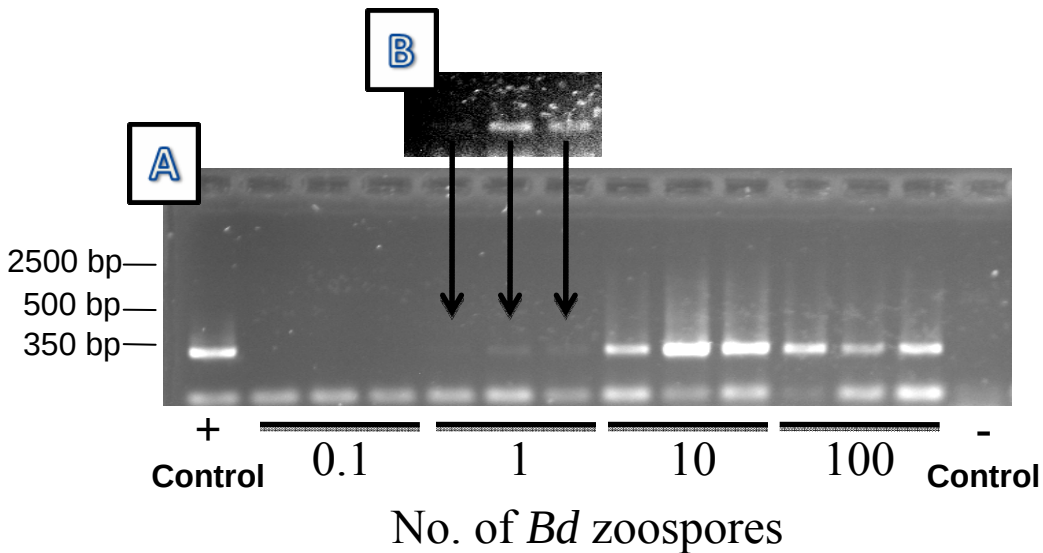


Figure 2b. Zoospores of *B. dendrobatidis* were serial-diluted and added to oral discs (A) and sterile swabs (B). Below: (A) Conventional PCR could detect as little as 1 zoospore per PCR reaction, which was approximately 10 zoospores per oral disc. (B) Approximately 100 zoospores were needed to detect *Bd* in cotton swabs.

