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A DNA-BASED ASSAY IDENTIFIES *BATRACHOCHYTRIUM DENDROBATIDIS* IN AMPHIBIANS

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ABSTRACT: Chytridiomycosis caused by *Batrachochytrium dendrobatidis* (Chytridiomycota) has been implicated in declines of amphibian populations on four continents. We have developed a sensitive and specific polymerase chain reaction–based assay to detect this pathogen. We isolated *B. dendrobatidis* from captive and wild amphibians collected across North America and sequenced the internal transcribed spacer regions of the rDNA cassette of multiple isolates. We identified two primers (Bd1a and Bd2a) that are specific to *B. dendrobatidis* under amplification conditions described in this study. DNA amplification with Bd1a/Bd2a primers produced a fragment of approximately 300 bp from *B. dendrobatidis* DNA but not from DNA of other species of chytrids or common soil fungi. The assay detected 10 zoospores or 10 pg of DNA from *B. dendrobatidis* and detected infections in skin samples from a tiger salamander (*Ambystoma tigrinum*), boreal toads (*Bufo boreas*), Wyoming toads (*Bufo baxteri*), and smooth-sided toads (*Bufo guttatus*). This assay required only small samples of skin and can be used to process a large number of samples.

Key words: Amphibians, anurans, *Batrachochytrium dendrobatidis*, chytridiomycosis, Chytridiomycota, diagnosis, disease, ITS, PCR.

INTRODUCTION

Batrachochytrium dendrobatidis (Longcore et al., 1999), a member of the fungal phylum Chytridiomycota (chytrids), is pathogenic to amphibians and causes chytridiomycosis in amphibians (Berger et al., 1998; Pessier et al., 1999). This disease has been implicated in population declines of amphibian species in North and Central America, Australia, and Europe (Berger et al., 1998; Daszak et al., 1999; Bosch et al., 2001). In adult amphibians, *B. dendrobatidis* colonizes only the keratinized skin cells and usually is found in isolated patches on the ventral side of the animal, with the heaviest infections proximal to the groin and on the webbing between the toes of the hind legs (Longcore et al., 1999; Pessier et al., 1999). Heavily infected animals and fully aquatic amphibians, such as *Xenopus* spp., can have infections on both the ventral and dorsal surfaces (Parker et al., 2002).

Diagnosis of chytridiomycosis is usually by histologic analysis, histochemical analysis, or both and is often difficult in individuals infected with only a few fungal thalli. Infection in anuran larvae is limited to the keratinized cells of the oral region and can result in deformed, eroded, or missing mouthparts, which can be used for diagnosis (Berger et al., 1998; Fellers et al., 2001; Vredenburg and Summers, 2001). Adult amphibians exhibit few clinical signs of chytridiomycosis until they are heavily infected and near death (Berger et al., 1998; Bradley et al., 2002; Parker et al., 2002). When heavily infected, some species shed skin that can be used for diagnosis; however, detection from shed skin is problematic, because animals with minor infections often do not shed enough infected skin to be useful. Most recently reported diagnoses of chytridiomycosis outbreaks have depended on histologic analyses of post mortem skin samples. A reliable screening method for living animals, including those with early or mild infections, is required to understand the ecology of *B. dendrobatidis* and the role of chytridiomycosis in amphibian population declines. In North America, for example, population declines of the boreal toad (*Bufo boreas*) in Colorado (USA; Muths et

al., 2003) and the Wyoming toad (*Bufo baxteri*) in Wyoming (USA; Carey et al., 2003) have been associated with chytridiomycosis. Both amphibian species are bred in captivity as part of reintroduction programs. A method is needed to determine whether animals are free of *B. dendrobatidis* infection before release and to ensure amphibians inhabiting areas targeted for reintroduction are free of this pathogen. Ideally, such a technique must be specific, sensitive, and reliable. A polyclonal antibody to *B. dendrobatidis* has been used in the development of immunohistochemical assays (Berger et al., 2002; Van Ells et al., 2003). This assay is an improvement on histopathology; however, the antibodies are reported to cross-react with other chytrids and to at least one genus of fungal ascomyceteous parasites (*Microsporum*), although none of these are amphibian parasites. In this paper, we describe a sensitive, polymerase chain reaction (PCR)–based assay that detects *B. dendrobatidis* with minimal cross-reaction with other fungi. The PCR-based assay employs a primer pair that specifically amplifies a portion of the rDNA cassette of *B. dendrobatidis.* Another advantage of this assay is that many samples can be processed within a single day with small amounts of skin tissue.

METHODS

Fungal cultures and DNA extraction

Batrachochytrium dendrobatidis cultures were isolated from infected amphibians (Table 1) and maintained as described by Longcore et al. (1999). Other chytrid species were isolated from the substrates listed in Table 2 and identified by cultural and microscopic techniques. Isolates of *B. dendrobatidis* were grown in 75 ml of 1% tryptone or TG (1% tryptone, 0.3% glucose) liquid medium in 125-ml, screw-top flasks at 23 C until colonies were of sufficient density for extraction (ca. 8–14 days). The other chytrid species were grown for DNA extraction in PmTG liquid medium (1 g peptonized milk, 1 g tryptone, 5 g glucose in 1,000 ml of water) at 23 C. Cultures in liquid medium were centrifuged for 10 min at 12,000 × G, and the resulting pellets were frozen with liquid nitrogen. After the liquid nitrogen evaporated, we extracted DNA with a standard hexadecyltrimethyl ammonium bromide method (Zolan and Pukkila, 1986) modified by adding 0.03 ng/ml RNase to the extraction buffer. DNA samples were purified with Qiaex II Gel Extraction Kit by the DNA desalting protocol (Qiagen Inc., Valencia, California, USA) and stored at −20 C until needed.

Developing *B. dendrobatidis*–specific primers

The universal fungal primers ITS5 and ITS4 (White et al., 1990), which anneal to conserved regions of the 28S and 18S rRNA genes, were used to amplify the 5.8S rRNA gene and the flanking internal transcribed spacer (ITS) regions ITS1 and ITS2 for *B. dendrobatidis* and other chytrid genera. Amplification reactions consisted of 10 ng DNA, 1 μM of each primer, 1.5 mM $MgCl_2$, 1× *Taq* buffer, 0.2 mM of each dNTP, and 0.8 units of *Taq* polymerase in a volume of 25 μl. The amplifications were performed in a Thermolyne Amplitron II thermocycler (Barnstead/Thermolyne, Dubuque, Iowa, USA) with the following steps: an initial denaturation at 93 C for 10 min followed by 30 cycles of 45 sec at 93 C, 45 sec at 57 C, and 1 min at 72 C. A final extension at 72 C for 10 min completed the amplifications.

The amplified DNA fragments were separated by electrophoresis through 0.8% agarose (GibcoBRL, Gaithersburg, Maryland, USA) gels in 1× TAE buffer (0.04 M Tris-acetate buffer, pH 8, 100 mM ethylenediaminetetraacetic acid). The resulting DNA bands were excised from the agarose and cleaned with the Qiaex II Gel Extraction Kit following the manufacturer's instructions. Fragments were sequenced directly with the ITS5 and ITS2 universal fungal primers (White et al., 1990) for the ITS1 region and with the ITS3 and ITS4 universal fungal primers (White et al., 1990) for the ITS2 region by the Sequencing Facility at the University of Maine (Orono, Maine, USA).

To identify a region that was unique to all isolates of *B. dendrobatidis,* sequences from *Batrachochytrium* isolates were aligned with Sequence Navigator (v.1.0, Applied Biosystems Inc., Foster City, California) or PileUp (GCG package, Accelrys, Burlington, Massachusetts, USA) and compared with the ITS sequences obtained from other chytrid species (Table 2), as well as to ITS sequences from *B. dendrobatidis* generated at other laboratories (Daszak, unpubl. data). The *B. dendrobatidis*–specific primers (Bd primers) Bd1a (5′-CAGTGTGCCATATGTCACG-3′) and Bd2a (5′-CATGGTTCATATCTGTCCAG-3′) were designed from the ITS1 and ITS2 regions, respectively. The specificity of the primers was initially tested by

TABLE 1. Hosts and locations from which *Batrachochytrium* isolates were obtained.

Host	Isolate no.[a]	Location of collection
Tiger salamander (*Ambystoma tigrinum*)	277	Santa Cruz County, Arizona
American toad (*Bufo americanus*)	282	Toledo Zoo, Indiana
Boreal toad (*Bufo boreas*)	273–276	Clear Creek County, Colorado
Smooth-sided toad (*Bufo guttatus*)	307	National Zoological Park, Washington, DC
Blue poison dart frog (*Dendrobates azureus*)	197	National Zoological Park, Washington, DC
Tomato frog (*Dyscophus guineti*)	203	Bronx Zoo, New York
Canyon tree frog (*Hyla arenicolor*)	229	Pima County, Arizona
Bango frog (*Limnodynastes dumerili*)	253	Melbourne, Australia (culture from L. Berger)
North American bullfrog	260–269	Near Montreal, Quebec
(*Rana catesbeiana*)	270–272	Marin County, California
Green frog	257, 286	Penobscot County, Maine
(*R. clamitans*)		
Mountain yellow-legged frog	213–217	Mono County, California
(*R. muscosa*)		
Pickerel frog	285	Penobscot County, Maine
(*R. palustris*)		
Northern leopard frog	254, 283	Penobscot County, Maine
(*R. pipiens*)	284	Commercial trade
Wood frog	258	Penobscot County, Maine
(*R. sylvatica*)		
Lowland leopard frog	231–238	Maricopa County, Arizona
(*R. yavapaiensis*)	226–228	Yavapai County, Arizona
	230	Pima County, Arizona
African clawed frog	225	Commercial trade
(*Xenopus tropicalis*)	239, 240, 245	Imported from Africa

[a] All cultures were isolated by J. E. Longcore, except 253, and are stored in the chytrid collection at the University of Maine, Orono.

amplification of DNA from other species of chytrids and some *B. dendrobatidis* isolates (JEL197, 215, or 274). Polymerase chain reaction amplifications were performed in 25 μl and consisted of 1 μM of each primer (Bd1a, Bd2a), different concentrations of $MgCl_2$, 1× *Taq* buffer, 0.2 mM of each dNTP, and 0.8 U of Platinum *Taq* polymerase (GibcoBRL). This *Taq* polymerase was chosen for its decreased nonspecific binding as a result of a polymerase-bound antibody that inhibits binding until the antibody is inactivated by heating at 93 C. To improve the specificity of the Bd primers, different concentrations of $MgCl_2$, ranging from 0.5 to 1.5 mM, were tested. A concentration of 0.9 mM $MgCl_2$ was optimum for specificity and strong amplification of *Batrachochytrium* DNA and was used in all subsequent reactions unless otherwise noted. The amplification conditions consisted of an initial denaturation at 93 C for 10 min followed by 30 cycles of 45 sec at 93 C, 45 sec at 60 C (the annealing temperature for the Bd1a and Bd2a primers), and 1 min at 72 C. A final extension at 72 C for 10 min completed the amplifications. The negative control reactions contained amplification reaction mix with distilled water instead of DNA. Positive control reactions to check for amplification of DNA (10 ng) or spores were performed with the ITS5 and ITS4 primers under the same amplification conditions as described above but with an annealing temperature of 57 C.

We further verified the specificity of the primers by attempting to amplify DNA from other species of chytrids (Table 2) and spores of common soil and plant fungi (species of *Pestalotia, Alternaria, Chalaropsis, Botrytis, Penicillium, Phomopsis,* and *Epicoccum*). We scraped spores from plates of the soil fungi with a sterile toothpick and agitated the toothpick in

TABLE 2. Identification of chytrid species, their habitat, and method used for their isolation.

Identification	Isolate no.[a]	Order	Substrate and habitat
Catenophlyctis variabilis	298	Blastocladiales	Baited on snakeskin in water containing soil; Maricopa county, Arizona
Diplochytridium sp.	72	Chytridiales	On dead green alga from small pond; Penobscot County, Maine
Podochytrium dentatum	30	Chytridiales	Baited on chitin in water and debris from Anderson Lake, Hancock County, Maine
Polychytrium aggregatum	109	Chytridiales	Baited on chitin with water and debris from bog, Cheboygan County, Michigan
Polychytrium aggregatum	190	Chytridiales	Baited on chitin with water and debris from Silver Lake, Sudbury, Ontario, Canada
Rhizophydium sp.[b]	142	Chytridiales	Baited on onion skin with water and debris from Mud Lake, Penobscot County, Maine
Rhizophydium sp.[b]	151	Chytridiales	Baited on pollen with water and debris from Pushaw Lake, Penobscot County, Maine
Gonapodya prolifera	183	Monoblepharidales	On duckweed in small pond; Penobscot County, Maine
Spizellomyces sp.	210	Spizellomycetales	Baited on pollen with water containing soil, Puerto Rico

[a] Numbers correspond to culture numbers in J. E. Longcore's collection.
[b] Clusters with *Batrachochytrium dendrobatidis* in analyses of 18S rDNA data (James et al., 2000).

5 μl of sterile distilled water in a 0.5-ml amplification tube. The tubes with spores were frozen at −20 C for 1 hr and then heated at 95 C for 5 min before adding amplification reaction mix. We loaded 10 μl of each amplification on 0.7% agarose (GibcoBRL)/0.9% Synergel (Diversified Biotech, Boston, Massachusetts) gels along with a low-mass DNA ladder (GibcoBRL) as a DNA size standard to separate the amplified products. We stained the DNA with Gelstar (BioWhittikar Molecular Applications, Rockland, Maine), visualized it under ultraviolet light, and photographed the gels with a ChemiImager™ 4400 (Alpha Innotech Corporation, San Leandro, California).

Detection limits of the DNA-based assay

To test the detection limits of the Bd primers, we amplified different concentrations of DNA and zoospores from isolates JEL197, 215, and 274 of *B. dendrobatidis.* We harvested zoospores from 1-wk-old cultures grown on 1% tryptone agar medium by flooding the dishes with 1–2 ml of water and collecting the released zoospores. Equal volumes of a portion of the zoospore solution (50 μl) and lactophenol were mixed together to kill the zoospores so they could be counted with a hemacytometer. From a 1×10^6 zoospores/ml stock solution, we diluted zoospores to different concentrations (10 μl in 0.5-ml amplification tubes) and froze the dilutions at −20 C. DNA was released from the zoospores by taking the tubes directly from −20 C, heating them at 90 C for 5 min, and placing them on ice until amplification. Amplification controls and conditions were as described above.

Detection of *B. dendrobatidis* in amphibians

We tested the assay on skin samples from an uninfected boreal toad from a Colorado breeding facility and the following infected animals: boreal toad, Wyoming toad, smooth-sided toad (*Bufo guttatus*), and tiger salamander (*Ambystoma tigrinum*). Infections were confirmed by microscopic examination of fresh or histologically prepared skin. Small pieces of skin were obtained by skin biopsies of thawed animal specimens (animals were shipped to our laboratory frozen), and skin samples were stored at −20 C.

We extracted DNA from skin samples with GeneReleaser (Bioventures Inc., manual version 03/14/96, Carlsbad, California) following a

protocol for extraction of DNA from small metazoans that includes proteinase K (Schizas et al., 1997). We placed 1–3 mm^2 of skin from the ventral surface or legs (cut into small pieces) in 0.5-ml amplification tubes. To each tube, we added 10 μl of 10× *Taq* buffer and 1 μl of proteinase K (1 mg/ml) and crushed the tissue with a pipette tip. Samples were incubated at 55 C for 3 hr and mixed by centrifuging at 16,000 × G for a few seconds. Samples were incubated at 100 C for 5 min to inactivate the proteinase K followed by the addition of 20 μl of GeneReleaser, and then the DNA was extracted with the use of the thermocycler program as described by the GeneReleaser manual (version 03/14/96). After the extraction program, the samples were centrifuged at 16,000 × G for 1 min, and the supernatant, which contained DNA, was transferred to a new 0.5-ml tube. As controls, DNA was extracted from fungi grown in liquid medium or harvested zoospores of *B. dendrobatidis* following a protocol for extracting DNA from yeast (Bio-Ventures Inc.). One microliter of supernatant was used in each reaction with the Bd-specific primers and the amplification conditions described above.

RESULTS

Development of *B. dendrobatidis*–specific primers

We sequenced and compared the 5′ portion of the ITS1 region from *B. dendrobatidis* isolates JEL197, 214, 215, 238, and 253 to the ITS1 sequence from other chytrids (JEL72, 151, 183, and 190) to find sequences that were conserved in *B. dendrobatidis* but not found in other chytrids. We did the same for the 5′ portion of the ITS2 region of *B. dendrobatidis* isolates JEL197, 213, 238, 239, 253, and 275 and other chytrids (JEL30, 52, 72, 109, 142, 151, 183, and 190). The 19-nucleotide primer Bd1a lies in a conserved 97-bp region of the ITS1, and the 20-nucleotide primer Bd2a in a conserved 50-bp region in the ITS2, of *B. dendrobatidis.* These regions were conserved in all of the *B. dendrobatidis* isolates sequenced and were not found in the ITS regions of the other chytrid species examined.

Specificity of the Bd primers

No matches to fungal sequences were found when the sequences of the Bd-specific primers were checked against archived ITS sequences by a BLAST search to the National Center of Biotechnology Information GenBank database (www.ncbi.nlm.nih.gov/BLAST). We tested the specificity of the Bd primers by amplifying DNA from different isolates of *B. dendrobatidis,* the closest known related chytrids (JEL151 and JEL142; James et al., 2000), other chytridialean species, and spores from common soil fungi. *Batrachochytrium dendrobatidis* isolates typically produced two bands with a strongly amplified band of approximately 600 bp and a weaker band of 900 bp when amplified with the ITS4 and ITS5 primers (Figs. 1, 2). DNA or spores of all of the other tested fungi amplified with the universal primers ITS4 and ITS5 produced one or two strong bands between 450 and 1,500 bp, which demonstrated that DNA could be successfully amplified (Fig. 1).

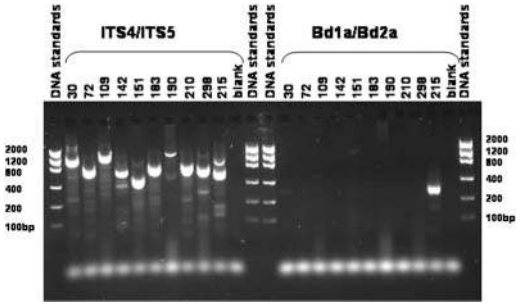

FIGURE 1. DNA of various chytrid genera (for identification and source see Table 2) including *Batrachochytrium dendrobatidis* (JEL215) amplified with the universal fungal primers ITS4 and ITS5 and the *B. dendrobatidis*–specific primers Bd1a and Bd2a. Blank reactions contained amplification reaction mix with 5 μl of water added instead of DNA.

The Bd primers produced a strongly amplified band of approximately 300 bp (referred to as 300 bp hereafter) and a weaker band of approximately 330 bp (330 bp hereafter) from isolates of *B. dendrobatidis* (Fig. 1). The 330-bp fragment was amplified only when greater amounts of DNA were used. With PCR conditions of 1.5 mM $MgCl_2$ and an annealing temperature of 60 C, other chytrid species (isolates JEL30, 142, 151, 183, 190, and 298)

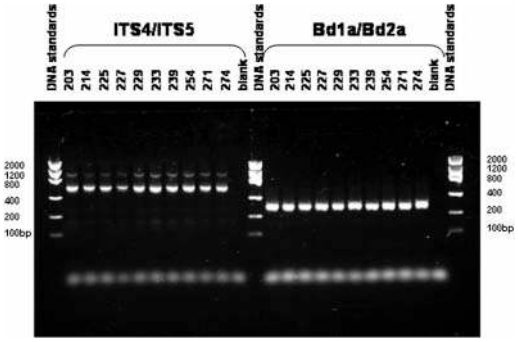

FIGURE 2. *Batrachochytrium dendrobatidis* isolates amplified with the universal fungal primers ITS4 and ITS5 and the *B. dendrobatidis*–specific primers Bd1a and Bd2a. Geographic location and specific host from which isolates were obtained are listed in Table 1. Blank reactions contained amplification reaction mix with 5 μl of water added instead of DNA.

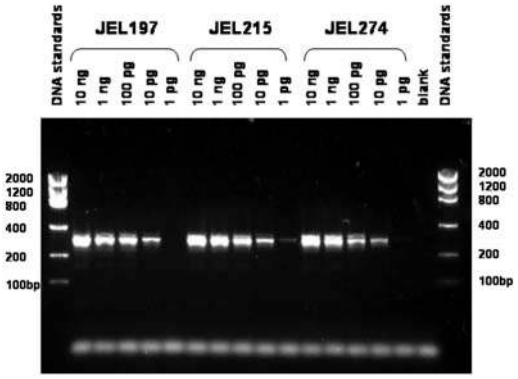

FIGURE 3. Different amounts of DNA (1 pg to 10 ng) from three *Batrachochytrium dendrobatidis* isolates amplified with the Bd-specific primers Bd1a and Bd2a. Blank reactions contained amplification reaction mix with 5 μl of water added instead of DNA.

sometimes produced bands of varying sizes, and some isolates occasionally produced faint bands of about 300 bp. When the $MgCl_2$ concentration in the PCR reactions was lowered to 0.9 mM, the majority of the non-*Batrachochytrium* species no longer amplified, even with repeated attempts. Isolate JEL30 was an exception and continued to yield an amplification product of about 300 bp; however, even using 10 ng of DNA, the band was much fainter than that of *B. dendrobatidis* (Fig. 1). JEL30 is the type isolate of *Podochytrium dentatum,* a saprobic, chitinophilic fungus that is unlikely to be associated with amphibian skin. Spores of the common fungal genera *Pestolatia, Alternaria, Chalaropsis, Botrytis, Penicillium, Phomopsis,* and *Epicoccum* produced bands ranging from 700 to 800 bp when amplified with the universal fungal primers (ITS4 and ITS5) and did not amplify with the Bd primers (data not shown). Amplifications of DNA from *B. dendrobatidis* isolates produced equally strong bands whether performed with 0.9 mM $MgCl_2$ (see *B. dendrobatidis* isolate JEL215 in Fig. 1) or with 1.5 mM $MgCl_2$ (Fig. 2).

Amplifications of DNA extracted from cultures of *B. dendrobatidis* isolated from a variety of captive and wild amphibians from the US (New York, California, Wisconsin, Arizona, Maine, and Colorado) and Africa (Table 1) all produced identical bands when amplified with the Bd-specific primers (Fig. 2). Amplifications of the DNA with the universal fungal primers produced bands whose intensity was as strong as that of amplifications with the Bd primers (Fig. 2). Fifty-two isolates of *B. dendrobatidis* from the JEL collection (Table 1) were tested for amplification with the Bd primers, and all produced identical DNA fragments of equal intensity as those in Figure 2 (data not shown).

Detection limits of the DNA amplification assay

To determine the minimum quantity of *B. dendrobatidis* DNA necessary for detection, we used the Bd primers to amplify from 1 pg to 10 ng of DNA from isolates JEL197, 215, and 274. The assay consistently detected 10 pg to 10 ng of DNA for all three isolates, and frequently a detectable band was produced from amplifications of 1 pg of DNA (Fig. 3). Typically, the intensity of the 300-bp band decreased as the amount of DNA in the amplification decreased, and the 330-bp band was visible only when 100 pg or greater amounts of DNA were used (Fig. 3).

We also tested a dilution series of zoospores (10,000, 1,000, 100, and 10 to ~1) from isolates JEL197, 215, and 274. A

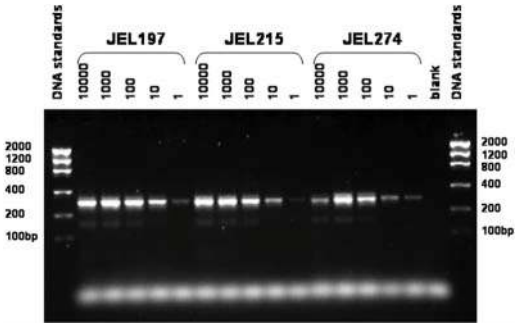

FIGURE 4. Different numbers of zoospores (~1–10,000) from three *Batrachochytrium dendrobatidis* isolates amplified with the Bd-specific primers Bd1a and Bd2a. Blank reactions contained amplification reaction mix with 5 µl of water added instead of DNA.

300-bp band resulted from amplifications of 10–10,000 zoospores of all three isolates, and occasionally, amplification of approximately one zoospore produced a detectable band (Fig. 4). Typically, the intensity of the bands decreased as the number of zoospores in the amplification decreased (Fig. 4). However, when the highest number of zoospores (10,000) was amplified, the intensity of the 300-bp band varied and was occasionally not as strong as with amplifications of lower numbers of zoospores (Fig. 4), possibly because of inhibition of the amplification by cell components of the zoospores.

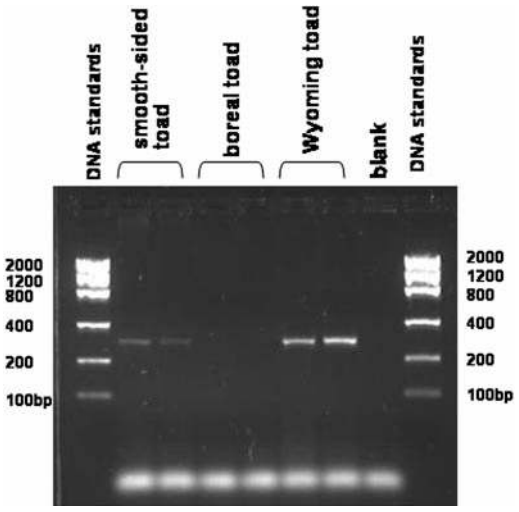

FIGURE 5. Amphibian tissue samples amplified with the Bd-specific primers Bd1a and Bd2a. Skin samples were taken from a smooth-sided toad and a Wyoming toad that were infected with *B. dendrobatidis* and from an uninfected boreal toad. Blank reactions contained amplification reaction mix with 5 µl of water added instead of DNA.

Summary of *Batrachochytrium dendrobatidis* detection assay

Minimum detection limit is 10 zoospores or 10 pg of DNA under conditions outlined below

Primers – Bd1a, 5'-CAGTGTGCCATATGTCACG-3'

-- Bd2a, 5'-CATGGTTCATATCTGTCCAG-3'

Amplification reactions – in 25 µl

- 1µM of each primer, 0.9 mM $MgCl_2$, 1 X Taq buffer, 0.2 mM of each dNTP and 0.8 units of Platinum Taq polymerase

Amplification program

- denaturation at 93 C for 10 min
- 30 cycles of: 45 s at 93 C, 45 s at 60 C and 1 min at 72 C
- final extension of 10 min at 72 C

Run 10 µl of reaction on 1.2 % agarose gel (or 0.9% agarose and 0.7% Synergel) to visualize bands

FIGURE 6. Summary of the polymerase chain reaction conditions for specific amplification of *Batrachochytrium dendrobatidis*.

Detection of *B. dendrobatidis* in amphibians

We used the Bd primers to determine whether we could detect *B. dendrobatidis* in amphibians. Pieces of skin from an infected Wyoming toad and from an infected smooth-sided toad produced single bands of 300 bp when amplified with the Bd primers (Fig. 5). No bands were detected in amplifications of skin from an uninfected juvenile boreal toad (Fig. 5). Skin from a tiger salamander and a boreal toad infected with *B. dendrobatidis* also produced single bands of 300 bp when amplified with the Bd primers (data not shown). Amplifying *Batrachochytrium* DNA from the above samples with either 0.9 or 1.5 mM concentrations of $MgCl_2$ yielded equally intense bands. A summary of the DNA-based assay for detecting *B. dendrobatidis* is presented in Figure 6.

DISCUSSION

We developed a DNA-based assay to detect *B. dendrobatidis* in infected amphibian skin. The primer pair Bd1a and Bd2a consistently amplified a 300-bp DNA fragment from the 52 isolates of *B. dendrobatidis* examined. These isolates were collected from infected animals from various locations in North America and from African clawed frogs (*Xenopus tropicalis*) recently imported from Africa (Table 1). The sequence of the primers occurred in the DNA of all isolates of *B. dendrobatidis* from North America, Central America, and Australia that have been sequenced. Our results, and the probability that *B. dendrobatidis* isolates from Australia and Central and North America are from a recently emerged clone (Morehouse et al., 2003), suggest that most, if not all, infections by *B. dendrobatidis* can be detected with the Bd1a and Bd2a primer pair developed in this study. Only one other tested chytrid, *P. dentatum*, which grows on chitin, produced a faint band of the correct size with our amplification conditions. This fungus could be a source of false positives when testing environmental samples, but this fungus would not grow on amphibians and thus is unlikely to cause false positives from amphibian samples.

The detection limit of the DNA-based assay is about 10 zoospores. We estimate a single zoosporangium in amphibian skin produces at least four to 150 zoospores, suggesting that if the sampling procedure is adequate, even minor infections of chytridiomycosis can be detected. In minor infections, only one to a few foci of *B. dendrobatidis* might exist in the entire amphibian, and a method to sample the largest possible skin area would be required to reduce the possibility of false negative results.

All amphibians with microscopically confirmed infections tested positive with the DNA-based assay. Only small (a few square millimeters) pieces of skin were needed to detect infection, allowing for the possibility of testing from live amphibians if toe clips or toe webbing can be collected. Our method of extracting DNA from tissue samples required a thermocycler but was quick and straightforward. This assay will allow multiple sample extractions and amplifications in a day. Researchers who need to detect *B. dendrobatidis* in artificially and naturally infected animals are now using this assay (Carey and Livo, pers. comm.). Further effort is needed to develop suitable DNA extraction methods to use the Bd primers to detect *B. dendrobatidis* from pond water or other environmental sources such as fish, reptiles, and nonvertebrate substrates.

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