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Batrachochytrium dendrobatidis haplotypes on the hellbender *Cryptobranchus alleganiensis* are identical to global strains

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ABSTRACT: To determine whether the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) found on the hellbender *Cryptobranchus alleganiensis* in the southern US is endemic or exotic, we identified the genetic type of this fungus using partial DNA sequences of the internal transcribed spacer (ITS) region. We identified 3 genetic types, which are found on Japanese amphibians other than the Japanese giant salamander *Andrias japonicus*, a species that belongs to the same family (Cryptobranchidae) as hellbenders. The fungus collected from hellbenders exhibited low genetic diversity and matched the common *Bd* genetic types which have been detected from around the world. These results support that the chytrid fungus on the hellbender is a novel pathogen, as proposed by previous studies. Although we have not observed disease symptoms directly linked to this fungus on this endangered salamander, further evaluation of the influence of this exotic fungus on this species is warranted.

KEY WORDS: Cryptobranchidae · Chytridiomycosis · Chytrids · ITS · Novel pathogen hypothesis · North America

INTRODUCTION

The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the amphibian disease chytridiomycosis and has been linked to declines in wild frog populations in Australia, New Zealand, North America, South America, and Europe (Berger et al. 1998, Lips 1999 Pessier et al. 1999, Bosch et al. 2001, Bradley et al. 2002, Ron et al. 2003, Weldon et al. 2004, Green & Dodd 2007, Skerratt et al. 2007). *Bd* is now widespread throughout many geographic regions and is known to occur all over the world including Asia, where amphibian declines caused by chytridiomycosis have not been reported (Une et al. 2008, Goka et al. 2009, Yang et al. 2009, Savage et al. 2011).

The disease chytridiomycosis was only recently discovered (Berger et al. 1998), and 2 hypotheses have been proposed for the origin of the chytrid fungus (Rachowicz et al. 2005, Pounds et al. 2006, Skerratt et al. 2007, Storfer et al. 2007, Lips et al. 2008, Kilpatrick et al. 2010). The first is the endemic pathogen hypothesis, which posits that *Bd* is endemic to each

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area and population declines result from changes in host susceptibility, pathogen virulence, environmental changes, or a combination of these factors (Morehouse et al. 2003, Weldon et al. 2004, Rachowicz et al. 2005, Morgan et al. 2007, Skerratt et al. 2007, Lips et al. 2008, James et al. 2009). The second, viz. the novel pathogen hypothesis, is that *Bd* was recently introduced to areas where amphibian population declines have been observed (Laurance et al. 1996, Skerratt et al. 2007).

Recently, genetic analyses of *Bd* have been used to attempt to distinguish between the novel and endemic pathogen hypotheses (Morehouse et al. 2003, Morgan et al. 2007, James et al. 2009). These studies revealed that geographically distant isolates appear genetically similar and that relatively little genetic variation exists globally. In addition to these genetic data, several studies indicated sudden appearances of *Bd* in Australia (Aplin & Kirkpatrick 2000), Central America (Lips et al. 2003, 2006), and North America (Ouellet et al. 2005, Padgett-Flohr & Hopkins 2009), and *Bd* is regarded as a novel pathogen in these areas.

Weldon et al. (2004) suggested that *Bd* had originated in Africa because chytridiomycosis was found in a *Xenopus laevis* specimen collected in 1938, and the infection rate of this species was stable without significant differences from the 1930s to 2000s. However, this African origin hypothesis has not been definitive because a source African population with higher genetic diversity has yet to be found (James et al. 2009).

Recently, Goka et al. (2009) conducted a phylogenetic analysis of *Bd* detected in Japanese amphibians, with some samples collected in other countries (USA, Ecuador, and Italy). Using partial DNA sequences of the ITS region, they revealed that the degree of genetic diversity of *Bd* detected in Japan is higher than that in foreign samples. They found 26 haplotypes of *Bd* within the Japanese Islands. Of these, 3 were specific to the Japanese giant salamander *Andrias japonicus*. These 3 haplotypes formed a monophyletic group and greatly diverged from other haplotypes. Thus, Goka et al. (2009) proposed that the 3 strains (B, J, and K) of *Bd* on giant salamanders appeared to have established a commensal relationship with this salamander without causing chytridiomycosis and concluded that Japan is one of the origins of the chytrid fungus and that the Japanese giant salamander is a natural host of *Bd*. This 'Chytrid out of Asia' hypothesis (Fisher 2009, Goka et al. 2009) has been supported by subsequent studies (Bai et al. 2012).

High infection rates in populations of hellbenders *Cryptobranchus alleganiensis* have been reported by several researchers (Briggler et al. 2007a, 2008 Gonynor et al. 2011, Souza et al. 2012). The genus *Cryptobranchus* is a member of the family Cryptobranchidae, which includes only 2 genera (*Cryptobranchus* and *Andrias*), and is represented by 2 subspecies *C. a. alleganiensis* and *C. a. bishopi*. Both subspecies have experienced severe population declines (Wheeler et al. 2003), and *C. a. bishopi* has recently been listed as an endangered species under the US Endangered Species Act.

Bodinof et al. (2011) investigated whether *Bd* occurred historically in hellbender populations in Missouri, USA, or whether it was a relatively novel occurrence by examining epidermal tissue from more than 200 archived hellbenders collected from 7 Missouri streams between 1896 and 1994. Their study detected no evidence for endemism of *Bd* in Missouri hellbender populations prior to 1969. However, because genetic strains of *Bd* in the hellbender have not been identified, the origin of *Bd* carried by this species has been unclear.

If *Bd* found on hellbenders is specific to this species and is related to the *Bd* strains carried by the Japanese giant salamander, we could infer a long co-evolutionary relationship between *Bd* and the cryptobranchid salamanders, supporting the endemic hypothesis. But if *Bd* found on hellbenders exhibits low diversity and matches the common type strains distributed globally, we can conclude that *Bd* on hellbenders in North America is a novel invasive species. Thus, identification of the genetic types and diversity of *Bd* carried by hellbenders provides a unique and powerful test to distinguish between the 2 competing hypotheses.

MATERIALS AND METHODS

Sample collection

To examine whether *Bd* was present in wild hellbender populations and to identify their haplotypes, field surveys were conducted during August 2009 in Arkansas and Tennessee, USA. *Cryptobranchus alleganiensis bishopi* were captured by hand by scuba divers in Arkansas, and *C. a. alleganiensis* were captured by hand by divers snorkeling and lifting rocks in Tennessee. Captured hellbenders were swabbed with a sterile cotton-tipped swab (Men-tip 1P1501, Nihon-Menbo) over the ventral surface of each foot 10 times. We collected 50 samples from 5 rivers

(Table 1). All swab samples were stored in 200 µl of 100% ethanol at room temperature until required for DNA analysis.

DNA extraction

Swab samples were first dried at 50°C for 2 h using an aluminum block heater. We then followed the extraction method described by Goka et al. (2001, 2009). Each swab was placed in a microtube containing 200 µl of lysis buffer (1 mg ml Proteinase K, 0.01 M NaCl, 0.1 M EDTA, 0.01 M Tris-HCl pH 8.0, and 0.5% Nonidet P-40). The microtube was then shaken at 15°C for 1 min using a vortex mixer. After removing the swab, the tube containing the extract was incubated at 50°C for 120 min and then at 95°C for 20 min. After incubation, the extract was diluted to 10% of its original concentration using TE buffer (0.001 M EDTA, 0.01 M Tris-HCl pH 8.0) and used as the source of DNA template for PCR assay.

Nested PCR assay

A nested PCR assay followed Goka et al. (2009). We amplified the target DNA using *Bd*18SF1 (5′-TTT GTA CAC ACC GCC CGT CGC-3′) and *Bd*28SR1 (5′-ATA TGC TTA AGT TCA GCG GG-3′) designed by Goka et al. (2009) for the first PCR step. We then amplified the first-round PCR products using *Bd*1a (5′-CAG TGT GCC ATA TGT CAC G-3′) and *Bd*2a (5′-CAT GGT TCA TAT CTG TCC AG-3′) designed by Annis et al. (2004) in the second amplification step. Polymerase chain reaction assays were conducted, as described in Goka et al. (2001), with 2 µl of each template DNA in a total reaction volume of 50 µl. The PCR reaction mix contained 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1.25 units of *Taq* DNA polymerase (Amplitaq Gold), and 0.5 mM of each primer. All PCR reagents were purchased from PerkinElmer Applied Biosystems. The conditions for the first amplification were an initial denaturation for 9 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C; and a final extension for 7 min at 72°C. The conditions for the second amplification were an initial denaturation for 9 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C; and a final extension for 7 min at 72°C. Each sample was tested twice. For each amplification, we included a positive control using DNA extracted from a swab taken from an Argentine horned frog *Ceratophrys ornata* (Une et al. 2008) and a negative control using TE buffer without any DNA. PCR products were separated on 6% polyacrylamide gels, and bands of DNA fragments were made visible by means of ethidium bromide staining under UV light. Each product of the second amplification was subcloned into a vector plasmid by using a pT7 Blue Perfectly Blunt Cloning Kit (Novagen, EMD Bioscience) and transformed into *Escherichia coli* in accordance with the manufacturer's protocol. The cloned fragments in 3 positive clones for each nested PCR product were sequenced using T7 promoter and U19 reverse primers on an ABI3730 Sequencer (Applied Biosystems). Using haplotype sequences from Goka et al. (2009), we identified haplotypes of *Bd* carried by hellbenders.

RESULTS

Of the 50 hellbender swab samples, 18 samples (36%) were identified as *Bd* positive (Table 1). Positive *Bd* samples were detected in all 5 rivers. Prevalence varied from 16 to 50%. The highest rate was observed in the Hiwassee River and the lowest in Tumbling Creek (Table 1).

Of 18 positive samples, 14 were identified as haplotype A of Goka et al. (2009) (AB435211), 2 were hap-

Table 1. Host species, sample collection sites, number of total samples, number of *Batrachochytrium dendrobatidis*-positive samples, and % prevalence with 95% confidence intervals in this study

Host species	Locality	N samples	N positive	Prevalence (95% CI)
Cryptobranchus alleganiensis bishopi	Eleven Point River, Arkansas, USA	9	2	22 (2.8–60.0)
C. a. alleganiensis	Hiwassee, Tennessee, USA	16	8	50 (24.6–75.3)
C. a. alleganiensis	Tumbling Creek, Tennessee, USA	6	1	16 (0.4–64.1)
C. a. alleganiensis	Little River, Tennessee, USA	9	3	33.3 (7.4–70.0)
C. a. alleganiensis	Beaverdam Creek, Tennessee, USA	10	4	40 (12.1–73.8)
Total		50	18	36 (22.9–50.8)

Table 2. *Cryptobranchus alleganiensis.* Sample numbers, collection locality, sampling method, and haplotype of *Batrachochytrium dendrobatidis*-positive samples. Eleven Point River is located in Arkansas; all other sites are in Tennessee. The 2 samples from Arkansas were *C. a. bishopi*, whereas all samples from Tennessee were *C. a. alleganiensis*

Sample number	Locality	Haplotype
AK10	EP07, Eleven Point River	A
AK11	EP18, Eleven Point River	A
TN01	Hiwassee	A
TN06	Hiwassee	A
TN07	Hiwassee	L
TN09	Hiwassee	A
TN11	Hiwassee	E
TN13	Hiwassee	L
TN14	Hiwassee	A
TN19	Hiwassee	A
TN25	Tumbling Creek	A
TN32	Little River	A
TN33	Little River	A
TN35	Little River	E
TN36	Beaverdam Creek	A
TN37	Beaverdam Creek	A
TN43	Beaverdam Creek	A
TN44	Beaverdam Creek	A

lotype E of Goka et al. (2009) (AB435214), and 2 were haplotype L of Goka et al. (2009) (AB435221) (Table 2). From 3 rivers (Eleven Point River of Arkansas, Tumbling Creek and Beaverdam Creek of Tennessee), only single strains (haplotype A) were detected, whereas multiple strains (haplotypes A, E, and L) were detected in the other 2 rivers (Hiwassee and Little River).

DISCUSSION

To evaluate the risk of disease posed by *Bd* infection to native amphibian populations, it is important to determine whether the infection strain is exotic or endemic. Causes of hellbender decline are mainly linked to habitat degradation or alteration, chemical contaminants, introduced species, commercial exploitation, diseases, and pathogens, such as *Bd* (Briggler et al. 2007b). *Bd* was first reported in *Cryptobranchus alleganiensis bishopi* from the North Fork of the White River, Ozark County, Missouri, USA (Briggler et al. 2007a), and is now geographically widespread in *C. a. bishopi* in the Ozark Highlands (Briggler et al. 2008). Our study has shown that chytrid strains found on both hellbender subspecies are not specific to cryptobranchids, but rather match common types distributed globally, supporting the exotic recent invasion hypothesis (Bodinof et al. 2011, Farrer et al. 2011).

All 3 haplotypes detected from hellbenders can be regarded as common types which have been detected from many different amphibian species and are genetically close to each other. Hapotype A differs from haplotype E at 1 indel region (continuous 10 bp insertion or deletion), and haplotype E differs from haplotpye L at 1 indel region (continuous 6 bp insertion or deletion; Fig. 1). However, these 3 haplotypes were distant (more than 10 substitutions, insertions, or deletions) from the specific haplotypes (B: AB435213, J: AB435220, and K: AB435221) observed on the Japanese giant salamander (Fig. 1 in this article; Fig. 6 in Goka et al. 2009; Fig. 2 in Bai et al. 2012).

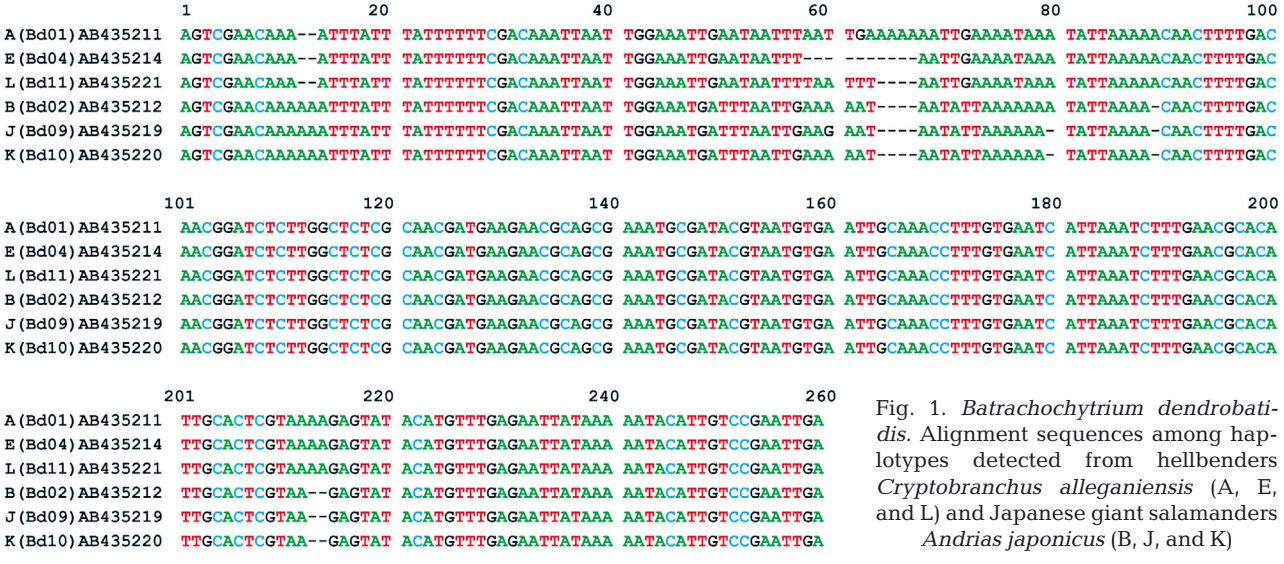

Fig. 1. *Batrachochytrium dendrobatidis.* Alignment sequences among haplotypes detected from hellbenders *Cryptobranchus alleganiensis* (A, E, and L) and Japanese giant salamanders *Andrias japonicus* (B, J, and K)

Several studies have revealed higher genetic diversity of *Bd* and a lower prevalence, and no clinical signs of chytridiomycosis in wild Asian amphibians. These studies found that some Asian amphibians carry the unique *Bd* strains which are divergent from other global strains and are endemic to Asia (Goka et al. 2009, Bai et al. 2012). This suggests that several Asian amphibians have established a commensal relationship with their specific *Bd* strains (Goka et al. 2009, Farrer et al. 2011, Savage et al. 2011, Swei et al. 2011, Bai et al. 2012), indicating that at least several Asian *Bd* strains are endemic, consistent with the 'Chytrid out of Asia' hypothesis. To deduce the history of spread of this pathogen, we must carefully examine the evolutionary relationship between *Bd* haplotypes. However, because only a short single locus, which does not include enough phylogenetic information, has been used for genetic analyses in recent studies (Goka et al. 2009, Bai et al. 2012, this study), it is difficult to elucidate their genetic relationships in detail. To clarify their genetic relationship and estimate their origin, further genetic surveys using new genetic markers are required. Although our results cannot conclusively support the 'Chytrid out of Asia' hypothesis, they are nevertheless quite suggestive.

Hellbender declines have been widespread and almost certainly involve multiple anthropogenic factors, posing a challenge to effective conservation. The best studied declines have occurred in the Ozark region, and have been characterized by low recruitment, poor body condition, impaired wound healing, and secondary infections (Wheeler et al. 2003, Briggler et al. 2007b). These factors point to stressed individuals which likely exhibit much higher susceptibility to exotic diseases such as *Bd*. We strongly recommend implementing long-term surveys of prevalence and virulence of *Bd* on hellbender populations throughout their range.

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