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Filling a gap in the distribution of *Batrachochytrium dendrobatidis*: evidence in amphibians from northern China

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ABSTRACT: Chytridiomycosis caused by *Batrachochytrium dendrobatidis* (*Bd*) has been recognized as a major driver of amphibian declines worldwide. Central and northern Asia remain as the greatest gap in the knowledge of the global distribution of *Bd*. In China, *Bd* has recently been recorded from south and central regions, but areas in the north remain poorly surveyed. In addition, a recent increase in amphibian farming and trade has put this region at high risk for *Bd* introduction. To investigate this, we collected a total of 1284 non-invasive skin swabs from wild and captive anurans and caudates, including free-ranging, farmed, ornamental, and museum-preserved amphibians. *Bd* was detected at low prevalence (1.1%, 12 of 1073) in live wild amphibians, representing the first report of *Bd* infecting anurans from remote areas of northwestern China. We were unable to obtain evidence of the historical presence of *Bd* from museum amphibians (n = 72). Alarmingly, *Bd* was not detected in wild amphibians from the provinces of northeastern China (>700 individuals tested), but was widely present (15.1%, 21 of 139) in amphibians traded in this region. We suggest that urgent implementation of measures is required to reduce the possibility of further spread or inadvertent introduction of *Bd* to China. It is unknown whether *Bd* in northern China belongs to endemic and/or exotic genotypes, and this should be the focus of future research.

KEY WORDS: Chytridiomycosis · *Andrias davidianus* · Museum specimens · Asia

INTRODUCTION

Chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been identified as a major driver of global amphibian population declines (Berger et al. 1998, Daszak et al. 2003, Walker et al. 2008, Cheng et al. 2011). *Bd* infects at least 695 species of amphibians (anurans, salamanders, and caecilians) from all continents where they exist and has been implicated in the enigmatic disappearance of many frog species in Australia, Europe, and North, Central, and South America (Berger et al. 1998, Lips et al. 2006, Walker et al. 2008, Vredenburg et al. 2010, Cheng et al. 2011, Soto-Azat et al. 2013, Olson & Ronnenberg 2014). However, negative impacts at the population level on other regions

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where *Bd* exists, such as Asia and Africa, have not been recorded (Kusrini et al. 2008, Goka et al. 2009, Yang et al. 2009, Bai et al. 2010, 2012, Soto-Azat et al. 2010, Savage et al. 2011, Swei et al. 2011). One possible explanation is that *Bd* is endemic in some areas but novel in others (Rosenblum et al. 2013). The endemic lineage BdAsia has been identified in China (Bai et al. 2012), Japan (Goka et al. 2009), and South Korea (Bataille et al. 2013).

Bd has been recently introduced to many regions, including Central America (Cheng et al. 2011, Velo-Anton et al. 2012), California, USA (Vredenburg et al. 2010, 2013), Australia (Skerratt et al. 2007, Murray et al. 2010), and Madagascar (Bletz et al. 2015), while in other regions, it has been present for a long time. For instance, Rodriguez et al. (2014) proposed that 2 lineages of *Bd* (BdGPL and BdBrazil) have been present in Brazil for more than 120 yr, and Rosenblum et al. (2013) suggested that BdGPL emerged at least 1000 yr ago. For Africa, the earliest known *Bd*-positive cases were confirmed in amphibians collected in the early 1930s (Soto-Azat et al. 2010). For Asia, the earliest detection of *Bd* by PCR was in samples collected in 1911 from the Korean Peninsula, although in Japan, evidence of *Bd* infection based on histology dates back to 1902 (Schloegel et al. 2012, Fong et al. 2015). Globally, the earliest known record of *Bd* infection found in a museum retrospective study is from a specimen of *Rana sphenocephala* collected in Illinois, USA, in 1888 (Talley et al. 2015).

In Asia, *Bd* has been described infecting wild and captive amphibians from many countries, including China, Japan, South Korea, Indonesia, Malaysia, Laos, Philippines, Sri Lanka, Kyrgyzstan, Vietnam, and India (Kusrini et al. 2008, Une et al. 2008, Goka et al. 2009, Yang et al. 2009, Bai et al. 2010, 2012, Savage et al. 2011, Swei et al. 2011, Bataille et al. 2013, Dahanukar et al. 2013, Zhu et al. 2014a). Nevertheless, extensive areas of central, northern, and western mainland Asia still lack information on the presence and impacts of *Bd* (Olson et al. 2013, Olson & Ronnenberg 2014). In China, *Bd* has been reported from 10 central and southern provinces of the country (Bai et al. 2010, 2012, Zhu et al. 2014a,b), with northern regions remaining poorly surveyed for the occurrence of this emerging disease.

Northeastern China is a mountain- and forest-dominated landscape and is bordered by Russia and North Korea. Here, amphibian diversity reaches 15 species (Fei et al. 2010). Two studies, one based on 191 *R. dybowskii* sampled from Heilongjiang Province in China (Wei et al. 2010), and the other based on 180 individuals of 3 amphibian species from the far eastern Russian Federation (Civis et al. 2013), have only found *Bd*-negative results. The northwestern region of China is an arid to semi-arid area, so the presence of amphibians here is limited to rivers, ponds, rice fields, and other natural wetlands. This region belongs to the mountains of Central Asia, and is considered 1 of the 35 global biodiversity hotspots (Mittermeier et al. 2011). To the north, this region borders Russia, Mongolia, and Kazakhstan, and *Bd* has not been previously surveyed in this vast territory.

Animal food markets and farms are considered critical for disease emergence and spread through the circulation of pathogens and host species (Li & Li 1998, Garner et al. 2006, Schloegel et al. 2009, Liu et al. 2013a). Many amphibians are commonly traded as food or for ornamental purposes, particularly around the most populous cities of China (Liu & Li 2009, Liu et al. 2012). The Critically Endangered Chinese giant salamander *Andrias davidianus* is raised for food in many provinces of China, such as Shaanxi, Zhejiang, and Beijing (Liang et al. 2004). The North American bullfrog *Lithobates catesbeianus* is widely raised for food in farms, from which it has escaped and has established feral populations in many regions across China (Li et al. 2006, 2011, Liu & Li 2009, Liu et al. 2012, 2013a). Other species, such as the Mexican axolotl *Ambystoma mexicanum*, the African clawed frog *Xenopus laevis*, and the Asiatic grass frog *R. chensinensis* are commonly traded in pet shops for ornamental purposes and are captive-bred in China. The latter 2 species are also commonly acquired to feed Asian arowana *Scleropages formosus*, a widely traded fish in Beijing. Unlike *A. mexicanum* and *X. laevis*, *R. chensinensis* is native to northern China.

To investigate the current and past distribution of *Bd* in northern China and to assess the potential role of invasive and traded amphibians as disease vectors, we investigated 1284 native and invasive anurans and caudates including free-ranging, farmed, ornamental, and museum-preserved amphibians for evidence of *Bd* infection.

MATERIALS AND METHODS

Wild amphibians

Sampling was conducted in 8 provinces (Fig. 1) throughout northern China from 2012 to 2014. A total of 1073 samples representing 19 species were collected during spring and autumn from various types of habitats, including park ponds, ephemeral pud-

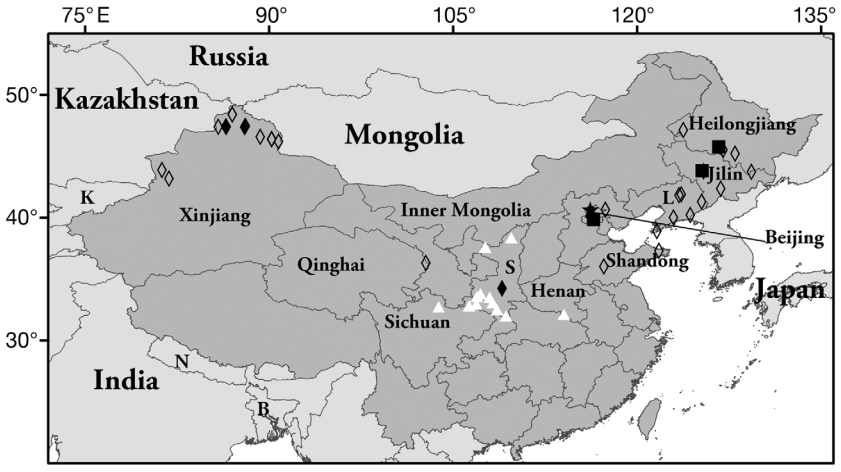

Fig. 1. Distribution of 1284 amphibians sampled from northern China surveyed for the presence of *Batrachochytrium dendrobatidis*. Rhombuses indicate wild amphibians; squares indicate amphibians from markets and pet shops; star represents amphibians farmed for food. Closed symbols indicate sites where *Bd* was detected. White triangles indicate sites where archived amphibians were collected. B: Bangladesh; K: Kyrgyzstan; N: Nepal; L: Liaoning Province; S: Shaanxi Province

dles, rice fields, man-made reservoirs, fish-breeding ponds, and natural wetlands. In addition to native species, we also collected free-ranging *Lithobates catesbeianus*, which have successfully invaded wide areas of China. All sampled amphibians were adults.

Farmed and traded amphibians

Fifty-two individual amphibians of 4 widely traded species, viz. *L. catesbeianus, Ambystoma mexicanum, Xenopus laevis*, and *Rana chensinensis*, were sampled from 1 food market and 2 pet shops in Beijing during the winter of 2013 and spring of 2015. We also sampled 18 *Andrias davidianus* from a salamander farm in Beijing during the winter of 2013. Finally, samples of 69 *L. catesbeianus* were obtained from 2 markets in Changchun (capital of Jilin Province) and Harbin (capital of Heilongjiang Province), during the spring of 2014. All sampled amphibians were adults.

Museum-preserved amphibians

We sampled 72 preserved amphibians archived at the National Zoological Museum (Beijing) and the Shanxi Institute of Zoology. These specimens were collected from Shanxi, Sichuan, and Henan Provinces between 1961 and 1990. All specimens were preserved in 10% buffered formalin and all were adults.

Sampling

All individual amphibians (wild, captive, and museum specimens) were sampled using the non-invasive skin swab technique described by Hyatt et al. (2007). To prevent cross-contamination, each sample was handled using a new pair of disposable gloves. In addition, for museum specimens preserved in jars containing 2 or more individuals, each was rinsed with 70% EtOH before sampling. All swabs were preserved in 70% EtOH in 1.5 ml centrifuge tubes and were ultimately stored at –20°C in the laboratory. For samples obtained from wild amphibians, strict biosecurity protocols were followed, including washing, disinfecting, and drying all equipment before entering a new location (Phillott et al. 2010).

Laboratory analysis

DNA extraction was done following Zhu et al. (2014a). Each swab was deposited into a microtube containing 200 µl of lysis buffer, which was prepared with the following concentration: 0.01 M NaCl, 0.1 M EDTA, 1 mg ml^{-1} Proteinase K, 0.01 M Tris-HCl (pH 8.0), and 0.5% Nonidet P-40. Each microtube was then shaken for 1 min with the use of a vortex mixer and then centrifuged for 1 min at 4208 × *g*. After removing the swabs, the microtubes were centrifuged again for 1 min at 4208 × *g* and later incubated first at 50°C for 2 h and subsequently at 95°C for 20 min. After incubation, the microtubes were centrifuged for 3 min at 16 831 × *g* at 4°C. Later, 10 µl of supernatant was diluted to 10% of its original concentration by adding H_2O and used as DNA template for the PCR assay.

The DNA template was amplified by a nested PCR assay (Goka et al. 2009, Bai et al. 2012, Zhu et al. 2014a). For the first amplification, we used the primers ITS1f and ITS4, which amplify the 5.8S rRNA gene along with the flanking internal transcribed spacer (ITS) of all fungi (White et al. 1990, Gaertner et al. 2009). For the second amplification, primers Bd1a and Bd2a were used to amplify the first-round PCR products (Annis et al. 2004). This PCR procedure was able to detect as little as 0.1 *Bd* zoospore equivalents. Total reaction volumes were 25 µl, consisting of 2 µl of DNA template, 10× PCR buffer (200 mM KCl, 100

mM $[NH_4]_2SO_4$, 200 mM Tris-HCl [pH 8.4], 20 mM $MgSO_4$, and PCR enhancer), 0.2 mM of each dNTP, 0.4 mM of each primer, and 1.25 U of TransStart *Taq* DNA polymerase (Beijing TransGen Biotech). For the first amplification, the conditions included an initial denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 59°C, 1 min at 72°C; and a final extension for 10 min at 72°C. For the second amplification, the conditions included an initial denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 65°C, 30 s at 72°C; and a final extension for 5 min at 72°C.

RESULTS

Bd infection was detected in 12 of 1073 (1.1%) wild amphibians, representing 5 of the 19 surveyed species. This is the first detection of *Bd* in northwestern China. In Xinjiang and in Shaanxi, 8 of 246 (3.3%) and 4 of 74 (5.4%) individuals, respectively, tested positive for *Bd*. Wild amphibians from the other 6 studied provinces (766 individuals in total) were negative for *Bd*, including 24 free-ranging *Lithobates catesbeianus* (all from Shandong). Wild amphibian species tested, provinces surveyed, and results of *Bd* assays are shown in Table 1.

Bd was also detected in 21 of 139 (15.1%) captive individuals sampled from farms, food markets, and pet shops. *Bd* was detected in *L. catesbeianus, Xenopus laevis*, and *Andrias davidianus,* with evidence of *Bd* obtained from all 4 surveyed captive settings (Table 2).

All 72 preserved amphibians examined tested negative for *Bd* (Table 3). Fig. 1 shows 42 sampling sites across northern regions of China from which amphibians were investigated for evidence of *Bd*.

Table 1. Wild amphibians tested for *Batrachochytrium dendrobatidis* infection in northern China

Province Sampling season	Species	No. examined	No. positive
Beijing			
Spring	*Bufo gargarizans*	21	0
	Pelophylax nigromaculatus	36	0
	Rana chensinensis	38	0
Heilongjiang			
Spring	*Bufo raddei*	13	0
	Rana amurensis	6	0
	Hyla ussuriensis	6	0
	Pelophylax nigromaculatus	171	0
Jilin			
Spring	*Bufo gargarizans*	16	0
	Pelophylax nigromaculatus	85	0
	Rana amurensis	27	0
	Rana dybowskii	20	0
Liaoning			
Spring	*Bombina orientalis*	17	0
	Bufo gargarizans	38	0
	Bufo raddei	23	0
	Hyla ussuriensis	42	0
	Pelophylax nigromaculatus	38	0
	Rana amurensis	1	0
	Rana emeljanovi	1	0
	Rana dybowskii	11	0
Autumn	*Glandirana emeljanovi*	14	0
	Bufo stejnegeri	1	0
	Rana dybowskii	2	0
	Bufo gargarizans	25	0
	Pelophylax nigromaculatus	6	0
Qinghai			
Autumn	*Bufo raddei*	19	0
	Bufo gargarizans	2	0
	Pelophylax nigromaculatus	3	0
	Rana kukunoris	6	0
Shandong			
Autumn	*Bufo gargarizans*	23	0
	Fejervarya limnocharis	1	0
	Lithobates catesbeianus	24	0
	Pelophylax nigromaculatus	14	0
	Rana culaiensis	3	0
Shaanxi			
Autumn	*Bufo gargarizans*	41	3
	Pelophylax nigromaculatus	33	1
Xinjiang			
Autumn	*Bufo bufo*	5	0
	Bufotes pewzowi	135	2
	Pelophylax terentievi	24	2
	Rana arvalis	38	4
Summer	*Pelophylax terentievi*	30	0
	Rana asiatica	2	0
	Bufo pewzowi	12	0
Total		1073	12

DISCUSSION

A greater understanding of the spatial epidemiology of *Bd* is crucial to assess the importance of various mechanisms that have either contributed to its rapid emergence or its ongoing spread (Olson et al. 2013). Moreover, recognizing species or populations susceptible to chytridiomycosis is essential to inform amphibian conservation management (Bletz et al. 2015). Thus, central and northern Asia remain as the greatest gaps in the knowledge of the global distribution of *Bd* (Swei et al. 2011, Olson & Ronnenberg. 2014).

Although intensively surveyed (723 individuals tested), evidence of *Bd* infection was not found in wild amphibians from northeastern China (Fig. 1). Our results are consistent with those of Wei et al. (2010), who, based on a survey of 191 *Rana dybowskii* (a species that is intensively hunted for human con-

Table 2. Amphibians tested for *Batrachochytrium dendrobatidis* infection from amphibian food markets, farms, and pet shops in northeastern China

City	Species	No. examined	No. positive
Markets			
Beijing	*Lithobates catesbeianus*	12	3
Changchun	*Lithobates catesbeianus*	36	2
Harbin	*Lithobates catesbeianus*	33	3
Pet shops			
Beijing-1	*Ambystoma mexicanum*	7	0
	Rana chensinensis	10	0
	Xenopus laevis	13	6
Beijing-2	*Xenopus laevis*	10	5
Farm			
Beijing	*Andrias davidianus*	18	2
Total		139	21

sumption), reported an absence of *Bd* in a northeastern province of China (Heilongjiang). James et al. (2015) stressed the importance in identifying regions and populations where *Bd* is absent or present at low prevalence ('cold spots'), to learn about key biotic and abiotic mechanisms underlying *Bd* distribution. For northeastern China, one of the most likely reasons why *Bd* appears to be absent in the wild is that it may never have dispersed there.

In contrast, our results from captive amphibians confirm the broad presence of *Bd* in the amphibian trade in Beijing (Bai et al. 2010), infecting *Lithobates catesbeianus* and *Xenopus laevis* (both with high invasive potential), as well as the threatened *Andrias davidianus*. In addition, we detected *Bd* from *L. catesbeianus* traded in Heilongjiang and Jilin, extending the current known distribution of *Bd* in Asia (albeit for captive amphibians) farther northeast. The presence of *Bd* in all surveyed farms, food markets, and pet shops calls for urgent implementation of measures by the Chinese animal health authority to reduce the likelihood of *Bd* spill-over due to escapes or low (if not non-existent) water and residue management of aquaculture settings (Allan & Gartenstein 2010). If *Bd* is truly absent from wild environments of northeastern China, as has been previously predicted by *Bd* niche modeling (Rödder et al. 2009, Liu et al. 2013b), many native amphibian species or populations from this region may be naïve to *Bd* and therefore possibly highly susceptible to the population effects of chytridiomycosis (Cheng et al. 2011).

Table 3. Preserved amphibians tested for *Batrachochytrium dendrobatidis* infection from 3 provinces of northern China. All specimens tested negative for *Bd*

Province Species	Sampling period (year/month)	No. examined
Shanxi		
Andrias davidianus	1986/09	10
Batrachuperus pinchonii	1980/10	15
Bufo gargarizans	1982/05	4
Bufo raddei	1983/09	3
Fejervarya limnocharis	1981/04	3
Liua tsinpaensis	1981/07	8
Microhyla ornata	1982/05	7
Nanorana quadranus	1980/09	4
Pelophylax nigromaculatus	1981/04	5
Rana chensinensis	1981/04	2
Sichuan		
Bufo gargarizans	1979/09	4
Henan		
Hyla immaculata	1961/09	7
Total		72

Our findings are the first report of *Bd* in wild amphibians from remote areas in Xinjiang, northwestern China (Fig. 1). All 8 positive individuals were collected from 2 sites in the Irtysh River Basin, extending by nearly 500 km the northern known distribution of *Bd* in Asia (Swei et al. 2011). The Irtysh River flows from the Altai Mountains on the Mongolian–Chinese border, northwest to Kazakhstan, and extends far north across Russia. Further *Bd* surveys downstream of this basin may continue to increase the distribution of *Bd* northward. *Bd* was also found in 4 individuals from Changle Park in downtown Xian, Shaanxi (Fig. 1). Xian is the most populous city in northwestern China, so human-assisted introduction of *Bd* to this area should not be ruled out.

None of the 72 archived amphibian specimens showed evidence of *Bd*, and thus we have no information on how long the pathogen may have been present in northern regions of China. The fixative agent formalin (in which the sampled amphibians were preserved) is capable of causing reversible cross-linking of DNA, possibly reducing the likelihood of *Bd* detection based on molecular assays (Soto-Azat et al. 2009). However, the methodology for DNA extraction and the nested PCR assay used here has recently proven to be successful in recovering and amplifying *Bd* genetic material from formalin-fixed amphibians (Zhu et al. 2014a). *Bd* was recently detected in specimens of *Rugosa emeljanovi* collected in 1911 from North Korea (Fong et al. 2015). Following a low expected detection rate of *Bd* from museum samples (Zhu et al. 2014a, Fong et al. 2015) and considering our small simple size, it is recommended that additional amphibian museum specimens be tested for *Bd* to validate our results. As the

specimens originated from only 3 provinces of north China, more specimens from other northern provinces should be tested in future

Overall, our findings extend the known distribution of *Bd* in Central Asia farther north. Extremely low *Bd* prevalence (1.1%) was found among wild amphibians of northwestern China, which is lower than the prevalence of *Bd* found in museum specimens (6%) and in samples from southern China (7.6%; Bai et al. 2012, Zhu et al. 2014a). Shin et al. (2014) suggested that swabbing often fails to detect *Bd* under conditions of low infection load. Therefore, our study may have underestimated the true prevalence of *Bd* in northern China.

Wild amphibians of northeastern China may be at a particular high risk to the effects of chytridiomycosis because of the potential historical absence of *Bd* from natural environments, compared to its extensive presence in the amphibian trade of this region (Bai et al. 2010). As a consequence, urgent actions are required to reduce the possibility of introductions of *Bd* to northeastern China (if it has not occurred already). The government should ban the transport of *L. catesbeianus* and *X. laevis* to this region. It is also necessary to inform the public about the impacts of releasing invasive amphibians. Investigations on the distribution of *Bd* farther north into Russia, but also research focused on isolation, DNA sequencing, and virulence testing, are recommended to further clarify the distribution and impacts of *Bd* on Asian amphibians.

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