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Original Contribution

Retrospective Survey of Museum Specimens Reveals Historically Widespread Presence of *Batrachochytrium dendrobatidis* in China

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Abstract: Chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), has been implicated in amphibian population declines worldwide. However, no amphibian declines or extinctions associated with *Bd* have been reported in Asia. To investigate the history of this pathogen in China, we examined 1,007 museum-preserved amphibian specimens of 80 species collected between 1933 and 2009. *Bd* was detected in 60 individuals (6.0%), with the earliest case of *Bd* infection occurring in one specimen of *Bufo gargarizans* and two *Fejervarya limnocharis*, all collected in 1933 from Chongqing, southwest China. Although mainly detected in non-threatened native amphibians, *Bd* was also found in four endangered species. We report the first evidence of *Bd* for Taiwan and the first detection of *Bd* in the critically endangered Chinese giant salamander (*Andrias davidianus*). *Bd* appears to have been present at a low rate of infection since at least the 1930s in China, and no significant differences in prevalence were detected between decades or provinces, suggesting that a historical steady endemic relationship between *Bd* and Chinese amphibians has occurred. Our results add new insights on the global emergence of *Bd* and suggest that this pathogen has been more widely distributed in the last century than previously believed.

Keywords: *Andrias davidianus*, *Batrachochytrium dendrobatidis*, China, Chytridiomycosis, Museum specimens

Introduction

Amphibian populations have experienced recent population declines in many regions of the world (Stuart et al. 2004). Chytridomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*), is considered to be an important driver of dramatic amphibian population declines and extinctions in Australia, Central and North America, and Europe (Berger et al. 1998; Longcore et al. 1999; Walker et al. 2008; Vredenburg et al. 2010). The decline of amphibians due to chytridiomycosis is considered "the most spectacular loss of vertebrate biodiversity due to disease in recorded history" (Skerratt et al. 2007). *Bd* infection has been demonstrated in over 500 species of

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amphibians from all continents where amphibians exist (Olson et al. 2013). In postmetamorphic susceptible amphibians, *Bd* infects the epidermis, causing hyperkeratosis, electrolyte and osmotic imbalances that can lead to death (Berger et al. 1998; Longcore et al. 1999; Voyles et al. 2009).

Despite considerable research efforts, the geographic origins of this pathogen and its patterns of global spread have still not been determined (Farrer et al. 2011). Two hypotheses have been proposed to explain the origin of *Bd*: the novel and the endemic pathogen hypotheses (Rachowicz et al. 2005). Early genetic studies supported the first, based on extremely low genetic diversity detected in strains of *Bd* collected from disparate regions of the world (Morehouse et al. 2003; James et al. 2009), and the rapid decline of amphibians coincident with the apparent introduction of *Bd* (Lips et al. 2006; Ryan et al. 2008; Cheng et al. 2011). For instance, a retrospective survey using museum specimens revealed that the arrival of a "*Bd* epidemic wave" that began in Mexico in the 1980s and subsequently spread to Central America was responsible for documented declines in amphibians from southern Mexico, Guatemala, and Costa Rica (Cheng et al. 2011). However, recent genomic data have demonstrated a higher genetic differentiation than previously recognized, including the existence of a globally widespread and several regional endemic strains of *Bd* (Goka et al. 2009; Farrer et al. 2011; Bai et al. 2012; Schloegel et al. 2012; Bataille et al. 2013, Rosenblum et al. 2013). Farrer et al. (2011) characterized three deeply diverged lineages of *Bd*: one of which may be endemic to Europe (*Bd*CH); one endemic to South Africa and then introduced to Mallorca (*Bd*CAPE); and a global pandemic lineage (*Bd*GPL). Additional studies have also found endemic strains associated with Asian native amphibians (Goka et al. 2009; Bai et al. 2012; Bataille et al. 2013) and Schloegel et al. (2012) characterized a new lineage of *Bd* (*Bd*Brazil), which has been found widespread in Brazil, USA, and Japan. More recently, Martel et al. (2013) isolated and characterized a well-supported new species of chytr id fungus, *Batrachochytrium salamandrivorans*, which has been associated with lethal chytridiomycosis and severe population decline of fire salamanders (*Salamandra salamandra*) in the Netherlands.

Weldon et al. (2004) first proposed that *Bd* originated in Africa and that the international trade in the African clawed frog (*Xenopus laevis*) might have contributed to the global spread of *Bd*, giving rise to the *Bd* "out of Africa" hypothesis. Currently, the earliest known record of *Bd* is from a specimen of the Fraser's African clawed frog (*Xenopus fraseri*) collected from Cameroon in 1933 (Soto-Azat et al. 2010). Both of these studies have also shown a consistently low historical prevalence of *Bd* in *Xenopus* spp. throughout the 20th century, suggesting a long history of coevolution between *Xenopus* spp. and *Bd* in Africa (Weldon et al. 2004; Soto-Azat et al. 2010).

In Asia, a potential historical *Bd* infection from museum specimens has been suggested to date back to as early as 1902, based on a *Bd* positive case in a museum specimen of the giant salamander (*Andrias japonicus*) from Japan (Goka et al. 2009), although this case has not been confirmed by PCR. Additionally, by sequencing the ribosomal DNA of *Bd*, three population genetic studies have found endemic lineages of *Bd* in Japan (Goka et al. 2009), China (Bai et al. 2012) and South Korea (Bataille et al. 2013). These results imply that *Bd* has had a long historical association with Asia; an argument from which a *Bd* "out of Asia" hypothesis has also been suggested (Fisher 2009; Goka et al. 2009; Bai et al. 2012). Three lines of evidence further sustain this hypothesis: (1) a low prevalence of *Bd* in wild amphibian populations, (2) all amphibians infected with *Bd* have shown no clinical signs of disease, and (3) no substantial chytridiomycosis-caused population declines have been reported in Asia (Kusrini et al. 2008; Goka et al. 2009; Yang et al. 2009; Rowley et al. 2010; Bai et al. 2010, 2012; Savage et al. 2011; Swei et al. 2011; Bataille et al. 2013).

However, the epidemic history of *Bd* in Asia is little known. Three PCR-based studies have assayed for *Bd* in archived amphibian specimens collected in Asia (Ouellet et al. 2005; McLeod et al. 2008; Zeng et al. 2011). Only one of these found *Bd*, detecting it in Asiatic toad (*Bufo gargarizans*) specimens, originally collected in 1980 in China (Zeng et al. 2011). To investigate the epidemic history of *Bd*, we undertook a widespread retrospective survey for *Bd* infection on more than 1,000 museum amphibian specimens collected in China in the last 80 years. We investigated the prevalence of *Bd* in museum specimens using a nested PCR assay and compared the differences in *Bd* infection between different time periods and provinces.

Materials and Methods

A total of 1,007 postmetamorphic wild-caught amphibians of 80 species collected between 1933 and 2009 were examined for evidence of *Bd* infection. These included 79

native and one alien species (*Lithobates catesbeianus*), which had been collected by many people for different purposes other than disease surveillance (Table 1). Fixation history of specimens is as follows: 967 specimens were always preserved in 10% buffered formalin, 39 specimens were originally fixed in 10% buffered formalin and then transferred into 95% ethanol (EtOH) in 2006, and only one specimen was preserved in ethanol since collection in 2009. Amphibians were collected from 14 provinces in China, mainly from the southwest regions of the country, where the highest amphibian diversity is found, but regions in the north, west, central, and east, and including the islands of Hainan and Taiwan were also represented.

Each specimen was handled using a new pair of disposable latex gloves to prevent possible cross-contamination. To decrease the chances of cross-contamination between specimens preserved in the same jars, each individual was rinsed with 70% EtOH before sampling. Each specimen was swabbed 30 times: five times on the ventral surface of the body, pelvic area, each ventral hind limb, and the plantar surface of each hind foot. After sampling, swabs were stored in 1.5 ml microcentrifuge tubes at −20°C.

DNA was extracted following the procedure described by Goka et al. (2009). Each swab was put into a microtube containing 150 µl of lysis buffer, which was prepared with the following proportions: 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. Each tube was shaken for 1 min using a vortex mixer and then centrifuged for 5 s at 2,000 rpm. After removing the swabs, the tubes were centrifuged again for 5 s and subsequently incubated first at 50°C for 2 h and later at 95°C for 20 min. After incubation, 10 µl of the supernatant was deposited in a 0.5 ml microcentrifuge tube containing 90 µl of TE buffer and then used as a DNA template for the PCR assay.

The DNA template was amplified using a nested PCR assay (Gaertner et al. 2009; Goka et al. 2009; Bai et al. 2012). The primers for the first amplification were 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). In the second amplification step, we used *Bd*1a and *Bd*2a to amplify the first-round PCR products (Annis et al. 2004).

The nested PCR assay was performed following the procedure described by Bai et al. (2012). This PCR procedure was optimized to achieve a sensitivity able to detect as little as 0.1 *Bd* zoospore equivalents per µl of extracted DNA (amount above which infection is indicated). Total reaction volumes were 25 µl, consisting of 2 µl of DNA template, 10× PCR Buffer (200 mM Tris–HCl [pH 8.4], 200 mM KCl, 100 mM $(NH4)_2SO_4$, 20 mM $MgSO_4$, and PCR enhancer), 0.4 mM of each primer, 0.2 mM of each dNTP, and 1.25 units of TransStart Taq DNA polymerase (Beijing TransGen Biotech, Beijing, China).

For the first amplification, the conditions were an initial denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 59°C, and 1 min at 72°C and a final extension for 10 min at 72°C. For the second amplification, the conditions were an initial denaturation for 5 min at 94°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 5 min at 72°C.

For each amplification, we included a positive control using DNA template solution containing 0.1 zoospore equivalents per µl and a negative control using TE buffer without any DNA. We then separated the PCR products (about 300 bp) on agarose gel electrophoresis (1.5% agarose gels). Each sample was tested in duplicate. Samples were regarded as being *Bd* positive if one out of two replicates returned a positive result (Cheng et al. 2011). Negative controls were run to avoid false positive.

Pearson χ^2 analyses were used to test differences in *Bd* prevalence between time periods and between Chinese provinces from which amphibians were collected. Confidence intervals (CI) were calculated by exact binomial probabilities. Statistical analyses were carried out using SPSS (v. 19.0). A map showing sample locations and our data on historical *Bd* distributions was produced using ArcGIS (v 9.3).

Results

Bd infection was detected in 60 of 1,007 (6.0%) amphibians examined, representing 21 of 80 (26.3%) species investigated. We report the first detection of *Bd* in 16 Chinese amphibian species, including the first detection of *Bd* in an amphibian collected from Taiwan (Table 1). *Bd* was previously known from *Babina pleuraden*, *Bufo gargarizans*, *Fejervarya limnocharis*, *Hyla annectans*, *Pelophylax nigromaculatus* (Bai et al. 2010, 2012). The earliest cases of *Bd* were found in one specimen of *B. gargarizans* and two specimens of the Asian grass frog (*Fejervarya limnocharis*), both collected from Chongqing in 1933.

All studied decades presented *Bd*-positive individuals, and significant differences between *Bd* prevalence and time periods were not found ($x^2 = 9.41$, $P = 0.23$; Table 2).

Table 1. Summary data on 1,007 archived amphibians collected from 14 provinces in China, examined for *Batrachochytrium dendrobatidis* (*Bd*) infection.

Taxon	Province	Sampling period	No. positives/ No. examined	Conservation status[#]
Amolops chunganensis	Sichuan	1974	0/4	LC
Amolops mantzorum	Sichuan, Yunnan	1962–1992	0/19	LC
Amolops ricketti	Fujian, Guangxi, Sichuan.	1964–1991	0/12	LC
Amolops torrentis	Hainan	1964	0/1	VU
Amolops viridimaculatus	Yunnan	1987	0/3	NT
Andrias davidianus	Chongqing, Guangdong, Sichuan.	1954–1986s, ND	1/17	CR
Babina adenopleura	Taiwan	no data	1/3	LC
Babina daunchina	Sichuan	1933–1991	1/28	LC
Babina pleuraden	Yunnan	1965–1989	1/2	LC
Batrachuperus pinchonii	Hubei, Sichuan	1979–1992	0/12	VU
Batrachuperus tibetanus	Sichuan	1941–1992	0/22	VU
Bombina maxima	Sichuan	1942–1992	0/13	NE
Buergeria japonica	Taiwan	no data	0/3	LC
Bufo bankorensis	Taiwan	no data	0/2	LC
Bufo gargarizans	Hubei, Sichuan	1933–1985	8/46	LC
Bufo tibetanus	Sichuan	1973	0/1	LC
Calluella yunnanensis	Guizhou	1959	0/1	LC
Chiromantis doriae	Hainan	1964	0/1	LC
Cynops cyanurus	Yunnan	1987	0/5	LC
Cynops orientalis	Anhui	1975	0/2	LC
Fejervarya limnocharis	Guangxi, Sichuan	1933–1996	5/36	LC
Hoplobatrachus tigerinus	Sichuan	1979	0/2	LC
Hyla annectans	Chongqing, Hubei, Sichuan, Yunnan	1935–2000	6/39	LC
Hyla immaculata	Guizhou	1963	1/5	LC
Hyla tsinlingensis	Chongqing, Hubei, Sichuan	1964–1998	0/9	LC
Hylarana guentheri	Guizhou, Sichuan	1933–1996	0/35	LC
Ichthyophis bannanicus	Guangxi	2006–2009	0/3	LC
Kaloula rugifera	Sichuan	1961	0/2	LC
Kaloula verrucosa	Sichuan, Yunnan	1942–1982	0/4	LC
Leptobrachium boringii	Sichuan	1957–1973	0/20	EN
Leptolalax alpinus	Yunnan	1987	0/5	EN
Leptolalax oshanensis	Sichuan	1966–1990	0/4	LC
Lithobates catesbeianus	Sichuan	1988	0/2	LC
Liua shihi	Chongqing, Hubei, Sichuan	1934–1998	2/26	NT
Microhyla butleri	Sichuan, Yunnan	1973–1991	0/17	LC
Microhyla heymonsi	Fujian, Taiwan	1964–1995	0/3	LC
Microhyla mixtura	Chongqing, Hubei, Sichuan	1979–1998	1/14	LC
Microhyla ornata	Guizhou, Sichuan, Yunnan	1943–1991	0/55	LC
Microhyla pulchra	Hainan	1964	0/1	LC
Nanorana maculosa	Yunnan	1982	0/1	EN
Nanorana parkeri	Tibet	1973	0/1	LC
Nanorana pleskei	Sichuan	1973–1987	0/6	NT
Nanorana quadranus	Hubei, Sichuan	1965–1998	1/21	NT
Nanorana unculuanus	Yunnan	1982	2/4	EN
Nanorana yunnanensis	Sichuan, Yunnan	1965–1992	1/12	EN

Table 1. continued

Taxon	Province	Sampling period	No. positives/ No. examined	Conservation status[#]
Occidozyga martensii	Hainan	1964	0/1	LC
Odorrana grahami	Sichuan	1934–1992	2/20	NT
Odorrana cf. livida⋆	Sichuan	1991	0/10	DD
Odorrana margaretae	Sichuan	1943–1991	2/36	LC
Odorrana schmackeri	Chongqing, Guangxi, Sichuan	1973–1986	4/27	LC
Oreolalax xiangchengensis	Sichuan	1992	0/9	LC
Paramesotriton caudopunctatus	Guizhou	1981	0/1	NT
Paramesotriton chinensis	Guizhou	1981	0/1	LC
Pelophylax hubeiensis	Hubei	1979	0/5	LC
Pelophylax nigromaculatus	Chongqing, Hubei, Sichuan, Yunnan	1934–1996	6/113	NT
Polypedates megacephalus	Sichuan, Yunnan	1963–2000	0/49	LC
Pseudepidalea viridis	Xinjiang	1962	0/1	LC
Pseudohynobius flavomaculatus	Hubei	1979	0/2	VU
Quasipaa boulengeri	Chongqing, Sichuan	1974–2000	2/34	EN
Quasipaa exilispinosa	Sichuan	1991	0/5	VU
Quasipaa spinosa	Sichuan	1942	0/3	VU
Rana chensinensis	Chongqing, Hubei, Shanxi, Sichuan	1933–1998	5/32	LC
Rana omeimontis	Guizhou, Sichuan	1941–1999	7/42	LC
Rana sauteri	Taiwan	1997	0/3	EN
Rana shuchinae	Yunnan	1982	0/2	LC
Rhacophorus chenfui	Sichuan	1973–1990	0/7	LC
Rhacophorus dennysi	Guangxi, Guizhou	1963–1986	1/2	LC
Rhacophorus dugritei	Chongqing, Hubei, Sichuan, Yunnan	1973–1998	0/13	LC
Rhacophorus omeimontis	Sichuan	1954–1990	0/10	LC
Scutiger boulengeri	Sichuan	1975	0/1	LC
Scutiger glandulatus	Sichuan	1987	0/1	LC
Scutiger mammatus	Sichuan	1975	0/1	LC
Scutiger muliensis	Sichuan	1992	0/9	EN
Tylototriton kweichowensis	Guizhou	No data	0/2	VU
Tylototriton verrucosus	Yunnan	1958–1982	0/5	LC
Xenophrys minor	Sichuan	1989–1996	0/10	LC
Xenophrys nankiangensis	Sichuan	1986	0/1	VU
Xenophrys omeimontis	Sichuan	1989	0/10	NT
Xenophrys shapingensis	Sichuan	1965	0/1	LC
Xenophrys spinata	Sichuan	1990–1991	0/14	LC
Total			60/1,007	

[#] Based on the IUCN Red List of Threatened Species, Version 2012.2.

⋆ According to Amphibian species of the World (ASW ver. 5.6, 2013), *Odorrana livida* is known only from the type locality (Myanmar) near the Thai border. So we use *Odorrana* cf. *livida* here instead.

NE not evaluated, *DD* data deficient, *LC* least concern, *NT* near threatened, *VU* vulnerable, *EN* endangered, *CR* critically endangered.

Infected amphibians were found in 7 of the 14 studied provinces in China. *Bd* was primarily detected in provinces located in the southwest of the country: Sichuan (42 of 775 individuals tested), Chongqing (4 of 61), Yunnan (4 of 57), and Guizhou (1 of 19), but it was also found in the central region of Hubei (5 of 41), the south region of Guangxi (2 of 18), and the island of Taiwan on the east coast of the country (1 of 13; Fig. 1). Differences in the prevalence of *Bd* infection among the 14 provinces surveyed were not significant ($x^2 = 5.33$, $P = 0.97$; Table 3). Additionally,

Table 2. *Batrachochytrium dendrobatidis* positive Chinese archived amphibian specimens classified by time period.

Time period	No. examined	No. positives	% positives (95% CI)
1930–1939	67	6	9.0 (3.4–18.5)
1940–1949	63	3	4.8 (1–13.3)
1950–1959	18	1	5.6 (0.1–27.3)
1960–1969	28	1	3.6 (0.1–18.3)
1970–1979	174	6	3.4 (1.3–7.4)
1980–1989	293	26	8.9 (5.9–12.7)
1990–1999	279	12	4.3 (2.2–7.4)
2000–2009	22	1	4.5 (0.1–22.8)
Total	944[a]	56	5.9 (4.5–7.6)

[a]The sampling dates of 63 specimens are unknown.
CI confidence interval.

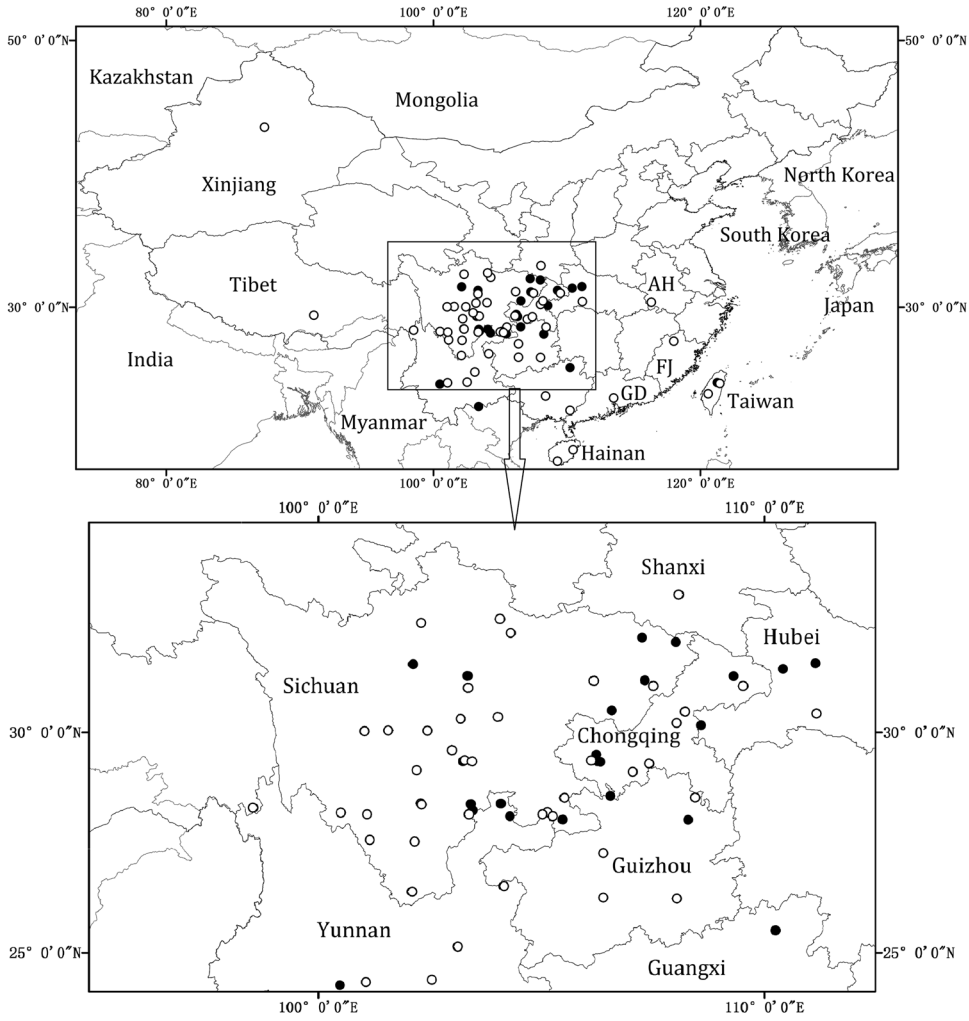

Figure 1. Map of China showing historical distribution of *Batrachochytrium dendrobatidis* (*Bd*) infection detected from amphibian museum specimens. *Filled circles* indicate sites where *Bd* was detected, and *open circles* indicate sites where *Bd* was not detected. *AH* Anhui, *FJ* Fujian, *GD* Guangdong.

Table 3. *Batrachochytrium dendrobatidis* positive archived Chinese amphibian specimens classified by province.

Province	No. examined	No. positives	% positives (95% CI)
Sichuan	775	42	5.4 (3.9–7.3)
Chongqing	61	4	6.6 (1.8–15.9)
Yunnan	57	4	7.0 (1.9–17.0)
Hubei	41	5	12.2 (4.1–26.2)
Guizhou	19	1	5.3 (0.1–26.0)
Guangxi	18	2	11.1 (1.4–34.7)
Taiwan	13	1	7.7 (0.2–36.0)
Hainan	4	0	0.0 (0.0–60.2)
Guangdong	3	0	0.0 (0.0–70.8)
Anhui	2	0	0.0 (0.0–84.2)
Fujian	2	0	0.0 (0.0–84.2)
Shanxi	2	0	0.0 (0.0–84.2)
Tibet	1	0	0.0 (0.0–97.5)
Xinjiang	1	0	0.0 (0.0–97.5)
Total	999[a]	59	5.9 (4.5–7.6)

[a]The locations of eight specimens are unknown.
CI confidence interval.

according to the IUCN Red List of Threatened Species (2012), four of the 21 infected species are classified as threatened. These include the Critically Endangered (CR) Chinese giant salamander (*Andrias davidianus*) and another three species of Endangered (EN) amphibians (Table 1).

Discussion

Our study expands the historical distribution of *Bd* in China based on a retrospective survey of amphibians over a long period (1933–2009). Although a low proportion of *Bd* infection was found (6.0%), this is remarkably similar to the overall prevalence (7.6%) detected from wild Chinese living amphibians in a previous study (Bai et al. 2012). Despite most of the examined specimens were entirely or at least initially fixed in formalin, the DNA extraction protocol and nested PCR assay used in this study, were successful in recovering and amplifying *Bd* genetic material from archived amphibians. The oldest *Bd*-positive specimens found in this study (three individuals collected in 1933) were stored in formalin for 79 years prior to testing. Similar results have been described by other recent studies (Cheng et al. 2011; Richards-Hrdlicka 2012), however, time of formalin fixation before testing has been considerably smaller (39 and 44 years, respectively). Formalin is known to be capable of degrading DNA, possibly reducing the likelihood of *Bd* detection (Soto-Azat et al. 2009); our study might, therefore, underestimate the true historical prevalence of *Bd* in native amphibians in China, but this only strengthens our findings on the historical widespread and long presence of *Bd* in the country.

We found the earliest known case of *Bd* infection in mainland Asia, in three specimens of two common and widespread Asian amphibians (*B. gargarizans* and *F. limnocharis*) collected in 1933 from southwest China. These three specimens were collected from the same area, but only the two *F. limnocharis* were collected during the same collection session and, therefore, kept in the same jar. It is possible that cross-contamination occurred between these individuals, either during field collection or while fixed and subsequently preserved, however, as the amount of amplified *Bd* DNA from museum amphibians is generally very low particularly if fixed in formalin (Soto-Azat et al. 2009, 2010; Vredenburg et al. 2013) and due to the measures put in practice to prevent it, probability of cross-contamination appears to be minimal.

Our study is also the first report of the presence of *Bd* in Taiwan, in a specimen of the olive frog (*Babina adenopleura*) collected from Yilan County in the north of the island. Only one previous study has assayed for *Bd* on amphibians in Taiwan, which investigated 20 frogs of 12

species in 2006; no *Bd*-positive result was obtained (Lehtinen et al. 2010). Nonetheless, the date of collection of the studied Taiwanese *B. adenopleura* specimens is unknown; thus, further retrospective and prospective epidemiological studies are required to determine the current and historical *Bd* status in Taiwan.

We also found a consistently low prevalence of *Bd* infection in Chinese amphibians over time, suggesting that a historical steady endemic relationship between *Bd* and native amphibians has occurred. The infection was not detected in seven provinces from which collected amphibians were available (Hainan, Guangdong, Anhui, Fujian, Shanxi, Tibet, Xinjiang),, however, each of these was represented by small sample sizes (<5 examined specimens). As a consequence, the absence of *Bd* from these and other non *Bd*-surveyed provinces of China cannot be ruled out. No significant differences were observed in the proportion of amphibians infected between provinces, indicating that *Bd* has been evenly distributed across much of China post 1933.

Bd infection was also detected for the first time in four rapidly declining Asian amphibian species: *A. davidianus* (CR), *Nanorana unculuanus* (EN), *N. yunnanensis* (EN), and *Quasipaa boulengeri* (EN). Whether chytridiomycosis is implicated as a contributing factor in the population declines described in these species is not known. Goka et al. (2009) found a high proportion of *Bd* infection in wild *A. japonicus* in Japan, with an apparently non-pathogenic species-specific endemic genotype of *Bd*. A drastic population decline, calculated to be $>80\%$ over the last three generations, has been described in *A. davidianus* (Gang et al. 2004). Whether wild *A. davidianus* in China are infected with endemic or a more virulent introduced pathogenic strain is unknown, therefore, an assessment of *Bd* as a threat to the world's largest amphibian species should be matter of future research.

Historical *Bd* infections found on archived specimens collected in Africa and Asia (Weldon et al. 2004; Goka et al. 2009; Soto-Azat et al. 2010) about 40 years before the beginning of the global amphibian population decline phenomenon (Stuart et al. 2004), should be considered as indicators of regions that might harbor novel endemic strains of *Bd* (Schloegel et al. 2012). *Bd* present in China since at least 1933, is unlikely to have been introduced from abroad. The international trades in *X. laevis* and *L. catesbeianus* are considered two important global vectors of *Bd* (Weldon et al. 2004; Garner et al. 2006; Liu et al. 2013). Goka et al. (2009) found that many alien strains of *Bd* including *Bd*GPL have been introduced into Japan via imported amphibians such as *L. catesbeianus*. It is also possible that *L. catesbeianus* vectored exotic *Bd* genotypes such as *Bd*GPL or *Bd*Brazil into South Korea and China when they were introduced (Bai et al. 2012; Schloegel et al. 2012; Bataille et al. 2013). As a result of bullfrog invasions, native frog abundance and species richness have declined on the Zhoushan Archipelago, China, though the role of *Bd* in these declines is not yet clear (Yiming et al. 2011).

The first reports of *L. catesbeianus* trade into China date back to the late 1950s and first establishments of wild populations did not occur until the early 1960s (Liu et al. 2010). In total, we detected 10 cases of *Bd* infection from museum specimens collected between 1933 and 1959. These early positive cases are consistent with infections with endemic strains of *Bd* (Bai et al. 2012), however, to clarify the origin of these, *Bd* DNA sequencing from museum-preserved material is required. Additionally, future retrospective studies on archived wild-caught *L. catesbeianus* might be useful to date the epidemic history of exotic *Bd* strain introductions to China.

Conclusion

Retrospective studies on archived amphibians can provide an important basis for our understanding of the global emergence and epidemiology of *Bd*. This study found the earliest known case of *Bd* infection on mainland Asia and confirms a historical widespread presence of *Bd* in China. We also found a consistently low prevalence of *Bd* infection in Chinese amphibians over time, suggesting that a historically steady endemic relationship between *Bd* and native amphibians has occurred. Independent of the strain of *Bd* involved, this pathogen appears to be more widely distributed in the last century than previously believed. Future studies on the historical and current presence of endemic and exotic strains of *Bd*, including isolation, partial DNA or whole genome sequencing, and virulence testing are required to understand the impacts of chytridiomycosis to native amphibians in China.

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