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Original Contribution

First Evidence of *Batrachochytrium dendrobatidis* in China: Discovery of Chytridiomycosis in Introduced American Bullfrogs and Native Amphibians in the Yunnan Province, China

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Abstract: Although the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), the etiological agent of amphibian chytridiomycosis, has been implicated in mass mortality and population declines on several continents around the world, there have been no reports on the presence of *Bd* infections in amphibians in China. We employed quantitative PCR and histological techniques to investigate the presence of *Bd* in introduced North American bullfrogs (*Rana catesbeiana*) (referred to hereafter as bullfrog) and native amphibians in bullfrog-invaded areas of the Yunnan Province, China. A total of 259 samples at five wild sites were collected between June and September in 2007 and 2008, including bullfrogs and four native amphibian species (*Rana pleuraden, Rana chaochiaoensis, Odorrana andersonii,* and *Bombina maxima*). In addition, 37 samples of adult bullfrogs were obtained from a food market. *Bd* infections were discovered in bullfrogs and three native amphibian species from all of the surveyed sites. Of the 39 *Bd*-positive samples, 35 were from wild-caught bullfrog tadpoles, postmetamorphic bullfrogs, *R. pleuraden, R. chaochiaoensis,* and *O. andersonii*, and four were from adult bullfrogs from the market. Our results provide the first evidence of the presence of *Bd* in Chinese amphibians, suggesting that native amphibian diversity in China is at risk from *Bd.* There is an urgent need to monitor the distribution of *Bd* in amphibians in China and understand the susceptibility of native amphibian species to chytridiomycosis. Strict regulations on the transportation of bullfrogs and the breeding of bullfrogs in markets and farms should be drafted in order to stop the spread of *Bd* by bullfrogs.

Keywords: chytridiomycosis, *Batrachochytrium dendrobatidis*, *Rana catesbeiana*, native amphibian species, infection, conservation

Introduction

Dramatic declines and extinctions of amphibian populations have been reported in many parts of the world (Houlahan et al., 2000; Stuart et al., 2004). The majority of these have been attributed to habitat loss, pollution, invasive species, and unsustainable harvesting, but a substantial proportion remain unexplained and is referred to as enigmatic population declines (Stuart et al., 2004). Recent studies suggest that an emerging infectious disease, chytridiomycosis, caused by the nonhyphal zoosporic

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chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), may be the driving factor behind these enigmatic declines (Berger et al., 1998; Lips, 1999; Bielby et al., 2008). Certainly, infection is often associated with mass mortalities of newly metamorphosed and adult anurans (and in some cases species extinctions) (Berger et al., 1998; Longcore et al., 1999; Bosch et al., 2001; Daszak et al., 2003; Lips et al., 2006; Schloegel et al., 2006). However, infections have also been detected in asymptomatic species commonly involved in the amphibian trade (e.g., *Xenopus laevis*, *Rana catesbeiana,* and *Bufo marinus*), leading many to consider these and other traded and introduced species as reservoir hosts and/or vectors of *Bd* (Daszak et al., 2004; Weldon et al., 2004; Garner et al., 2006; Fisher and Garner, 2007; Walker et al., 2008).

Batrachochytrium dendrobatidis infects amphibians in Australia, the Americas, Europe, and Africa and has most recently been detected in Asian amphibians (Berger et al., 1998; Lips, 1999; Bosch et al., 2001; Garner et al., 2005, 2006; David et al., 2008; Kusrini et al., 2008; Yumi et al., 2008; Yang et al., 2009). In Asia, reports of the presence of *Bd* are few: To date, published reports include a survey of captive amphibians in the pet trade in Japan (Yumi et al., 2008), a study showing an extremely low prevalence of *Bd* in wild populations of four native amphibian species in Indonesia (Kusrini et al., 2008), and an investigation showing that three of seven native anuran species were infected with *Bd* in South Korea (Yang et al., 2009). Only two studies have described attempts to detect *Bd* in Chinese amphibians. The first included a survey of a limited number of animals from mainland China archived in museum collections as part of a larger study examining historical distributions of *Bd* in North America (Ouellet et al., 2005). The second involved a survey of both wild and imported amphibians in Hong Kong (Rowley et al., 2007). Neither study detected the presence of *Bd* in Chinese amphibians.

The results of these studies do not mean that Chinese amphibians are not at risk of acquiring *Bd*. China has one of the largest national amphibian trades in the world, with tens of thousands of frogs being bred, consumed, and exported each year (Daszak et al., 2006; Warkentin et al., 2009). *Rana catesbeiana*, the North American bullfrog, is the most commonly farmed amphibian in China. Introduced into Taiwan in 1924 and onto mainland China in 1959, it has been bred widely for local consumption and export in many provinces of China and beyond (Li et al., 2006, 2009; Liu and Li, 2009). Escapes are common and naturalized populations of bullfrogs have been found in the Yunnan, Hunan, Hubei, Guizhou, and Zhejiang provinces of China (Li et al., 2006; Liu and Li, 2009). China is home to 333 described amphibian species, 28% of which are considered to be threatened with extinction (IUCN, 2008). Thus, the potential threat of *Bd* carried by bullfrogs to China's amphibian diversity is great, and the consequences could be catastrophic. As a first step towards assessing the possible threat *Bd* may pose to China's amphibians, we surveyed introduced bullfrogs and native amphibian species in bullfrog-invaded areas of the Yunnan Province. Our aims were: (1) to determine if *Bd* is present in bullfrogs in the wild and in markets and in native amphibian species in the wild, and (2) to determine the burden of infection of *Bd*-infected frogs and tadpoles.

Materials and Methods

Study Area

This survey was conducted at five sites in four regions in the Yunnan Province: Kunming, Qujing, Chenggong, and Lugu Lake (Figure 1; Table 1), where feral populations of bullfrogs were found. The climate in these regions is represented by a subtropical humid monsoon climate zone. The vegetation includes subtropical evergreen broad-leaved forests in Kunming, Qujing, and Chenggong, and subalpine coniferous forests in Lugu Lake. The daily maximum air temperature in July (the hottest month) in these four regions is generally less than 30°C, and the mean annual precipitation is approximately 900–1000 mm.

Sample Collection

We collected amphibian tadpoles and postmetamorphic individuals during the rainy seasons (June through September) of 2007 and 2008 (Table 1). We chose populations so as to cover a wide array of habitat types (Table 2). All of the sites have been invaded by bullfrogs, although the precise dates for the introduction of bullfrogs into these sites are unknown. We captured individual bullfrogs and native species in Kunming and Lugu Lake, but only *Rana pleuraden* at Qujing and Chenggong due to the low number and difficulty of capturing bullfrogs in these regions. We captured postmetamorphic frogs manually or with long-handled nets; tadpoles were collected using long-handled nets. We also sampled adult bullfrogs at a food market in Qujing. We sampled no more than 10

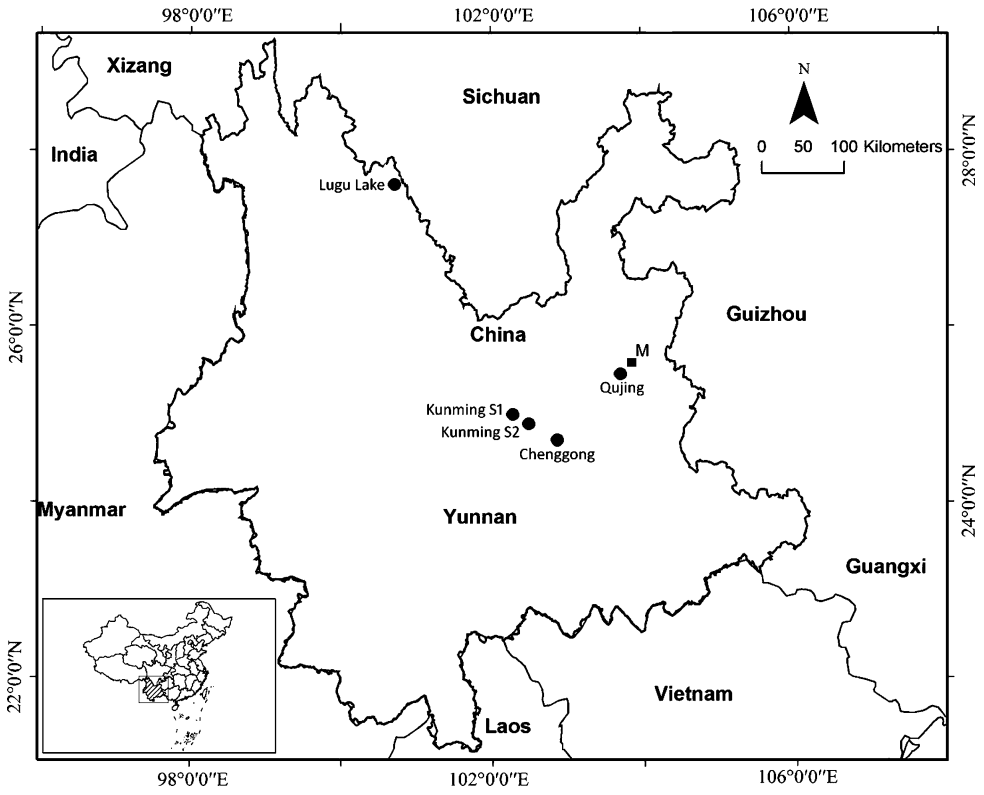

Figure 1. Map of sample locations for Chytridiomycosis in the Yunnan Province, China. A closed circle indicates a site where *Batrachochytrium dendrobatidis* was detected; the closed square denotes the market that was surveyed in Qujing (see text).

frogs from one container used to hold live frogs. To prevent the transfer of infected materials among sites (or in the market) and animals, we rinsed our boots and equipment with 5% bleach before entering each location, and all of the frogs were handled with nonpowdered latex gloves that were discarded after examining each animal. We clipped the top one-third part of the third toe of the right hind-foot from each postmetamorphosed frog. We then released the frog at its capture site the following morning. We euthanized bullfrog tadpoles captured with ethyl ether and excised mouth parts from the dead tadpoles after euthanasia. Each tissue sample from toe clips and mouth parts was preserved separately in 70% EtOH in 2-ml screw-cap microcentrifuge tubes. We brought the samples to the laboratory and stored at –20°C.

Laboratory Analysis

We extracted and amplified all of the samples following the procedure of Boyle et al. (2004), with slight modifications so as to allow for various tissue types and volumes (Garner et al., 2006). We constructed standard curves using 100, 10, 1 and 0.1 *Bd* zoospore standards. We tested each sample in duplicate, and recorded the sample as positive if both replicates were amplified and indicated the presence of *Bd*. We also estimated the *Bd* infection burdens as the mean genomic equivalent (GE) score per sample for the two replicates. As DNA extractions were diluted by one-tenth and only one-tenth of DNA dilutions were taken for PCR assay, we corrected the scores by a factor of 100 [Kerry Kriger, personal communication] and considered a GE of 0.1 as the minimum acceptable value for a positive score. We calculated the prevalence as the proportion of individuals testing positive at 0.1 GE, and calculated 95% confidence intervals (CI) for each prevalence rate using the two-tailed CI for proportions (Garner et al., 2006).

For histological examination, we prepared tissue samples of the toe-clips after they were analyzed by qPCR assay, and fixed (Berger et al., 1999), sectioned (5-μm thickness), and stained them with hematoxylin and eosin (H&E). We observed for diagnostic signs of infection with *Bd* on the sections stained (Berger et al., 1999).

Sample locations were merged into a site if they were separated from other sample locations by less than 2 km (Muths et al., 2008; Skerratt et al., 2008). Such sample locations were combined into five sites: Kunming S1, Kunming S2, Qujing, Chenggong, and Lugu Lake. The infection prevalence was calculated as the number of positive individuals divided by the total number of

Table 1. Prevalence and Average Genomic Equivalents of *Bd* in American Bullfrogs and Native Amphibian Species in the Yunnan Province, China

Species	Sites (elevation, m)	Latitude (°S)	Longitude (°E)	No. of Positives[a]	No. of samples[a]	Mean GE ± SE	Prevalence (%) by site (95% CI)
Lithobates catesbeianus	Kunming S1 (1890)	24.962	102.849	19 (A, 17; J, 2)	47 (A, 42; J, 5)	386.9 ± 168.8	40.43 (25.86–54.99)
	Kunming S2 (1890)	24.959	102.810	1 (L)	30 (L)	81.7 ± 81.7	3.33 (0–10.15)
	Lugu Lake (2900)	27.711	100.856	1 (J)	20 (A, 18; J, 2)	0.4 ± 0.4	5.00 (0–15.47)
	Market in Qujing (1800)	25.509	103.867	2 (A)	9 (A)	22.2 ± 20.2	10.8 (0.31–21.31)
				1 (A)	9 (A)	96.1 ± 96.1	
				1 (A)	9 (A)	0.4 ± 0.4	
				0	10 (A)	0	
Rana pleuraden	Lugu Lake (2900)	27.711	100.856	0	15 (A, 14; J, 1)	0	0
	Qujing (1800)	25.435	103.749	3 (A, 2; J, 1)	24 (A, 17; J, 7)	16.9 ± 11.7	12.5 (0–26.77)
	Chenggong (2000)	24.771	102.800	2 (A)	31 (A)	14.8 ± 12.8	6.45 (0–15.61)
Odorrana andersonii	Kunming S1 (1890)	24.962	102.849	1 (A)	20 (A)	8.5 ± 8.5	5.00 (0–15.47)
Rana chaochiaoensis	Kunming S1 (1890)	24.962	102.849	8 (A, 6; L, 2)	20 (A, 16; L, 4)	44.1 ± 22.4	40.00 (16.48–63.52)
Bombina maxima	Lugu Lake (2900)	27.711	100.856	0	15 (A, 13; J, 2)	0	0

Bd Batrachochytrium dendrobatidis, *GE* genomic equivalent (including positive samples and negative samples, *GE* represents the burden of infection with *Bd*).

[a]*Capital letters* and *number* in parentheses indicate the life stage and number of samples examined or positive in a given location: *A* Adult, *J* Juvenile, *L* Larva.

Table 2. Prevalence and Genomic Equivalents of *Bd* in Wild Frog Populations Collected from the Five Sites According to Breeding Habitat

Species	Sites (elevation, m)	Breeding habitat[a]	No. of positives	No. of samples	Mean GE ± SE	Prevalence (%) by breeding habitat (95% CI)
Lithobates catesbeianus	Kunming S1 (1890)	PP	16	33	480.0 ± 234.2	48.48 (30.49–66.48)
		EP	3	14	167.4 ± 121.7	21.43 (0–46.01)
	Kunming S2 (1890)	PP	1	30	81.7 ± 81.7	3.33 (0–10.15)
	Lugu Lake (2900)	PP	1	20	0.4 ± 0.4	5.00 (0–15.47)
Rana pleuraden	Lugu Lake (2900)	PP	0	12	0	0
		EP	0	3	0	0
	Qujing (1800)	PP	1	6	3.4 ± 3.4	16.67 (0–59.51)
		EP	1	12	16.5 ± 16.5	8.33 (0–26.67)
		PS	1	6	44.3 ± 44.3	16.67 (0–59.51)
	Chenggong (2000)	PP	1	7	27.3 ± 27.3	14.29 (0–49.24)
		ES	0	10	0	0
		PS	1	14	2.2 ± 2.2	7.14 (0-22.57)
Odorrana andersonii	Kunming S1 (1890)	PP	0	7	0	0
		PS	1	13	8.5 ± 8.5	7.69 (0–24.45)
Rana chaochiaoensis	Kunming S1 (1890)	PP	6	11	66.6 ± 38.9	54.55 (19.46–89.63)
		EP	1	3	11.3 ± 11.3	33.3 (0–100)
		PS	1	6	19.2 ± 19.2	16.67 (0–59.51)
Bombina maxima	Lugu Lake (2900)	EP	0	3	0	0
		T	0	12	0	0

Bd Batrachochytrium dendrobatidis, *GE* genomic equivalent (including positive samples and negative samples).

[a]Breeding habitat: *EP* ephemeral pond, *ES* ephemeral stream, *PP* permanent pond, *PS* permanent stream, *T* terrestrial.

individuals sampled. The arithmetic mean genomic equivalent (GE) detected in individuals sampled (including positive samples and negative samples) was used to represent the intensity of infection of these individuals (Kriger et al., 2007), which is believed to be more pertinent than the mean load of infected individuals only. We did not try to detect differences in the prevalence and intensity of *Bd* infection among the species or sites due to the low statistical power of small sample sizes for each species at a site.

Results

A total of 259 specimens from four genera, including five species, were sampled (Table 1). All of the samples came from live individuals. We did not find any dead amphibians during this study. We detected the presence of *Bd* infection in 39 out of 259 (15.06%) samples comprising four species: *R. catesbeiana*, *R. chaochiaoensis, R. pleuraden,* and *O. andersonii*. Fourteen of the 95 (14.74%) individuals of three native species were *Bd*-positive, and 21 of 97 (21.65%) bullfrogs (30 tadpoles and 67 postmetamorphosed bullfrogs) in the wild tested positive. Chytrid infections in wild amphibian populations occurred at all five sites. Four of 37 adult bullfrogs (10.8%) from three of four containers in the market of Qujing tested positive for *Bd* (Table 1).

For all of the species combined, *Bd* infection was detected in all three of the life stages (adult, juvenile, and larva) in our survey (Table 1). Mean GEs for individual positive samples ranged from 3.9 to 7449.0 (GE mean = 598.3, median = 147.0, n = 39). These samples came from individuals in different habitats (Table 2). Three species were tested positive for *Bd* infection at Kunming S1, and bullfrogs tested positive at Kunming S1 and Lugu Lake; *Rana pleuraden* tested positive at Qujing and Chenggong.

We prepared 36 sections from 18 toe clips for histological examination, which had tested positive for *Bd* by qPCR analysis. We did not find any evidence for the presence of *Bd* in these sections.

Discussion

We present evidence of *Bd*-infected captive and wild-caught introduced bullfrogs and native amphibian species in the Yunnan Province. To our knowledge, this is the first report regarding *Bd* infection in amphibians in China. *Bd* appeared to be widespread at five sites between 1800–2900 m in elevation, and infect multiple species found in a permanent stream, an ephemeral stream, an ephemeral pond, and a permanent pond. Overall, we found a moderate *Bd* infection rate (15.18%, n = 259) and low zoospore loads (598.3 GEs). We used toe clips rather than swabbing as samples, which may underestimate the prevalence rate of *Bd* infection in postmetamorphic individuals in this study. As *Bd* infection distributes in skins, sampling a small section of skin from toe clips could yield a false negative more than swabbing or brushing a large area of the feet and ventral sides (Boyle et al., 2004; Hyatt et al., 2007; Soto-Azat et al., 2009). Previous studies have shown that histological examination can detect relatively intense infections of individuals in naturally infected species (Berger et al., 1998; Garner et al., 2006). We did not find typical histological signs of *Bd* in qPCR-positive samples, possibly because the zoospore loads in the samples might have been too low to be detected by a histological examination. The qPCR generally is a more sensitive technique for detecting *Bd* infection in amphibians than histological examination (Kriger et al., 2006; Hyatt et al., 2007).

We observed a widespread prevalence of *Bd* infection in apparently healthy native amphibians in the study areas, as observed in previous studies (Rachowicz and Vredenburg, 2004; Woodhams et al., 2007). It is possible that native frogs with symptoms of chytridiomycosis had already died before we surveyed the area, so we could not collect such individuals. In addition, there may be species-specific susceptibility to chytrid infection (Blaustein et al., 2005; Rollins-Smith et al., 2006). Some of the native amphibian species may show innate resistance to chytridiomycosis and may therefore act as reservoir hosts, given their capacity to maintain infection but not show clinical signs of disease.

Schloegel et al. (2009) recently showed that bullfrogs in markets in the United States that had been imported from mainland China or Taiwan were infected with *Bd*, implying that the bullfrogs had been infected in China. The results of this study confirm their concern. An approximate 10.8% prevalence of *Bd* in bullfrogs was found in the Qujing market. This prevalence was comparable to those of natural bullfrog populations at Lugu Lake and Kunming S1, but lower than the 17–100% prevalence in markets in most cities surveyed in the United States and the 14–100% prevalence reported for populations in other countries (Garner et al., 2006). All bullfrogs sampled in the Qujing market came from bullfrog farms. It is generally believed

that the prevalence of *Bd* in frog farms should be high due to the rearing of amphibians at high densities (Hanselmann et al., 2004). Since wildlife trade per se does not amplify the prevalence of pathogens in bullfrogs (Schloegel et al., 2009), the prevalence of *Bd* must have been low in the bullfrog farms in Yunnan. This finding strikingly contrasts with the common-sense understanding of the high prevalence of *Bd* in frog farms. More samples are needed to determine the prevalence of *Bd* in bullfrog markets and farms in Yunnan.

Our results suggest that trade in bullfrogs for human consumption could be an important contributor to the spread of *Bd* in China and internationally. As bullfrog legs from China have been exported to Europe, Mexico, the United States, Malaysia, and other Asian countries or regions (Warkentin et al., 2009), international trade in bullfrog legs could potentially spread *Bd* to native amphibian populations in these countries. Within China, there is a high probability that bullfrogs escaping from transportation facilities for live amphibians or from bullfrog farms and markets come into contact with local amphibian species and spread the disease to local amphibian populations. Since bullfrogs have been widely bred in farms and sold in markets in China and have also invaded the wild in large numbers (Li et al., 2006; Liu and Li, 2009), the *Bd* carried by bullfrogs may have spread widely to native amphibian populations across China, thereby threatening native amphibian diversity.

Current conservation efforts should focus on monitoring the distribution and spread of *Bd* in China. Because both the bullfrogs in markets and farms and the release of zoospore-contaminated water from bullfrog containers into drains can potentially spread *Bd* (Schloegel et al., 2009), strict regulations on the transportation of bullfrogs and the breeding of bullfrogs in markets and farms should be drafted to stop the spread of *Bd* by bullfrogs. There is an urgent need for understanding the susceptibility of native amphibian species to chytridiomycosis.

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