A survey for Batrachochytrium salamandrivorans in Chinese amphibians

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Abstract For the last decade, chytridiomycosis was considered to be caused by a single species of fungus, Batrachochytrium dendrobatidis (Bd), but a second chytrid species, Batrachochytrium salamandrivorans (Bs), was recently isolated from an infected Salamandra salamandra in the Netherlands. To date, Bs has only been found in the Netherlands. To assess whether Bs is present in China, we analyzed a total of 665 samples, including 425 wild samples, 41 preserved specimens, and 199 captive samples, from 30 different species, including both urodeles and anurans. Our sample sites covered 15 provinces in China. All of the samples tested negative for Bs, resulting in a 95% confidence limit for a prevalence of 0.6%. The absence of Bs observed in this large-scale survey in China has significant implications for amphibian conservation and for border trade management strategies intended to control amphibian diseases. We strongly recommend the continued close monitoring of Bs to verify the status of this potentially devastating amphibian fungus in China [Current Zoology 60 (6): 729–735, 2014].

Keywords Amphibian decline, Asia, Batrachochytrium salamandrivorans, China, Chytridiomycosis, Batrachochytrium dendrobatidis.

The emerging infectious disease chytridiomycosis has been identified as a major factor contributing to the decline and extinction of amphibian species at the global scale (Berger et al., 1998; Daszak et al., 2003; Lips et al., 2006; Walker et al., 2008; Vredenburg et al., 2010; Cheng et al., 2011). For the last decade, Batrachochytrium dendrobatidis (Bd) was thought to be the only fungus that caused chytridiomycosis. It was first recognized as a pathogenic organism in 1998 (Berger et al., 1998) and was described as a species of chytrid in 1999 (Longcore et al., 1999). This chytrid fungus has been found on every continent except Antarctica, and it is known to have affected more than 500 species of amphibians worldwide (Fisher et al., 2009; Olson et al., 2013).

However, a second chytrid species, Batrachochytrium salamandrivorans (Bs), was recently isolated from infected Salamandra salamandra in the Netherlands (Martel et al., 2013). Currently, Salamandra salamandra is the only reported host of Bs, which has brought Salamandra salamandra close to extinction in the Netherlands (Spitzen-van der Sluijs et al., 2013). The natural hosts of Bs remain unknown, but this species may have different amphibian hosts with Bd. For example, in an experimental setting, it has been shown that Bs fails to infect midwife toads, which are highly susceptible to Bd infection (Martel et al., 2013). The current molecular screening tests used for Bd are not able to accurately detect Bs. Therefore, it is inappropriate to estimate the distribution of Bs using the Bd mapping framework (Olson et al., 2013). Consequently, there is an urgent need to screen different amphibian species for Bs around the world to evaluate the distribution and potential risk of Bs emerging across the globe.

Two molecular methods have been developed to identify Bs infection. One method is a single-round polymerase chain reaction (PCR) assay in which a region of the 5.8S ribosomal RNA gene of Bs is amplified by a pair of specific primers developed by Martel et al. (2013). The other method is a duplex real-time PCR analysis, which is more sensitive than the single-round
PCR method, with a detection limit of 0.1 genomic equivalents of Bs zoospores (Blooi et al., 2013). For Bd (sister species of Bs) detection, a nested PCR assay has demonstrated more sensitivity than real-time PCR (Goka et al., 2009; Bai et al., 2012). Therefore, we conducted a sensitivity test to determine the sensitivity of the nested PCR assay in detecting Bs.

Commercial trade in amphibians is considered an important factor contributing to the spread of chytridiomycosis, with studies reporting the presence of Bd in pet stores, food markets, introduced amphibians, and the laboratory animal trade (Daszak et al., 2003; Garner et al., 2006; Weldon et al., 2007; Bai et al., 2012; Schloegel et al., 2012; Liu et al., 2013b). Therefore, it is extremely important to assess the risk of Bs spreading through amphibian trade. Additionally, museum collections can further our understanding of the history of emerging pathogens (Soto-Azat et al., 2010). For example, Bd has been present for many years on several continents where endemic lineages of Bd have been found. The earliest known record of Bd was collected in 1894 in Brazil, where Bd-GPL and Bd-Brazil have been present for a long time (Rodriguez et al., 2014). In Africa, the oldest Bd-positive specimens were collected in 1933 (Weldon et al., 2004). Bd-CAPE was found in South Africa and Europe (Farrer et al., 2011). In Asia, the earliest evidence of chytridiomycosis dates back to as early as 1902, but this finding has not yet been confirmed by PCR (Schloegel et al., 2012). These findings indicate that Bd has had a worldwide presence for a long time. Therefore, as the sister species of Bd, it is necessary to investigate the presence of Bs in preserved specimens to understand the history of this chytrid fungus and determine whether this is an old or new pathogen.

China’s large area and complicated terrestrial landscapes provide diverse climates and vegetation patterns that offer various habitats for amphibians (Xie et al., 2007). To date, Bd has been detected in 10 provinces of China (Bai et al., 2010; Bai et al., 2012; Zhu et al., 2014). However, the distribution and prevalence of the emerging Bs remain unknown, and there have been virtually no reports of Bs outside of the Netherlands. In the present study, we collected amphibians from the field, markets, aquaculture farms and museums in order to provide the first evaluation of possible Bs infections in Chinese amphibians.

1 Materials and Methods

1.1 Samples from wild amphibians

We conducted field samplings from 2007 to 2013 in 10 provinces, including Yunnan and Sichuan, which are located in a region recognized as a global biodiversity hotspot with many endemic and endangered amphibian species (Myers et al., 2000). In the Liaoning, Jilin, and Heilongjiang provinces, we collected native amphibian species in the summer when the daily maximum temperature exceeded 30°C. In the Xinjiang and Yunnan provinces, we collected samples during autumn when the daily maximum temperature rarely exceeded 25°C. Individuals from other provinces were collected during spring before the daily maximum temperature exceeded 25°C. In addition to native species, we also collected the introduced American bullfrog (Lithobates catesbeianus, referred to as the bullfrog), which is a known introduced species that has successfully invaded a wide area of China (Li et al., 2006; Liu and Li, 2009; Li et al., 2011; Liu et al., 2012; Liu et al., 2013a). A total of 20 bullfrog tadpoles were collected in the field using long-handled nets during daylight hours, while 26 post-metamorphic bullfrogs were collected by hand at night. Each sample was handled using a fresh pair of disposable latex gloves to prevent cross-contamination (Goka et al., 2009). Additionally, we rinsed our boots and equipment with 5% bleach before entering each location to prevent cross-contamination among the sites (Longcore et al., 2007).

We used two methods to sample Bs from the collected individuals: (i) epithelial swabbing (260 samples) in which each individual was sampled for Bs using the swab technique (Hyatt et al., 2007), and (ii) histology of phalanges (165 samples) in which the top of the third toe of the right hind foot was clipped (Bai et al., 2010). All of the post-metamorphic amphibians were released at their capture site after sampling. For tadpoles, the collected specimens were euthanized with ethyl ether, and then the mouthparts were collected. Both the swabs and tissue samples from the toe clips and mouthparts were preserved separately in 75% EtOH in 1.5-ml microcentrifuge tubes and were stored at –20°C in the laboratory (Bai et al., 2010).

1.2 Samples from amphibians in farms and food markets

The amphibians collected from the markets in Beijing were sampled for Bs using the swab technique (Hyatt et al., 2007). In the other 7 provinces, individuals collected from the farms and food markets were sampled by toe clipping (Bai et al., 2010). Captive samples collected from farms in Huzhou, Zhejiang province were sampled during summer. Other samples were collected during winter and spring. Both swab and tissue
samples from toe clips and mouthparts were preserved separately in 75% EtOH in 1.5-ml microcentrifuge tubes and were stored at –20°C in the laboratory (Bai et al., 2010).

1.3 Samples from preserved amphibians

We used the swab technique to sample the preserved amphibians (Hyatt et al., 2007). These amphibian specimens were collected from the field across 8 provinces between the years of 1957 and 2006. All of the specimens were preserved in 10% buffered formalin at the Chongqing Museum of Natural History. The sampling season was unknown for all but 16 specimens (nine in spring, three in summer, three in autumn, and one in winter). To prevent possible cross-contamination between specimens preserved in the same jars, each individual was rinsed with 70% EtOH before sampling (Cheng et al., 2011).

1.4 Laboratory analysis

1.4.1 Extraction of DNA from swab samples and histology samples

DNA was extracted from the swabs following the protocol described by Goka et al. (2009). Each toe sample (approx. 1 to 3 mg wet weight) was placed into a microcentrifuge tube containing 150 µl of lysis buffer [1 mg/ml proteinase K, 0.1 M EDTA, 0.01 M NaCl, 0.01 M Tris-HCl (pH 8.0) and 0.5% Nonidet P-40]. The tubes were centrifuged for 30 sec and then incubated at 50°C for 2 hrs and then at 95°C for 20 min. After incubation, 10 µl of supernatant was diluted to 10% of its original concentration in TE buffer and used as a DNA template for PCR.

1.4.2 Nested PCR assay

We diluted the DNA template solutions, which were provided by Prof. An Martel at Ghent University, to 100, 10, 1, 0.1, 0.01 and 0.001 genomic equivalents of Bs zoospores (GE) in each PCR reaction. We conducted nested PCR to detect Bs in these samples.

All DNA templates extracted from swab and histology samples were amplified using nested PCR. The primers used 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 the primers STerF and STerR to amplify the first-round PCR products (Martel et al., 2013).

For the first amplification, the total reaction volume was 25 µl and contained 2 µl of DNA template, 10x PCR Buffer (200 mM Tris-HCl [pH 8.4], 20 mM MgSO₄, 200 mM KCl, 100 mM (NH₄)₂SO₄ and PCR enhancer), 0.2 mM of each dNTP, 0.4 µM of each primer, and 1.25 units of TransStart Taq DNA polymerase (Beijing TransGen Biotech, Beijing, China). The PCR conditions were as follows: 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 total reaction volume was 20 µl and contained 1.5 µl of first-round PCR product, 10x PCR Buffer (200 mM Tris-HCl [pH 8.4], 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 200 mM KCl and PCR enhancer), 0.2 mM of each dNTP, and 0.8 units of TransStart Taq DNA polymerase (Beijing TransGen Biotech, Beijing, China). The PCR amplification was performed under the following conditions: 10 min at 93°C, followed by 30 cycles of 45 s at 93°C, 45 s at 61°C, 60 s at 72°C, and 10 min at 72°C.

For each amplification step, we included a positive control using a DNA template solution containing 0.1 zoospore equivalents per µl and a negative control using TE buffer without any DNA. Each sample was tested in duplicate.

2 Results

The sensitivity test revealed that nested PCR could detect Bs with as little as 0.01 GE (Fig. 1). Therefore, the nested PCR assay is more sensitive than the realtime PCR assay described by Blooi et al. (2013).

A total of 665 samples representing 30 species were collected from museums, food markets, farms, and the field. There were 425 individuals representing 18 species sampled from the field, 199 captive individuals representing 7 species collected from 6 farms and 7 food markets, and 41 individuals representing 9 species collected from museums (Tables 1, 2, 3, Fig. 2).

The nested PCR results revealed that all of the samples in our study area were negative for Bs, with a 95% confidence limit for a prevalence of 0.6%.

3 Discussion

To date, Bs has only been found in the Netherlands (Martel et al., 2013). The present study focused on Chinese amphibians and represents the first effort to test for Bs in another region of the world. Our sample sites were located across a wide latitude range, between 22.26° N and 48.43° N. Moreover, we sampled a total of 30 amphibian species including both urodele (the original infected taxa in the Netherlands, Martel et al., 2013) and anuran species. All 665 specimens sampled in our study area were negative for Bs. Although we cannot state conclusively that Bs is absent in Chinese amphibians
Fig. 1  Results of the sensitivity test for nested PCR

‘100, 10, 1, 0.1, 0.01 and 0.001’ represent genomic equivalents of zoospores (GE) in each PCR reaction. N’ represents the negative control, which used TE buffer instead of DNA template in the amplification reaction.

Table 1  List of wild amphibian populations from ten provinces of China tested for Batrachochytrium salamandrivorans infection

| Province     | Sample season | Species                  | No. examined |
|--------------|---------------|--------------------------|--------------|
| Beijing      | spring        | Rana chensinensis        | 15           |
|              |               | Bufo gargarizans         | 15           |
|              |               | Pelophylax nigromaculatus | 20          |
| Heilongjiang | summer        | Rana amurensis           | 3            |
|              |               | Pelophylax hubeiensis    | 4            |
|              |               | Fejervarya limnocharis   | 3            |
|              |               | Pelophylax nigromaculatus | 4           |
|              |               | Pelophylax nigromaculatus | 7           |
|              |               | Fejervarya limnocharis   | 1            |
|              |               | Microhyla ornata         | 2            |
| Hubei        | spring        | Pelophylax nigromaculatus | 20          |
|              |               | Fejervarya limnocharis   | 1            |
|              |               | Microhyla ornata         | 1            |
| Hunan        | spring        | Hoplobatrachus tigerinus | 2            |
|              |               | Fejervarya limnocharis   | 6            |
|              |               | Pelophylax nigromaculatus | 1           |
|              |               | Microhyla ornata         | 1            |
| Jilin         | summer        | Pelophylax nigromaculatus | 20          |
| Liaoning     | summer        | Pelophylax nigromaculatus | 20          |
| Sichuan      | spring        | Pelophylax nigromaculatus | 5           |
| Xinjiang     | autumn        | Bufo pfezowi             | 53           |
|              |               | Pelophylax ridibunda     | 24           |
|              |               | Rana arvalis             | 38           |
|              |               | bufo bufo                | 5            |
| Yunnan       | autumn        | Lithobates catesbeianus  | 16           |
|              |               | chaochiaoensis           | 15           |
|              |               | Babina plesuaden         | 18           |
|              |               | Bombina maxima           | 7            |
|              |               | Microhyla ornata         | 10           |
| Zhejiang     | spring        | Lithobates catesbeianus  | 30           |
|              |               | Pelophylax nigromaculatus | 10          |
|              |               | Hyla chinesis            | 10           |
|              |               | Fejervarya limnocharis   | 10           |
|              |               | Microhyla ornata         | 10           |
|              |               | Bufo gargarizans         | 10           |
|              |               | Pelophylax plancyi       | 10           |
| **Total**    |               |                          | **425**      |

Table 2  List of amphibians from food markets and farms in China tested for Batrachochytrium salamandrivorans infection

| Cities       | Sample season | Species                  | No. examined |
|--------------|---------------|--------------------------|--------------|
| Farms        |               |                          |              |
| Beijing      | winter        | Andrias davidianus       | 18           |
| Huarong      | spring        | Lithobates catesbeianus  | 20           |
| Hougang      | spring        | Lithobates catesbeianus  | 18           |
| Huzhou-M1    | summer        | Lithobates catesbeianus  | 10           |
| Huzhou-M2    | summer        | Lithobates catesbeianus  | 10           |
| Zhangjiajie  | spring        | Lithobates catesbeianus  | 10           |
| Food markets |               |                          |              |
| Beijing-M1   | spring        | Lithobates catesbeianus  | 12           |
| Beijing-M2   | winter        | Ambystoma mexicanum      | 7            |
|              |               | Rana chensinensis        | 10           |
|              |               | Xenopus laevis           | 13           |
| Beijing-M3   | winter        | Xenopus laevis           | 10           |
| Dali         | spring        | Lithobates catesbeianus  | 7            |
| Panzhuhua    | spring        | Xenopus laevis           | 2            |
|              |               | Lithobates catesbeianus  | 3            |
| Shijiazhuang | winter        | Cynops orientalis        | 5            |
|              |               | Xenopus laevis           | 5            |
|              |               | Cynops orientalis        | 4            |
|              |               | Lithobates catesbeianus  | 5            |
|              |               | Bufo gargarizans         | 5            |
| Zhengzhou    | winter        | Xenopus laevis           | 5            |
|              |               | Cynops orientalis        | 5            |
|              |               | Lithobates catesbeianus  | 15           |
|              |               | Total                    | **199**      |

Based on these results, we can assume that the prevalence of Bs across our study regions is only 0.6% according to a 95% confidence limit if we assume that all sampled species and individuals have an equal likelihood of carrying this pathogen. The exact reasons for our inability to detect Bs in the present study are unclear. Temperature may be a potential reason. The optimal temperature for Bs growth is
Table 3  List of preserved amphibians from six provinces of China tested for Batrachochytrium salamandrivorans infection

| Species                  | Province          | Sampling period        | No. examined |
|--------------------------|-------------------|------------------------|--------------|
| Andrias davidianus       | Sichuan, Chongqing| 1973.11, 1986.02, no data | 6            |
| Batrachuperus pinchonii  | Sichuan, Hubei    | 1979, 1992, no data    | 6            |
| Batrachuperus tibetanus  | Sichuan           | 1982                   | 1            |
| Cynops cyanurus          | Yunnan            | 1987.03                | 4            |
| Ichthyophis bannanicus   | Guangxi           | 2006.09                | 2            |
| Liu shihi                | Chongqing, Hubei  | 1957.06, 1979.05, 1998 | 14           |
| Paramesotriton chinensis | Guizhou           | 1981.06                | 1            |
| Tylototriton kweichowensis | Guizhou        | no data                | 2            |
| Tylototriton verrucosus  | Yunnan            | 1958.06, 1982          | 5            |
| Total                    |                   |                        | 41           |

10°C to 15°C under laboratory conditions, and Bs will die when the environmental temperature exceeds 25°C (Martel et al., 2013). Most of the wild amphibians were sampled in the spring, but the daily maximum temperature of these sampling sites in the summer often exceeded 30°C. Previous studies have detected the sister species Bd in 10 of the provinces sampled in our study (Bai et al., 2010; Bai et al., 2012; Zhu et al., 2014). It has been suggested, however, that there is a difference in thermal niches between Bd and Bs, which prefers a substantially lower thermal limit (Martel et al., 2013). Therefore, Bs may be more sensitive to high temperatures. Therefore, we suggest that future studies focus on regions at higher latitudes and elevations with lower temperature conditions that may be more suitable for Bs.

Moreover, we did not find Bs present in any of the captive amphibians from the farms, markets or museums. This finding further demonstrates that Bs may be absent in Chinese amphibians. Farms and markets, however, are considered prime locations for the spread of wildlife pandemics through the circulation of introduced host species for amphibian diseases (Li and Li, 1998; Garner et al., 2006; Schloegel et al., 2009; Liu et al., 2013a). Therefore, to control the possible inadvertent introduction of an emerging disease, including Bs, to naïve populations, the amphibians in pet stores and food markets should be regularly screened using non-invasive methods. Additionally, human activities are also considered an important factor in the spread of exotic amphibian species (Li et al., 2006; Liu et al., 2014), and the government should implement strict management policies to control the introduction of alien species into the wild. Finally, we strongly recommend...
that wild amphibians be periodically sampled and tested for Bs to intensively monitor for this potentially devastating amphibian fungus.

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