Asian Herpetological Research 2017, 8(3): 190–198
DOI: 10.16373/j.cnki.ahr.170010

ORIGINAL ARTICLE

Amphibians Testing Negative for B*atrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* on the Qinghai-Tibetan Plateau, China

Supen WANG[1#], Wei ZHU[1,2#], Liqing FAN[1,2,3#], Jiaqi LI[4] and Yiming LI[1,2*]

[1] *Key Laboratory of Animal Ecology and Conservation Biology*, *Institute of Zoology*, *Chinese Academy of Sciences*, *Beijing* 100101, *China*
[2] *University of Chinese Academy of Sciences*, 19 *Yuquan Road*, *Beijing* 100049, *China*
[3] *Institute of Plateau Ecology*, *Agriculture and Animal Husbandry College of Tibet University*, *No.* 8 *Xueyuan Street*, *Bayi Town*, *Linzhi County*, *Xizang Province* 860000, *China*
[4] *Nanjing Institute of Environmental Sciences under Ministry of Environmental Protection of China. No.* 8 *Jiang Wang Miao Street*, *Nanjing* 210042, *China*

Abstract A disease caused by the fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) is responsible for recent worldwide declines and extinctions of amphibian populations. The Qinghai-Tibetan Plateau (QTP) is aglobal biodiversity hotspot, yet little is known about the prevalence of *Bd* and *Bsal* in this region. In this study, we collected 336 non-invasive skin swabs from wild amphibians (including an exotic amphibian species) on the QTP. In addition, to assess the historical prevalence of *Bd* and *Bsal* on the QTP, we collected 117 non-invasive skin swabs from museum-archived amphibian samples (from 1964–1982) originating from the QTP. Our results showed all samples to be negative for *Bd* and *Bsal*. The government should ban the potentially harmful introduction of non-native amphibian species to the QTP and educate the public about the impacts of releasing exotic amphibians from chytrid-infected areas into native environments of the QTP.

Keywords Chytridiomycosis, Amphibians, invasive species, museum specimens, Qinghai-Tibetan Plateau

1. Introduction

Chytridiomycosis, an emerging lethal fungal disease caused by the chytrid fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been implicated in declines and extinctions of amphibian species worldwide (Fisher *et al*., 2012; Kilpatrick *et al*., 2010). A number of studies have shown that *Bd* can infect all major amphibian orders (Anura, Caudata and Gymnophiona) and cause mortality (Berger *et al*., 1998; Davidson *et al*., 2003; Gower *et al*., 2013). To date, *Bd* has been detected in over 600 amphibian species worldwide, and declines in more than 200 amphibian species have resulted from *Bd* infection (Fisher *et al*., 2009; Olson *et al*., 2013; Skerratt *et al*., 2007). Previous studies have shown that *Bd* occurs on every continent except for Antarctica (Fisher *et al*., 2009) and that *Bd* has played a major role in the collapse of amphibian populations in the Neotropics, Australia, Europe, the USA, and east Africa (Berger *et al*., 1998; Cheng *et al*., 2011; Lips *et al*., 2006; Rosa *et al*., 2013; Vredenburg *et al*., 2010; Walker *et al*., 2008). *Bd* was first identified in 1997, later named in 1999 (Longcore *et al*., 1999), and was the only known *Batrachochytrium* species until Martel *et al*. (2013) discovered a new species of chytrid fungal pathogen, *Batrachochytrium salamandrivorans* (*Bsal*), in fire salamanders (*Salamandra salamandra*). Compared with *Bd*, *Bsal* exhibits a narrower host range, limited to Caudata, and its distribution is currently restricted to only four European countries (the United Kingdom, Belgium,

[#] These authors contributed equally to this paper.
[*] Corresponding author: Prof. Yiming LI, from Institute of Zoology, Chinese Academy of Sciences, with his research focuses on conservation biology and ecology of amphibians.
E-mail: liym@ioz.ac.cn
Received: 6 February 2017 Accepted: 4 July 2017

the Netherlands and Germany) (Cunningham *et al*., 2015; Martel *et al*., 2013, 2014; Sabino-Pinto *et al*., 2015) and a country in Asia (Vietnam) (Laking *et al.*, 2017). Two previous studies have shown that *Bsal* displays higher pathogenicity and causes more mortality in infection experiments in newts and salamanders (Martel *et al*., 2013, 2014). Furthermore, *Bsal* has been implicated in the collapse of the *S. salamandra* population in the Netherlands (Spitzen-van der Sluijs *et al*., 2013) as well as that of some amphibians in captive collections in the UK (Cunningham *et al*., 2015).

In Asia, *Bd* has been reported to infect wild amphibians from 11 countries, Indonesia, Japan, South Korea, China, Malaysia, Kyrgyzstan, Laos, The Philippines, India, Sri Lanka, and Vietnam, without a clear geographic pattern (Bai *et al*., 2010, 2012; Bataille *et al*., 2013; Dahanukar *et al*., 2013; Goka *et al*., 2009; Kusrini *et al*., 2008; Savage *et al*., 2011; Swei *et al*., 2011; Yang *et al*., 2009; Zhu *et al*., 2014a, 2016). In China, the presence of *Bd* has been assessed in 24 provinces (Zhejiang, Hubei, Hunan, Guizhou, Sichuan, Yunnan, Fujian, Guangxi, Chongqing, Guangdong, Taiwan, Hainan, Anhui, Tibet, Beijing, Heilongjiang, Jilin, Liaoning, Qinghai, Shandong, Shaanxi, Xinjiang, Shanxi, Henan) (Bai *et al*., 2010, 2012; Zhu *et al*., 2014a, 2016), with positive results and a discontinuous distribution for 11 (Shaanxi, Xinjiang, Beijing, Heilongjiang, Jilin, Sichuan, Yunnan, Chongqing, Guizhou, Hubei, Taiwan). Compared with *Bd*, little research on *Bsal* has been conducted in Asia. Only one survey of *Bsal* in China has been conducted (Zhu *et al*., 2014b); in this survey, 665 individual amphibians of 30 species (including urodeles and anurans) from 13 provinces and two municipalities in China were tested. None of the tested samples were positive for *Bsal*. In summary, surveys for *Bd* and *Bsal* have been made in several provinces in China, but the Qinghai-Tibetan Plateau (QTP) has not been surveyed for this emerging disease.

The QTP is the highest (approximately 4500 m above sea level on average) and most extensive plateau in the world, covering an area of 2.5×10^6 km^2 (Zhou *et al*., 2006).The QTP is an important biodiversity hotspot, crossing three biodiversity hotspots together with its adjacent areas: Indo-Burma, the Himalayas, and the mountains of southwestern China (Mittermeier *et al*., 2011). The extensive variation in the topography and climate of the QTP supports a number of different habitats and numerous species (Mittermeier *et al*., 2011). To date, there are 56 amphibian species reported on the QTP, including the salamanders *Batrachuperus tibetanus*, *Andrias davidianus* and *Batrachuperus karlschmidti* and 53 anuran species (Amphibia China, 2016). Furthermore, some studies have revealed that the QTP has been experiencing faster warming than low-elevation regions at the same latitude (Liu and Chen, 2000; Qin *et al*., 2009; Wei and Fang, 2013). Thus, chytridiomycosis may become more prevalent due to global warming (Di Rosa *et al*., 2007).

Therefore, in the present study, we collected 453 native and invasive anurans and caudates, including wild and museum-preserved amphibians, to assess the current and historical distribution of *Bd* and *Bsal* on the QTP.

2. Materials and Methods

2.1 Wild amphibians This study was conducted at three locations on the QTP (Figure 1): Lhasa, Nyingchi, and Ruoergai, from 2012 to 2015. A total of 228 individuals of native amphibians representing the two most widespread species on the QTP (*Nanorana parkeri* and *Rana kukunoris*) were collected during the spring and autumn from various habitat types, including ephemeral puddles, man-made reservoirs, and natural wetlands. In addition to native species, we also collected 108 individuals of the black-spotted frog (*Pelophylax nigromaculatus*), which has successfully invaded several areas of the QTP (Wang *et al*., 2017; Zhang *et al*., 2015).

2.2 Museum-preserved amphibians Historical samples from 1964–1982 were obtained from the herpetological collections of the Shanxi Institute of Zoology. All of these specimens (117 individuals of 11 species) came from the QTP, were preserved in 10% buffered formalin, and were adults. The sampling time ranged from March to August except at three sampling sites.

2.3 Sampling All individuals were sampled using the non-invasive skin swab technique (Hyatt *et al*., 2007). Each wild specimen was handled using a fresh pair of disposable gloves to prevent cross-contamination. To control potential transmission of disease between wild amphibians from different zones, strict biosecurity protocols were followed, employing procedures including washing, disinfecting, and drying all equipment before leaving a sampling location (Phillott *et al*., 2010). Each museum specimen was rinsed with 70% EtOH before sampling because these specimens are preserved in jars containing 2 or more individuals. We preserved all of the obtained swabs in 70% EtOH in 1.5 ml microcentrifuge tubes. These microcentrifuge tubes were kept in the dark and transferred to cool storage during field work and

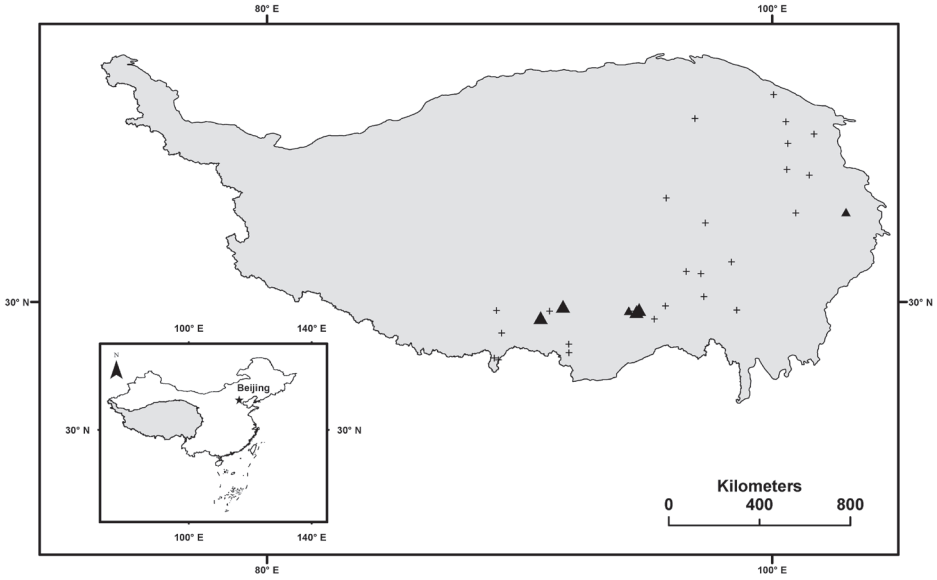

Figure 1 Distribution of the 453 individual amphibians sampled on the QTP to determine the presence of *Batrachochytrium salamandrivorans* and *Batrachochytrium salamandrivorans*. The gray area indicates the Qinghai-Tibet Plateau. Triangles denote the sites where wild samples were collected (large triangles: >50 individuals; smaller triangles, <20 individuals). Crosses denote the sites where preserved samples were collected.

were finally stored at −20°C in the laboratory for further analysis.

2.4 Laboratory analysis Scientific names were standardized following the taxonomy of Amphibia Web (www.amphibiaweb.org). DNA was isolated from the swabs using published methods (Goka *et al*., 2009). First, we deposited each swab in a microtube containing 200 µl of lysis buffer (containing 0.01 M NaCl, 0.01 M Tris-HCl (pH 8.0), 0.1 M EDTA, 0.5% Nonidet P-40 and 1 mg ml^{-1} proteinase K). Second, we used a vortex mixer to shake each microtube for 1 min. Each tube was then centrifuged at 4208 × g for 1 min. Third, the supernatants were transferred to a new tube. Fourth, the tubes were incubated for 2 h at 50°C and subsequently for 20 min at 95°C. Fifth, the microtubes were centrifuged at 16 831 × g for 3 min at 4°C. Finally, we extracted the supernatant, which was subsequently diluted 1:10 with 0.25 × TE buffer and used as the DNA template for PCR.

To test for *Bd* and *Bsal*, we employed the nested PCR technique to amplify the *Bd*-specific internal transcribed spacer sequence ITS1-5.8S-ITS2. Each sample was tested twice. In the first PCR step, the primer pair Bd18SF1 (5'-TTTGTACACACCGCCCGTCGC-3') and Bd28SR1 (5'-ATATGCTTAAGTTCAGCGGG-3') was used (White *et al*., 1990; Gaertner *et al*., 2009); in the second PCR step, Bd1a (5'-CAGTGTGCCATATGTCACG-3') and Bd2a (5'-CATGGTTCATATCTGTCCAG-3') were employed for *Bd* identification (Annis *et al*., 2004). STerF and STerR were used for *Bsal* identification (Gaertner *et al*., 2009; Martel *et al*., 2013; White *et al*., 1990). PCR was performed following published procedures (Bai *et al*., 2010; Zhu *et al*., 2014b, 2016). The first PCR amplification conditions for *Bd* were 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 59°C, and 1 min at 72°C and a final 10 min at 72°C. The second PCR amplification conditions for *Bd* were 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C and a final 5 min at 72°C.The first PCR amplification conditions for *Bsal* were 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 59°C, and 1 min at 72°C and a final 10 min at 72°C. The second PCR amplification conditions for *Bsal* were 10 min at 93°C, followed by 30 cycles of 45 s at 93°C, 45 s at 61°C, and 60 s at 72°C and a final 10 min at 72°C. The positive control used a DNA template solution (*Bd* DNA template solutions from our lab and *Bsal* DNA template solutions provided by Prof. Martel at Ghent University) containing 0.1 zoospore equivalents per µl, and the negative control contained TE buffer without any DNA. PCR amplification products were examined by 1% agarose gel electrophoresis.

We calculated pathogen prevalence for both *Bd* and *Bsal* as the number of infected animals divided by the sample size. In addition, we calculated the 95% Clopper-Pearson binomial confidence intervals in R version 2.15 (R Development Core Team, 2012).

3. Results

Overall, we swabbed 453 individuals comprising 24.1% of all extant QTP amphibian species (13/58) from 32 sites across the QTP (Table S1; Figure 1). A total of 336 field samples from two native species (*N. parkeri* and *R. kukunoris*) and an alien species (*P. nigromaculatus*) of amphibians were collected on the QTP (Figure 1; Table 1). All wild samples tested negative for *Bd* and *Bsal*. In total,117 individuals of 11 species (one Caudata and 116 anurans) were sampled from museums (Table 2). All preserved specimens tested also negative for *Bd* and *Bsal*.

Table 1 Wild amphibians tested for *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* infection on the Qinghai-Tibet Plateau, China. All specimens tested negative for *Bd* and *Bs*.

Sampling site	Species	No. examined	Elevation (m)	Latitude (°S)	Longitude (°E)	95% Confidence Interval
Nyingchi-1	*Nanorana parkeri*	54	3700	29.704	94.729	0–6.6
Nyingchi-2	*Nanorana parkeri*	18	3300	29.613	94.622	0–18.53
Nyingchi-3	*Nanorana parkeri*	51	3200	29.671	94.335	0–6.98
Lhasa-1	*Nanorana parkeri*	100	4000	29.836	91.718	0–3.62
Lhasa-2	*Pelophylax nigromaculatus*	108	3600	29.378	90.829	0–3.36
Ruoergai	*Rana kukunoris*	5	3500	33.573	102.936	0–52.18
Total	*Nanorana parkeri*	223				0–1.64

4. Discussion

We sampled 453 amphibians (both urodele and anuran species) from the QTP. We failed to detect *Bd* and *Bsal* in any of the samples of the 13 species that were tested. To our knowledge, this was the first study to investigate the presence of *Bd* and *Bsal* on the QTP at this scale.

We assumed that the 95% confidence limit for *Bd* and *Bsal* prevalence for *R. kukunoris* is 52.18%. Considering the recommendation of Skerratt *et al*. (2008), our sampling required >59 individuals in a site to detect *Bd* at a lowinfection rate, and we needed greater sampling for *R. kukunoris* to provide strong evidence for prevalence in this region. Compared to other studies onthe genera *Nanorana* and *Pelophylax* at China (Swei *et al*., 2011; Zhu *et al*., 2014b, 2016), *Bd* and *Bsal* generally occur at a low prevalence, and our results are consistent with these studies.

We did not observe *Bd* and *Bsal* in high altitude localities (850m–4900m) on the QTP. There have been several studies that have noted a correlation between the prevalence of *Bd* and elevation. For example, Muths *et al*. (2008) found that the probability of *Bd* prevalence in amphibians was negatively correlated with elevation in the Rocky Mountains, USA. Grundler *et al*. (2012) reported that the prevalence of *Bd* showed a positive correlation with elevation in the Atlantic Coastal Forest of Brazil. In contrast, Knapp *et al*. (2011) suggested that there was no relationship between elevation and *Bd* prevalence in the Sierra Nevada, USA). Overall, the available results indicate that the relationship between elevation and *Bd* prevalence is complicated. The present study failed to detect an influence of elevation on the distribution of *Bd*. According to James *et al*. (2015), regions where *Bd* is absent or is present at a low prevalence (‘cold spots’) may be common. Thus, one explanation for why *Bd* could be absent on the QTP is that it might never have dispersed there. However, the specific reasons for our failure to detect *Bd* on the QTP remain uncertain and will require additional study. Although we only tested one historical sample of a salamander for *Bsal*, this study is the first to report the prevalence of *Bsal* on the QTP. Targeted (or focused) monitoring on the QTP will be required, particularly because *Bsal* is suspected to be endemic to Asia (Martel *et al*., 2014).

We did not find evidence of *Bd* or *Bsal* in the 117 archived amphibian specimens. Some caution should be exercised in interpreting our results. First, previous studies have shown that formalin can cause reversible cross-linking of DNA. Thus, formalin may reduce the likelihood that the pathogen will be detected based on molecular assays when the sampled amphibians are preserved in this way (Soto-Azat *et al*., 2009). However, previous researchers have used similar methodologies for DNA extraction, and nested PCR has proven to be successful in amplifying *Bd* from formalin-fixed

Table 2 Historical specimens tested for *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* infection on the Qinghai-Tibet Plateau. "- -" indicate no data. All specimens tested negative for *Bd* and *Bs*.

Sampling site	Species	Museum number	No. examined	Collection Date	Elevation (m)	Latitude (°S)	Longitude (°E)
Qinghai1	*Bufo raddei*	640014–640024	11	1964/08	3250	37.147	100.541
Qinghai2	*Bufo raddei*	800002–800005	4	1980/03	2800	37.266	96.941
Qinghai3	*Bufo raddei*	730008–730012	5	1973/06	--	38.213	100.057
Qinghai4	*Scutiger boulengeri*		2	--	--	36.274	100.615
Qinghai5	*Scutiger boulengeri*		3	--	--	33.132	97.350
Qinghai6	*Scutiger boulengeri*	820005–820006	2	1982/07	3450	35.029	101.468
Qinghai7	*Scutiger boulengeri*	820007–820018	12	1982/07	3200	35.251	100.578
Qinghai8	*Bufo gargarizans*	810005–810009	5	1981/03	--	36.649	101.652
Qinghai9	*Rana chensinensis*	710004–710010	7	1971/08	3600	33.527	100.935
Qinghai10	*Andrias davidianus*		1	--	--	34.123	95.796
Tibet1	*Scutiger boulengeri*	730024	1	1973/06	4100	27.713	89.156
Tibet2	*Scutiger boulengeri*	730025	1	1973/06	3250	31.593	98.389
Tibet3	*Scutiger boulengeri*	730026–730027	2	1973/06	4900	28.332	91.941
Tibet4	*Scutiger boulengeri*	770195–770196	2	1977/07	3900	29.675	89.085
Tibet5	*Scutiger boulengeri*	770254–770255	2	1977/07	4200	30.215	97.296
Tibet6	*Scutiger boulengeri*	730028	1	1973/06	4200	27.792	88.999
Tibet7	*Scutiger boulengeri*	770263–770267	5	1977/06	4000	29.679	98.592
Tibet8	*Xenophrys omeimontis*	770268–770271	4	1977/07	850	29.330	95.332
Tibet9	*Scutiger mammatus*	730041–730045	5	1973/06	3550	31.133	97.175
Tibet10	*Scutiger mammatus*	730046–730047	2	1973/06	3900	31.218	96.600
Tibet11	*Nanorana conaensis*	770516–770537	22	1977/06	3000	27.995	91.956
Tibet12	*Scutiger nyingchiensis*	770795	1	1977/06	3040	29.643	94.356
Tibet13	*Scutiger nyingchiensis*	732032	1	1973/08	2700	29.854	95.767
Tibet14	*Nanorana parkeri*	730046–730052	7	1973/08	--	28.782	89.289
Tibet15	*Nanorana parkeri*	740053	1	1974/06	3700	29.644	91.176
Tibet16	*Polypedates megacephalus*	770787–770794	8	1977/08	1200	--	--

amphibians (Zhu *et al*., 2014a). Therefore, the effects of formalin should not have affected our results. Second, we only sampled 117 archived amphibian specimens from the museum. This small sample size mayhave been responsible forthe failure to detect *Bd* and *Bsal* (Zhu *et al*., 2016). Therefore, to confirm our results, we should in the future testadditional amphibian museum specimens originating from the QTP that have been preserved and stored under better conditions.

Introducing non-native infected animals into native amphibian populations plays an important role in the spread of chytridiomycosis (Fisher and Garner, 2007). *Bd* has been detected in *P. nigromaculatus*in provinces adjacent to the QTP (e.g.,Chongqing, Sichuan, and Yunnan) (Zhu *et al*., 2014a). Nonetheless, the exotic frog *P. nigromaculatus* (Wang *et al*., 2017) tested negative for *Bd* and *Bsal* in the present study. To prevent the introduction of *Bd* and *Bsal* to native amphibians, we should suggest that the government ban the potentially harmful introduction of exotic species to the QTP. Moreover, the public needs to be educatedon the impacts of releasing exotic amphibians into native environments.

Although *Bd* and *Bsal* do not appear to be present in the examined amphibian populations, more surveys will need to be conducted to determine whether *Bd* and *Bsal* are present on the QTP. Because *Bd* and *Bsal* may be transmitted through the pet trade, future surveys should also target potentially vulnerable geographic areas, such as cities and regions with coastal ports or other centers of transportation on the QTP, such as Nyingchi

city, Lhasa city, and Xining city (Kolby *et al*., 2014). According to a previous study, *Bsal* primarily infects Caudata species (Martel *et al*., 2013, 2014). Thus, future surveys should target Caudata species found on the QTP, such as *B. tibetanus*, *A. davidianus* and *B. karlschmidti*. Moreover, our sampling sites were located in a narrow range compared to the entire area of the QTP (Figure 1). However, the QTP is at high risk of biological invasions (Li *et al*., 2016) and according to the pruned model, there is an appropriate distribution of *Bd* (Liu *et al*., 2013). Therefore, we suggest that more survey studies encompassing a greater scope of the QTP are needed to assess *Bd* and *Bsal*. Finally, as some non-amphibians can carry *Bd* (such as crayfish and waterfowl) (Garmyn *et al*., 2012; McMahon *et al*., 2013) and it is not known whether *Bsal* utilizes alternative hosts, future research should investigate potential non-amphibian hosts of *Bd* and *Bsal*.

Acknowledgements We thank Hong CHEN at the Shaanxi Institute of Zoology for access to specimen collections. We are also grateful to two anonymous reviewers for helpful comments on the draft of the manuscript. This research was funded by China Postdoctoral Science Foundation (2016M601132).The collection and handling of frogs were conducted by the Animal Care and Use Committee of Institute of Zoology, Chinese Academy of Sciences (Project Nos. 2012/5, 2013/4, 2014/5 and 2015/2).

References

Amphibia China. 2016. The database of Chinese amphibians. Kunming, Yunnan. Retrieved from http://www.amphibiachina.org

Annis S. L., Dastoor F. P., Ziel H., Daszak P., Longcore J. E. 2004. A DNA-based assay identifies *Batrachochytrium dendrobatidis in amphibians*. J Wildl Dis, 40: 420–428

Bai C. M., Garner T. W. J., Li Y. M. 2010. First evidence of *Batrachochytrium dendrobatidis* in China: Discovery of Chytridiomycosis in introduced American bullfrogs and native amphibians in the Yunnan Province, China. Ecohealth, 7: 127–134

Bai C. M., Liu X., Fisher M. C., Garner T. W. J., Li Y. M. 2012. Global and endemic Asian lineages of the emerging pathogenic fungus *Batrachochytrium dendrobatidis* widely infect amphibians in China. Divers Distrib, 18: 307–318

Bataille A., Fong J. J., Cha M., Wogan G. O. U., Baek H. J., Lee H., Min M. S., Waldman B. 2013. Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. Mol Ecol, 22: 4196–4209

Berger L., Speare R., Daszak P., Green D. E., Cunningham A. A., Goggin C. L., Slocombe R., Ragan M. A., Hyatt A. D., McDonald K. R., Hines H. B., Lips K. R., Marantelli G., Parkes H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci U S A, 95: 9031–9036

Cheng T. L., Rovito S. M., Wake D. B., Vredenburg V. T. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proc Natl Acad Sci U S A, 108: 9502–9507

Cunningham A. A., Beckmann K., Perkins M., Fitzpatrick L., Cromie R., Redbond J., O'Brien M. F., Ghosh P., Shelton J., Fisher M. C. 2015. Emerging disease in UK amphibians. Vet Rec, 176: 468–468

Dahanukar N., Krutha K., Paingankar M. S., Padhye A. D., Modak N., Molur S. 2013. Endemic Asian chytrid strain infection in threatened and endemic anurans of the northern Western Ghats, India. PLOS ONE, 8: e77528

Davidson E. W., Parris M., Collins J. P., Longcore J. E., Pessier A. P., Brunner J. 2003. Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum)*. Copeia, 2003: 601–607

Di RosaI., Simoncelli F., Fagotti A., Pascolini R. 2007. Ecology: the proximate cause of frog declines? Nature, 447: E4–5; discussion E5–6

Fisher M. C., Garner T. W. J. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. Fungal Biol Rev, 21: 2–9

Fisher M. C., Garner T. W. J., Walker S. F. 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annu Rev Microbiol, 63: 291–310

Fisher M. C., Henk D. A., Briggs C. J., Brownstein J. S., Madoff L. C., McCraw S. L., Gurr S. J. 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature, 484: 186–194

Gaertner J. P., Forstner M. R. J., O'Donnell L., Hahn D. 2009. Detection of *Batrachochytrium dendrobatidis* in endemic salamander species from Central Texas. Ecohealth, 6: 20–26

Garmyn A., Van Rooij P., Pasmans F., Hellebuyck T., Van den Broeck W., Haesebrouck F., Martel A. 2012. Waterfowl: Potential environmental reservoirs of the chytrid fungus *Batrachochytrium dendrobatidis*. PLOS ONE, 7: e35038

Goka K., Yokoyama J., Une Y., Kuroki T., Suzuki K., Nakahara M., Kobayashi A., Inaba S., Mizutani T., Hyatt A. D. 2009. Amphibian chytridiomycosis in Japan: Distribution, haplotypes and possible route of entry into Japan. Mol Ecol, 18: 4757–4774

Gower D. J., Doherty-Bone T., Loader S. P., Wilkinson M., Kouete M. T., Tapley B., Orton F., Daniel O. Z., Wynne F., Flach E., Müller H., Menegon M., Stephen I., Browne R. K., Fisher M. C., Cunningham A. A., Garner T. W. J. 2013. *Batrachochytrium dendrobatidis* infection and lethal Chytridiomycosis in caecilian amphibians (Gymnophiona). Ecohealth, 10: 173–183

Gründler M. C., Toledo L. F., Parra-Olea G., Haddad C. F. B., Giasson L. O. M., Sawaya R. J., Prado C. P. A., Araujo O. G. S., Zara F. J., Centeno F. C., Zamudio K. R. 2012. Interaction between breeding habitat and elevation affects prevalence but not infection intensity of *Batrachochytrium dendrobatidis* in Brazilian anuran assemblages. Dis Aquat Organ, 97: 173–184

Hyatt A. D., Boyle D. G., Olsen V., Boyle D. B., Berger L., Obendorf D., Dalton A., Kriger K., Heros M., Hines H., Phillott R., Campbell R., Marantelli G., Gleason F., Coiling A. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Dis Aquat Organ, 73: 175–192

James T. Y., Toledo L. F., Rödder D., da Silva Leite D., Belasen A. M., Betancourt-Román C. M., Jenkinson T. S., Soto-Azat C., Lambertini C., Longo A. V., Ruggeri J., Collins J. P., Burrowes P. A., Lips K. R., Zamudio K. R., Longcore J. E. 2015. Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: Lessons from the first 15 years of amphibian chytridiomycosis research. Ecol Evol, 5: 4079–4097

Kilpatrick A. M., Briggs C. J., Daszak P. 2010. The ecology and impact of chytridiomycosis: An emerging disease of amphibians. Trends Ecol Evol, 25: 109–118

Knapp R. A., Briggs C. J., Smith T. C., Maurer J. R. 2011. Nowhere to hide: impact of a temperature-sensitive amphibian pathogen along an elevation gradient in the temperate zone. Ecosphere, 2

Kolby J. E., Smith K. M., Berger L., Karesh W. B., Preston A., Pessier A. P., SkerrattL. F. 2014. First evidence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in Hong Kong amphibian trade. PLOS ONE, 9: e90750

Kusrini M. D., Skerratt L. F., Garland S., Berger L., Endarwin W. 2008. Chytridiomycosis in frogs of Mount Gede Pangrango, Indonesia. Dis Aquat Organ, 82: 187–194

Laking A. E., Ngo H. N., Pasmans F., Martel A., Nguyen T. T. 2017. *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. Sci Rep, 7: 44443

Li X., Liu X., Kraus F., Tingley R., Li Y. 2016. Risk of biological invasions is concentrated in biodiversity hotspots. Front Ecol Environ, 14: 411–417

Lips K. R., Brem F., Brenes R., Reeve J. D., Alford R. A., Voyles J., Carey C., Livo L., Pessier A. P., Collins J. P. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci U S A, 103: 3165–3170

Liu X. D., Chen B. D. 2000. Climatic warming in the Tibetan Plateau during recent decades. Int J Climatol, 20: 1729–1742

Liu X., Rohr J. R., Li Y. 2013. Climate, vegetation, introduced hosts and trade shape a global wildlife pandemic. Proc Biol Sci, 280: 20122506

Longcore J. E., Pessier A. P., Nichols D. K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia, 91: 219–227

Martel A., Blooi M., Adriaensen C., Van Rooij P., Beukema W., Fisher M. C., Farrer R. A., Schmidt B. R., Tobler U., Goka K., Lips K. R., Muletz C., Zamudio K. R., Bosch J., Lötters S., Wombwell E., Garner T. W. J., Cunningham A. A., Spitzen-van der Sluijs A., Salvidio S., Ducatelle R., Nishikawa K., Nguyen T. T., Kolby J. E., Van Bocxlaer I., Bossuyt F., Pasmans F. 2014. Wildlife disease. Recent introduction of a chytrid fungus endangers Western palearctic salamanders. Science, 346: 630–631

Martel A., Spitzen-van der Sluijs A., Blooi M., Bert W., Ducatelle R., Fisher M. C., Woeltjes A., Bosman W., Chiers K., Bossuyt F., Pasmans F. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci U S A, 110: 15325–15329

McMahon T. A., Brannelly L. A., Chatfield M. W. H., Johnson P. T. J., Joseph M. B., McKenzie V. J., Richards-Zawacki C. L., Venesky M. D., Rohr J. R. 2013. Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. Proc Natl Acad Sci U S A, 110: 210–215

Mittermeier R. A., Turner W. R., Larsen F. W., Brooks T. M., GasconC. 2011. Global biodiversity conservation: The critical role of hotspots. 3–22. In Zachos F. E., Habel J. C. (Eds.), Biodiversity hotspots. Berlin, Germany: Springer

Muths E., Pilliod D. S., Livo L. J. 2008. Distribution and environmental limitations of an amphibian pathogen in the Rocky mountains, USA. Biol Conserv, 141: 1484–1492

Olson D. H., Aanensen D. M., Ronnenberg K. L., Powell C. I., Walker S. F., Bielby J., Garner T. W. J., Weaver G., Bd Mapping Group, Fisher M. C. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis,* the amphibian chytrid fungus. PLOS ONE, 8: e56802

Phillott A. D., Speare R., Hines H. B., Skerratt L. F., Meyer E., McDonald K. R., Cashins S. D., Mendez D., Berger L. 2010. Minimising exposure of amphibians to pathogens during field studies. Dis Aquat Organ, 92: 175–185

Qin J., Yang K., Liang S. L., Guo X. F. 2009. The altitudinal dependence of recent rapid warming over the Tibetan Plateau. Clim Change, 97: 321–327

R Development Core Team. 2012. R: A language and environment for statistical computing. Vienna, Austria: R Foundation Statistical Computing. Retrieved from http://www.R-project.org/

Rosa G. M., Anza I., Moreira P. L., Conde J., Martins F., Fisher M. C., Bosch J. 2013. Evidence of chytrid-mediated population declines in common midwife toad in Serra da Estrela, Portugal. Anim Conserv, 16: 306–315

Sabino-Pinto J., Bletz M., Hendrix R., Perl R. B., Martel A., Pasmans F., Lötters S., Mutschmann F., Schmeller D. S., Schmidt B. R., Veith M., Wagner N., Vences M., Steinfartz S. 2015. First detection of the emerging fungal pathogen *Batrachochytrium salamandrivoransin* Germany. Amphib Reptil, 36: 411–416

Savage A. E., Grismer L. L., Anuar S., Onn C. K., Grismer J. L., Quah E., Muin M. A., Ahmad N., Lenker M., Zamudio K. R. 2011. First Record of *Batrachochytrium dendrobatidis* infecting four frog families from peninsular Malaysia. Ecohealth, 8: 121–128

Skerratt L. F., BergerL., Hines H. B., McDonald K. R., Mendez D., Speare R. 2008. Survey protocol for detecting chytridiomycosis in all Australian frog populations. Dis Aquat Organ, 80: 85–94

Skerratt L. F., Berger L., Speare R., Cashins S., McDonald K. R., Phillott A. D., Hines H. B., Kenyon N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. Ecohealth, 4: 125–134

Soto-Azat C., Clarke B. T., Fisher M. C., Walker S. F., Cunningham A. A 2009. Non-invasive sampling methods for the detection of *Batrachochytrium dendrobatidis* in archived amphibians. Dis Aquat Organ, 84: 163–166

Spitzen-van der Sluijs A., Spikmans F., Bosman W., de Zeeuw M., van der Meij T., Goverse E., Kik M., Pasmans F., Martel A. 2013. Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. Amphib Reptil, 34: 233–239

Swei A., Rowley J. J. L., Rödder D., Diesmos M. L. L., Diesmos A. C., Briggs C. J., Brown R., Cao T. T., Cheng T. L., Chong R. A., Han B., Hero J. M., Hoang H. D., Kusrini M. D., Le D. T., McGuire J. A., Meegaskumbura M., Min M. S., Mulcahy D. G., Neang T., Phimmachak S., Rao D. Q., Reeder N. M., Schoville S. D., Sivongxay N., Srei N., Stöck M., Stuart B. L., Torres L. S., Tran D. T., Tunstall T. S., Vieites D., Vredenburg V. T. 2011. Is chytridiomycosis an emerging infectious disease in Asia? PLOS ONE, 6: e23179

Vredenburg V. T., Knapp R. A., Tunstall T. S., Briggs C. J. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proc Natl Acad Sci U S A, 107: 9689–9694

Walker S. F., Bosch J., James T. Y., Litvintseva A. P., Oliver Valls J. A., Piña S., García G., Rosa G. A., Cunningham A. A., Hole S., Griffiths R., Fisher M. C. 2008. Invasive pathogens threaten species recovery programs. Curr Biol, 18: R853–R854

Wang S., Fan L., Liu C., Li J., Gao X., Zhu W., Li Y. 2017. The origin of invasion of the alien *Pelophylax nigromaculatus* in Tibet, China. Curr Zool. doi:10.1093/cz/zow1117

Wei Y. Q., Fang Y. P. 2013. Spatio-temporal characteristics of global warming in the Tibetan Plateau during the last 50 years based on a generalised temperature zone-elevation model. PLOS ONE, 8: e60044

White T., Bruns T., Lee S., Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, 18: 315–322

Yang H., Baek H., Speare R., Webb R., Park S., Kim T., Lasater K. C., Shin S., Son S., Park J., Min M., Kim Y., Na K., Lee H., Park S. 2009. First detection of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in free-ranging populations of amphibians on mainland Asia: survey in South Korea. Dis Aquat Organ, 86: 9–13

Zhang H., Fu H., Li W. 2015. *Pelophylax nigromaculatus* record in Qinghai Province was confirmed. Chin J Zool, 50: 145–147

Zhou S. Z., WangX. L., WangJ., XuL. B. 2006. A preliminary study on timing of the oldest Pleistocene glaciation in Qinghai-Tibetan Plateau. Quatern Int, 154–155: 44–51

Zhu W., Bai C., Wang S., Soto-Azat C., Li X., Liu X., Li Y. 2014a. Retrospective survey of museum specimens reveals historically widespread presence of *Batrachochytrium dendrobatidis in China*. Ecohealth, 11: 241–250

Zhu W., Fan L., Soto-Azat C., Yan S., Gao X., Liu X., Wang S., Liu C., Yang X., Li Y. 2016. Filling a gap in the distribution of Batrachochytrium dendrobatidis: Evidence in amphibians from northern China. Dis Aquat Organ, 118: 259–265

Zhu W., Xu F., Bai C. M., Liu X., Wang S. P., Gao X., Yan S. F., Li X. P., Liu Z. T., Li Y. M. 2014b. A survey for *Batrachochytrium salamandrivorans* in Chinese amphibians. Curr Zool, 60: 729–735

Appendix

Table S1 Information of amphibian species in QTP.

Species	Sampling species	Number of sampling	Species	Sampling species	Number of sampling
Amolops aniqiaoensis			*Nanorana medogensis*		
Amolops chayuensis			*Nanorana parkeri*	+	331
Amolops gerbillus			*Nanorana pleskei*		
Amolops medogensis			*Nanorana polunini*		
Amolops monticola			*Nasutixalus medogensis*		
Amolops nyingchiensis			*Odorrana zhaoi*		
Andrias davidianus	+	1	*Pelophylax nigromaculatus*	+	108
Batrachuperus karlschmidti			*Philautus kempii*		
Batrachuperus tibetanus			*Polypedates megacephalus*	+	8
Bufo gargarizans	+	5	*Rana kukunoris*	+	5
Bufo tuberculatus			*Rana chensinensis*	+	7
Bufotes zamdaensis			*Rhacophorus bipunctatus*		
Duttaphrynus cyphosus			*Rhacophorus burmanus*		
Duttaphrynus himalayanus			*Rhacophorus maximus*		
Feihyla vittata			*Rhacophorus translineatus*		
Gracixalus medogensis			*Rhacophorus tuberculatus*		
Ingerana borealis			*Rhacophorus verrucopus*		
Kurixalus verrucosus			*Scutiger boulengeri*	+	33
Liurana alpina			*Scutiger maculatus*		
Liurana medogensis			*Scutiger mammatus*	+	7
Liurana reticulata			*Scutiger nyingchiensis*	+	2
Liurana xizangensis			*Scutiger sikimmensis*		
Megophrys medogensis			*Scutiger spinosus*		
Megophrys pachyproctus			*Scutiger wuguanfui*		
Megophrys zhangi			*Strauchbufo raddei*	+	20
Nanorana blanfordii			*Theloderma andersoni*		
Nanorana chayuensis			*Theloderma baibengensis*		
Nanorana conaensis	+	22	*Theloderma moloch*		
Nanorana liebigii			*Xenophrys omeimontis*	+	4

[+] indicates sampling species. Information of amphibian species in QTP based on Amphibia China 2016, http://www.amphibiachina.org.