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# Early-spring survey for *Batrachochytrium dendrobatidis* in wild *Rana dybowskii* in Heilongjiang Province, China

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ABSTRACT: *Batrachochytrium dendrobatidis* has been investigated worldwide because of its importance in population declines in multiple species of amphibians; however, little is known regarding the disease status of all native amphibian species in China. The present study is the first survey of chytridiomycosis in free-ranging amphibian populations in China, and it examined the possible presence of *B. dendrobatidis* in *Rana dybowskii* in northeastern China (Heilongjiang Province). *R. dybowskii* is mainly distributed in the northeast part of China and is intensively hunted for human consumption, making populations vulnerable to extirpation in the event of additional stresses from disease epidemics. The survey was performed in early spring of 2008, using a PCR assay, histological examination of skin samples, and zoospore culture. In total, 191 frogs were examined; thus, a 95% confidence limit for prevalence of 1.57% was selected. Our results demonstrate that *R. dybowskii* is currently free from chytridiomycosis in Heilongjiang, even though the natural conditions of the sampling sites are suitable for the occurrence of *B. dendrobatidis*. Central and local governments should implement strict management measures to prevent the escape of non-native commercial amphibian species into this area, which might endanger local populations of native species.

KEY WORDS: *Batrachochytrium dendrobatidis* · Free-range · *Rana dybowskii* · Detection · China

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## INTRODUCTION

*Batrachochytrium dendrobatidis*, which belongs to the new genus *Batrachochytrium* (Phylum Chytridiomycota, Class Chytridiomycetes, Order Chytridiales; Longcore et al. 1999), has been identified as the causative agent of an emerging infectious disease in amphibians (Daszak et al. 2000) and may account for mass mortality, population declines, and extinction of various species of amphibians globally (Daszak et al. 2003). Since the first report of this disease (Berger et al. 1998), infections of *B. dendrobatidis* have been recorded in more than 90 amphibian species from Australia, New Zealand, Europe, Africa, and South, Central, and North America, from a broad range of habitats (Herrera et al. 2005).

Contrary to western countries, few investigations on the disease have been conducted in Asia. McLeod et al. (2008) revealed that *Batrachochytrium dendrobatidis* was not found in amphibians in Thailand in the last 60 yr. Rowley et al. (2007) also reported a negative result for the disease in 274 individuals of 4 native amphibian species and 137 imported amphibians in Hong Kong. The first case of *B. dendrobatidis* in Asia was reported in Japan among 45 exotic frogs of 18 species kept for breeding by a private owner (Une et al. 2008). At the same time, *B. dendrobatidis* was detected in 4 samples from *Rhacophorus javanus*, *Rana chalconota*, *Leptobrachium hasseltii*, and *Limnonectes microdiscus* in Indonesia (Kusrini et al. 2008). It is believed that *B. dendrobatidis* has not yet invaded in China (Xie et al. 2007).

The large land area of China and its highly varied terrain give rise to a wide array of climates and vegetation patterns that offer a variety of habitats (Xie et al.

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2007), which are exploited by 399 amphibian species (6.28% of the global species richness). However, few historical records are available on size changes of amphibian populations in most areas of China, and fungal diseases among natural populations have never been studied properly.

*Rana dybowskii* has long been hunted intensively for food consumption and scientific utilization. It is a typical inhabitant of forests in the mountain ranges of northeastern China, occupying a range of habitats from lowlands to areas above 1800 m.

Heilongjiang Province covers about 454 800 km$^2$ and borders on Russia and North Korea. The anuran community comprises 9 species belonging to 5 families (Bombinatoridae, Bufonidae, Hylidae, Ranidae, Microhylidae). This region may be more susceptible to climate warming than any other area in China (Cheng et al. 2004), and chytridiomycosis may become more prevalent under conditions of global warming (Di Rosa et al. 2007). The purpose of the present work was to reveal the existence of chytridiomycosis and its prevalence in wild populations of *Rana dybowskii* in northeastern China.

## MATERIALS AND METHODS

The study sites were the Kalunshan Forest Farm (Heihe), Shanhe Provincial Nature Reserve for *Rana dybowskii* (Acheng), the Shuangyi Forest Farm (Hebei Forest Bureau), and the Dumuhe Forest Farm (Dongfanghong Forest Bureau), which are situated in the Lesser Xingan Mountains, the Zhangguangcai Mountains, and the Wandashan Mountains, where *R. dybowskii* is mainly distributed in Heilongjiang (Fig. 1). In each of the 4 regions of collection, we chose 3 sampling sites located more than 3 km from each other on the banks of streams or ponds. At each sampling site, 15 samples were collected randomly. Site details are listed in Table 1. Another 11 frogs from Tieli submitted by the local forest farm were also examined (Fig. 1). These mountain areas are surrounded by ponds and streams of different sizes, offering ample habitat diversity. The climate is temperate and cold temperate continental monsoon climate; precipitation occurs throughout the year, though more intensely in summer and autumn. Frosts begin in mid-autumn and snow occurs in winter, and the minimum temperatures are recorded in January (Yu et al. 2009).

A detailed survey of the area during the early spring (5 to 15 April 2008) was conducted and frogs were captured on banks after hibernation. Once collected, the specimens were brought to the laboratory, where their general condition was assessed. Ventral and toe skin was excised and kept for patho-histological, molecular biological examinations and spore cultures separately. After samples were fixed in 10% formalin and embedded in paraffin, 5 µm thick tissue sections were cut and stained with hematoxylin and eosin according to routine protocols to detect the presence of chytrid fungus. The diagnosis of chytridiomycosis was made according to the guidelines provided by Berger et al. (1999). PCR was performed as described by Annis et al. (2004). Isolate culture followed the protocol suggested by Longcore (2000).

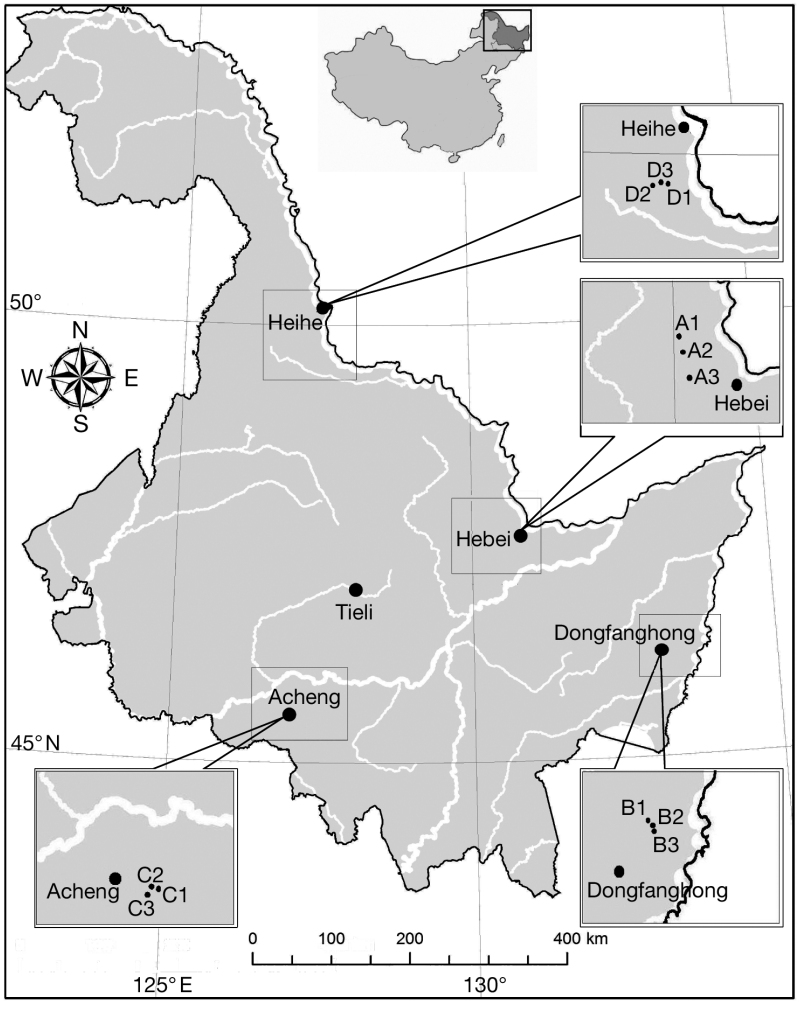


Fig. 1. Sampling regions in Heilongjiang Province, China

Table 1. *Rana dybowskii*. Clinical symptoms upon gross necropsy

| Regions | Location | Elevation (m) | No. with lesions /no. examined | Collection no. | Clinical signs |
|---|---|---|---|---|---|
| Hebei | 47° 59′ 45″ N, 130° 06′ 28″ E | 273 | 1/15 | A106 | Skin trauma on ventral surface and limbs |
| Forest | 47° 52′ 51″ N, 130° 10′ 36″ E | 226 | 1/15 | A204 | Skin ulcers on dorsal and ventral part |
| Bureau | 47° 39′ 58″ N, 130° 18′ 22″ E | 169 | 2/15 | A303 | Edema under skin |
| | | | | A312 | Small white spots on surface of liver |
| Dongfang-hong | 46° 24′ 11″ N, 133° 26′ 38″ E | 186 | 2/15 | B114 | Skin ulcers on fore-limbs |
| | | | | B115 | Trauma on ventral skin |
| Forest | 46° 21′ 15″ N, 133° 30′ 12″ E | 87 | 0/15 | | |
| Bureau | 46° 21′ 15″ N, 133° 30′ 12″ E | 103 | 1/15 | B308 | Small white spots on surface of liver |
| Acheng City | 45° 28′ 49″ N, 127° 35′ 36″ E | 343 | 1/15 | C111 | Hematoma under skin and congestion of intestinal tract |
| | 45° 28′ 54″ N, 127° 33′ 52″ E | 351 | 2/15 | C208 | Swollen spleen and gall, congestion of intestinal tract |
| | | | | C209 | Congestion in lung and hypodermis |
| | 45° 27′ 10″ N, 127° 31′ 55″ E | 322 | 2/15 | C307 | Congestion of skin |
| | | | | C315 | Edema under skin, grey color of liver with blutene chloaide |
| Heihe | 49° 51′ 19″ N, 127° 11′ 04″ E | 153 | 1/15 | D107 | Trauma on ventral skin |
| City | 49° 51′ 28″ N, 127° 02′ 15″ E | 360 | 2/15 | D201 | Congestion in hypodermis |
| | | | | D206 | Small skin ulcers on dorsal part |
| | 49° 52′ 07″ N, 127° 04′ 07″ E | 309 | 1/15 | D305 | Yellow spots on liver |

## RESULTS

Results of PCR, patho-histological examination, and zoospore culture indicated no evidence of *Batrachochytrium dendrobatidis* infection in wild *Rana dybowskii* populations in our study area. The results of the laboratory diagnosis of the specimens' general condition are shown in Table 1.

## DISCUSSION AND CONCLUSIONS

Our results from 191 sampled individuals provide no evidence to support the presence of the chytrid fungus in the population of *Rana dybowskii* in the area under investigation.

We assumed that a 95% confidence limit for prevalence is 1.57%. Considering that in other regions, *Batrachochytrium dendrobatidis* generally occurs at greater than 5% prevalence when endemic and weather conditions are favorable (Rowley et al. 2007), it appears that *Rana dybowskii* is free from *B. dendrobatidis*.

The early-spring environmental conditions (e.g. temperature, altitude, latitude) of the survey area are suitable for the occurrence of *Batrachochytrium dendrobatidis* according to previous records (Berger et al. 2004, Ouellet et al. 2005, Drew et al. 2006, Kriger et al. 2007). Temperature appears to be associated with outbreaks of fungal skin infections in amphibians. The optimal temperature for the development of the fungus is 17 to 25°C under laboratory conditions (Piotrowski et al. 2004); however, *B. dendrobatidis* can grow within a wide range of temperatures (4 to 25°C; Piotrowski et al. 2004). A previous study also illustrated that the chytrid fungus grows best at cooler temperatures (Longcore et al. 1999). At the sampling sites, the multi-year average temperature at the beginning of April is between 1 and 5°C with a fluctuation of 7.5°C (http://database.ce.cn/gqzlk/sgl/hlj/hldl/200711/14/t20071114_13597020.shtml); thus the average temperature is still within the range of temperatures that support the existence of *B. dendrobatidis*. The wide fluctuation of temperature in early spring here may disturb the immune systems of amphibians and may increase the probability of disease occurrence (Clem et al. 1991, Maniero & Carey 1997).

The collection sites were situated between 87 and 360 m in elevation and between 45° 27′ 10″ and 49° 52′ 07″ N in latitude. Given discoveries of *Batrachochytrium dendrobatidis* across broad geographical ranges (Bosch et al. 2001, Beard & O'Neill 2005), it is reasonable to believe that elevation and latitude are not key factors preventing chytridiomycosis from being prevalent in our samples and thus the factors that prevent *B. dendrobatidis* from being prevalent need further analysis.

Taking behavioral ecology into consideration, the cooler season may have decreased the activity of amphibians in our study. The lack of available hosts for the fungus to infect during this season may have been a reason why no cases of *Batrachochytrium dendrobatidis* were detected, even though other environmental variables were optimal for fungal growth. As in most

cases of wildlife epidemiological research, susceptibility to pathogens is always an important factor for detection. The unknown susceptibility of *Rana dybowskii* to *B. dendrobatidis* prevents us from drawing definite conclusions regarding the lack of chytridiomycosis prevalence in Heilongjiang.

This is the first investigation of chytrid fungus in wild *Rana dybowskii* in China. The negative result suggested that in the investigated area, *R. dybowskii* is currently free from *Batrachochytrium dendrobatidis*, which is noteworthy from the aspects of conservation biology and conservation medicine. The lack of positive results in this area of China adjacent to North Korea provides evidence that *B. dendrobatidis* has not yet extended north along the Korean Peninsula into China. Combining this with the fact that bullfrogs, which are carriers of *B. dendrobatidis* (Daszak et al. 2004), have been introduced into Heilongjiang Province for food consumption, it raises protection and monitoring demands for native populations and the greater wildlife community. Central and local governments should implement strict management measures to prevent the escape of non-native commercial amphibian species into this area, which might endanger local populations of native species.

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