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SHORT NOTE

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Sampling for *Batrachochytrium dendrobatidis* in Russia

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Batrachochytrium dendrobatidis (*Bd*), a fungus causing the amphibian disease chytridiomycosis, is decimating populations around the globe. Nevertheless, the biggest continent, Asia, has been studied only infrequently and huge areas remain unsampled. We collected samples in the far eastern part of the Russian Federation. No amphibians were detected to be positive for *Bd*. Our results correspond with presumptions of low prevalence in the north of Asia and show that *Bd*-free areas are still to be found and harbour naïve and potentially susceptible populations.

Key words: Bufo gargarizans, chytridiomycosis, far east, infectious disease, *Rana dybowskii*, *Salamandrella keyserlingii*

Chytridiomycosis, the disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al., 1998; Longcore et al., 1999), has been linked to amphibian population declines in various taxa and regions. Mass mortality has been observed in Australia, America and Europe (Berger et al., 1998; Bosch et al., 2001; Ouellet et al., 2005; Lips et al., 2006). Although recently published articles are beginning to build our knowledge as to the presence of *Bd*, Asia remains little explored from the perspective of *Bd* research (Swei et al., 2011; Bai et al., 2012).

Current field data on *Bd* in Asia show its presence in China (Bai et al., 2010; 2012), Japan (Une et al., 2008; Goka et al., 2009), Indonesia (Kusrini et al., 2008; Swei et al., 2011), Malaysia (Savage et al., 2011; Swei et al., 2011), South Korea (Yang et al., 2009; Swei et al., 2011), as well as Kyrgyzstan, Laos, Philippines, Sri Lanka and Vietnam (Swei et al., 2011). Some studies, however, have failed to find the pathogen in wild populations (e.g., Rowley et al., 2007; Wei et al., 2010; Swei et al., 2011) or museum specimens (McLeod et al., 2008). Owing to the size of the Asian continent, huge areas remain unsampled. Therefore, we carried out one of the first attempts within the Russian Federation as a whole to assess *Bd* occurrence and the very first in Primorskiy Kray.

The study area is bordered by the People's Republic of China to the west, North Korea to the south-west, and the Sea of Japan to the south, east and south-east. The northern part of the study area links to the Khabarovsk area. Sampling sites were mostly small ponds and river basins.

Sample collection was carried out by skin swabbing live amphibians during the spring months (April and May) in two successive years (2010 and 2011) at seven remote locations (Fig. 1, Table 1). The sampling technique is an acknowledged methodology (Hyatt et al., 2007). At each locality, we endeavoured to reach a minimum of 30 samples per species in order to detect *Bd* with acceptable probability (DiGiacomo & Koepsell, 1986). Individuals were captured by hand or using nets

Fig. 1. Localities of amphibian populations in the Russian Federation testing negatively for *Bd* (circles) during 2010–2011.

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Table 1. *Batrachochytrium dendrobatidis* sampling from adult specimens collected in Russia (Primorskiy Kray) during 2010 and 2011. Site number: corresponding site numbers as shown in Fig. 1, Lat.: latitude, Long.: longitude, Elev.: elevation, Species (no. test. / no. pos.): species and number of tested and positive animals, *Rd*: *Rana dybowskii*, *Bg*: *Bufo gargarizans*, *Sk*: *Salamandrella keyserlingii*.

Year	Map site no.	Site	Site description	Lat. °N	Long. °E	Elev. (m)	Family	Species (no. test. / no. pos.)
2010	1	Gornotaezhnoe	Blind stream branch, river ponds	44.59082	132.89023	127	Ranidae, Bufonidae, Hynobiidae	*Rd*(26/0); *Bg*(2/0); *Sk*(1/0)
2010	2	Duboviy Klyuch river basin	Smaller flatland ponds	43.78819	132.64839	42	Bufonidae	*Bg*(25/0)
2010	3	Kamenushka	Meandering stream with blind branches	43.66333	132.37547	100	Ranidae, Bufonidae	*Rd*(27/0); *Bg*(1/0)
2010	4	Kulkyn Klyuch	Slope near a stream	43.50665	134.67878	112	Ranidae	*Rd*(35/0)
2011	5	12 km west of Malaya Kema City	Warnambol, flooded track, small streams	45.51194	136.93906	177	Ranidae	*Rd*(34/0)
2011	6	5 km north of Terney city	Serebryanka river basin	45.08925	136.58872	24	Ranidae	*Rd*(24/0)
2011	7	6 km east of Novaya Moskva City	Kamenchin Klyuch river basin	43.35687	132.69892	154	Ranidae	*Rd*(5/0)

while wearing disposable gloves. Standard precautionary hygienic measures were taken (Wellington & Haering, 2008). Sampled individuals were photographed and returned to the place of their capture.

Samples were at first processed by standard protocols for *Bd* detection using TaqMan-probe-based real-time quantitative polymerase chain reaction (qPCR, Boyle et al., 2004), with addition of bovine serum to exclude PCR inhibition (Garland et al., 2010). As some objections to the sensitivity of the probe used have previously been raised (Goka et al., 2009), we checked for undetected DNA amplification during the qPCR procedure by standard electrophoresis on 1.5% agarose gel. If fragments of any length were detected, then the original sample was re-processed using the PCR method described by Annis et al. (2004). All laboratory procedures were carried out at the Department of Biology and Wildlife Diseases, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. In this laboratory, samples from captive, wild and museum amphibians are routinely processed. Quantification standards were provided by the Institute of Zoology, Zoological Society of London.

In total, 180 samples (117 in 2010 and 63 in 2011) from three amphibian species were analyzed (Fig. 1, Table 1). No presence of *Bd* was detected in any of these samples collected. The real-time qPCR showed no growth in fluorescence, although in several cases DNA fragments were detected by electrophoresis. These PCR products differed in size (approximately 400 bp) compared to the fragment targeted by specific primers (around 150 bp). After processing these samples by standard PCR with no positive outcomes, we regarded them as negative. Amplification of DNA in the real-time qPCR process probably resulted from TaqMan probes acting as a third primer in the reaction and thus amplifying DNA sequences not specific to *Bd*. Based upon the reported high sensitivity and specificity of both methods to detect *Bd* (Annis et al., 2004; Boyle et al., 2004), we can assume that all samples were truly free of *Bd*. As no positive samples were detected, the overall prevalence is 0% (the 95% confidence interval using Sterne's exact method is 0.00–2.08%).

In other studies, Swei et al. (2011) considered more than 3,000 samples from 15 countries to determine an overall prevalence of 2%. Goka et al. (2009) collected more than 2,100 field samples from the Japanese archipelago, and found an overall prevalence of around 4% (with positivity clustering on *Andrias japonicus*) while also detecting new phylogenetic lineages. Most recently, Bai et al. (2012) reported a prevalence of 8%

from China (2,075 samples). They detected a potentially basal phylogenetic lineage and supported Goka et al.'s (2009) proposition that Asia may be the source of some *Bd* lineages.

The aforementioned studies along with our negative results show that the occurrence of *Bd* in Asia is not universal, and that there are still areas free of the pathogen. Niche modelling has proven to be a suitable tool for assessing the potential distribution of *Bd* (Rödder et al., 2009; Swei et al., 2011), and it is encouraging that the outputs from those models correspond with our findings.

We were unable to detect *Bd* using a combination of two DNA-based detection methods and a sufficiently large dataset. Nevertheless, and particularly in the case of emerging diseases, presenting verified negative *Bd* datasets in amphibian populations is as important as sharing data for positive, declining or moribund populations. Truly naïve populations have crucial importance in the case of *Bd* conservation measurements, and such populations should be intensively studied and rigorously protected.

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