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Evidence of *Batrachochytrium dendrobatidis* Infection in Amphibians from Serbian Lowlands

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ABSTRACT: We investigated presence of *Batrachochytrium dendrobatidis* in the Republic of Serbia. Seven out of 88 samples (8%) tested positive, all belonging to the frog genus *Pelophylax*. Two positive sites were located directly on the Danube River. The Danube River could be an important disease corridor, and distribution of *Batrachochytrium dendrobatidis* along this river should be further explored.

Batrachochytrium dendrobatidis (*Bd*), a fungus that can cause chytridiomycosis in amphibians, has been detected across much of the European continent. In an effort to manage and understand this devastating pathogen and its impacts, the Risk Assessment of Chytridiomycosis to the European Amphibian Biodiversity project was initiated by the European Union in order to compile data on amphibian species for western countries (Fisher et al. 2012). Investigators in 20 European countries have recorded data on the presence of *Bd* in over 45 species of amphibians, with prevalence varying among species and geographic regions (e.g., Baláž et al. 2014a). However, eastern and southeastern European regions remain largely understudied (Vörös et al. 2013; Reshetnikov et al. 2014).

We evaluated the presence of *Bd* in the Republic of Serbia (Fig. 1). The country lies on the Balkan Peninsula in southeastern Europe. Serbia is generally divided into three altitudinal regions, distinctive in geographic and ecological characteristics: 1) the Pannonian region (plain) in Vojvodina Province, up to 200 m in elevation, 2) the Peripannonian region (lowlands and hills), from 200 to 600 m in elevation, and 3) the Mountain-valley region, from 600 m to 2,650 m in elevation (Stevanović and Stevanović 1995). We conducted this study primarily in the Pannonian region (localities 1–10) and at two additional sites in the higher-elevation region south of the Sava River, locality 11 in the Peripannonian region and locality 12 in the Mountain-valley region (Fig. 1).

Localities within the Pannonian region (1–10) either were directly on the banks of the Danube River (i.e., localities 2, 3, and 7) or were a part of wetlands, floodplain marshes, or oxbow lakes near the Danube River (localities 1, 4, 5, 6, 8, 9, and 10). These localities represented both federally protected (seven sites) and unprotected areas (three sites). Six localities (1, 2, 4, 6, 9, and 10) were protected nature reserves of Serbia, whereas locality 5 was affected by sewage and agricultural runoff, and locality 7 near Belgrade was an extremely anthropogenically burdened area. Locality 11 in Peripannonian region represented a small aquatic ecosystem at an elevation of 400 m, while locality 12 in the Mountain-valley region represented water troughs of mowed meadows at an average elevation of 930 m.

Each site was visited on a single occasion between April and July of 2015. We opportunistically collected amphibians by hand. To prevent cross contamination at a single site, vinyl gloves were replaced every time a new individual was caught. To prevent cross contamination among the sites, all field equipment, including footwear, was treated with commercial bleach (final concentration: 5% NaOCl). Animals were swabbed with sterile cotton tips with a plastic handle according to the protocol of Kriger et al. (2007), after which the tips were placed in 1.5 mL sterile O-ring tubes (Lake Charles Man-

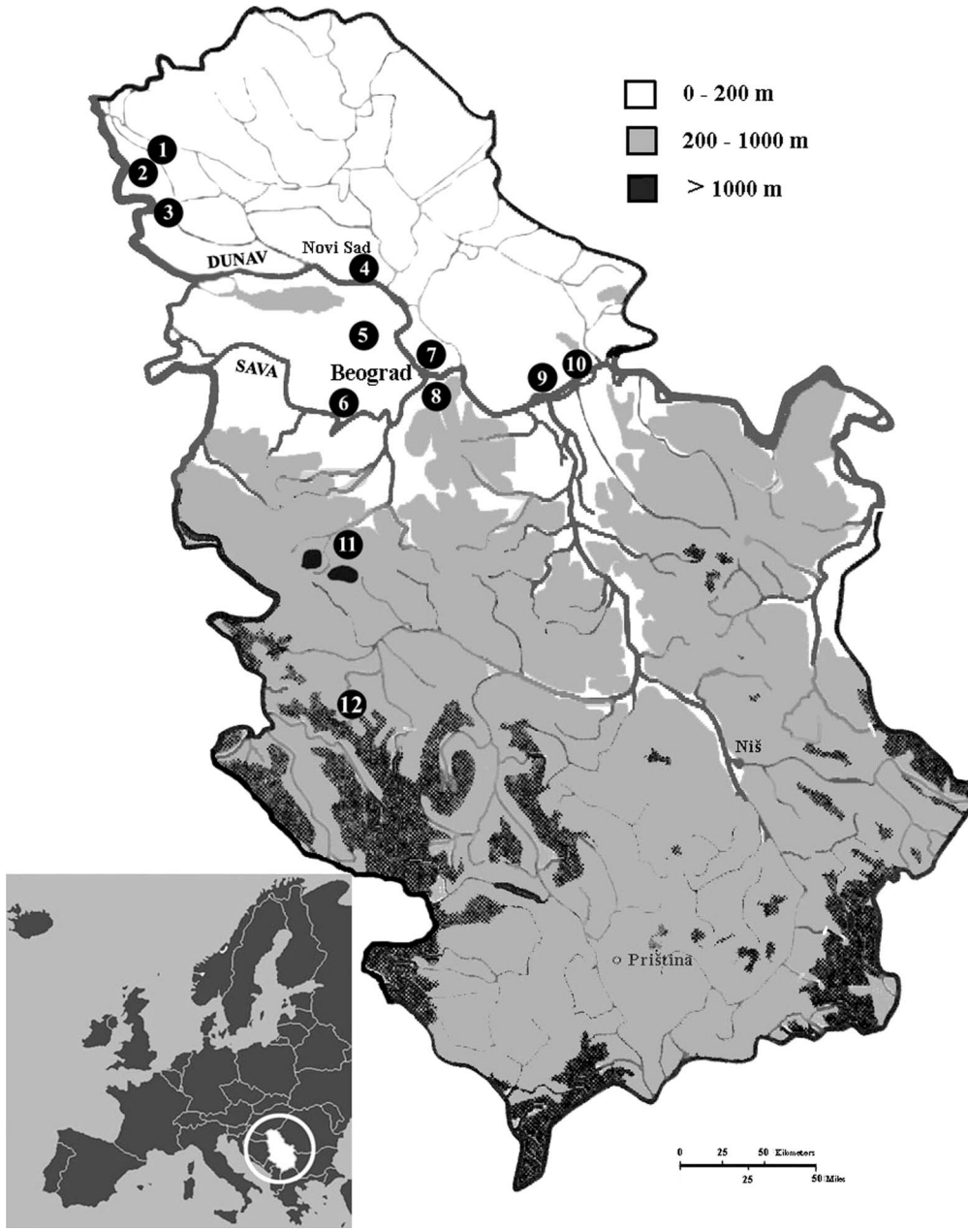

FIGURE 1. Map of the Republic of Serbia showing 12 localities where amphibians were screened for the fungus *Batrachochytrium dendrobatidis*. The map shows three regions, the Pannonian region from 0 to 200 m of elevation, the Peripannonian region at 200 to 600 m of elevation, and the Mountain-valley region at 1000 m of elevation.

ufacturing, Lake Charles, Louisiana, USA). Samples did not experience the field conditions for longer than 12 h. Upon return from the field, all samples were stored at –80 C until laboratory testing.

The DNA was extracted using a PrepMan assay (Life Technologies, Carlsbad, California, USA), and the presence of *Bd* was assessed using a real-time Taqman *q*PCR assay (Life Technologies, Carlsbad, California, USA).

TABLE 1. Results from the first surveillance of *Batrachochytrium dendrobatidis* (*Bd*) in Serbia, including locality number (corresponding to localities shown in Fig. 1), survey month, species surveyed, sample size per species with number of infected individuals in parenthesis, and mean zoospore equivalents among infected individuals. Each locality was surveyed on a single occasion in 2015. Overall, *Bd* was found in four locations and among three species of *Pelophylax* (Ranidae).

Locality							
No.	Name	Latitude	Longitude	Month	Species	*n* Tested (*n* positive)	Zoospore equivalents
1	Kupisina	45°43′27″N	18°56′38″E	May	*Bombina bombina*	5 (0)	0
	Kupisina	45°43′27″N	18°56′38″E	May	*Pelophylax kl. esculentus*	1 (0)	0
	Kupisina	45°43′27″N	18°56′38″E	May	*Pelophylax lessonae*	5 (1)	1,634
	Kupisina	45°43′27″N	18°56′38″E	May	*Pelophylax ridibundus*	4 (1)	237
2	Apatin	45°40′46″N	18°57′30″E	May	*Bufo bufo*	1 (0)	0
	Apatin	45°40′46″N	18°57′30″E	May	*Pelophylax kl. esculentus*	1 (0)	0
	Apatin	45°40′46″N	18°57′30″E	May	*Pelophylax lessonae*	4 (3)	1,050
3	Karavukovo	45°28′51″N	19°5′22″E	May	*Bufo bufo*	1 (0)	0
	Karavukovo	45°28′51″N	19°5′22″E	May	*Pelophylax kl. esculentus*	3 (1)	4,260
	Karavukovo	45°28′51″N	19°5′22″E	May	*Pelophylax lessonae*	2 (0)	0
4	Kovilj	45°11′30″N	20°4′4″E	July	*Pelophylax kl. esculentus*	4 (0)	0
	Kovilj	45°11′30″N	20°4′4″E	July	*Pelophylax lessonae*	3 (0)	0
	Kovilj	45°11′30″N	20°4′4″E	July	*Pelophylax ridibundus*	1 (0)	0
5	Jarkovci	45°2′36″N	20°1′39″E	May	*Pelophylax kl. esculentus*	6 (1)	485
	Jarkovci	45°2′36″N	20°1′39″E	May	*Pelophylax ridibundus*	3 (0)	0
6	Obedska Bog	44°44′9″N	19°59′53″E	May	*Pelophylax kl. esculentus*	2 (0)	0
	Obedska Bog	44°44′9″N	19°59′53″E	May	*Pelophylax lessonae*	13 (0)	0
7	Krnjaca	44°50′4″N	20°30′41″E	June	*Bombina bombina*	1 (0)	0
	Krnjaca	44°50′4″N	20°30′41″E	June	*Pelophylax kl. esculentus*	10 (0)	0
	Krnjaca	44°50′4″N	20°30′41″E	June	*Pelophylax lessonae*	4 (0)	0
8	Zuce	44°40′52″N	20°33′5″E	June	*Pelophylax kl. esculentus*	1 (0)	0
	Zuce	44°40′52″N	20°33′5″E	June	*Pelophylax* sp.	3 (0)	0
9	Deliblato Sands I	44°47′56″N	21°14′5″E	June	*Pelophylax kl. esculentus*	1 (0)	0
	Deliblato Sands I	44°47′56″N	21°14′5″E	June	*Pelophylax lessonae*	4 (0)	0
10	Deliblato Sands II	44°51′14″N	21°18′16″E	June	*Pelophylax kl. esculentus*	1 (0)	0
11	Tupanci	44°16′4″N	19°45′25″E	April	*Ichthyosaura alpestris*	2 (0)	0
12	Zlatibor	43°55′5″N	19°38′22″E	July	*Pelophylax ridibundus*	1 (0)	0
	Zlatibor	43°55′5″N	19°38′22″E	July	*Rana dalmatina*	1 (0)	0

The probe ChytrMGB2 was used with two species-specific primers ITS1-3 Chytr and 5.8S Chytr (Boyle et al. 2004; Garland et al. 2010; Kilburn et al. 2010). We ran each sample in triplicate and compared the results to a regression line based on a consecutive 10-fold dilution of five standards. Using the JEL423 strain as a genome reference and following the suggestions of Rebollar et al. (2014), we considered that one copy of the amplified gene fragment (ITS1–5.8S) was equal to the equivalent of 0.045 zoospores. Values above this zoospore number were considered positive for the presence of *Bd*. We ensured that quantifications had an efficiency between 95% and 100% (with an average of 98.15%) and an R^2 value higher than 0.997. Triplicates of the positive samples were averaged to estimate zoospore equivalents per sample.

We collected swabs from 88 amphibians, of which 77 belonged to the genus *Pelophylax* (Table 1). Other samples belonged to the genera *Bombina* (*n*=6), *Bufo* (*n*=2), *Ichthyosaura* (*n*=2), and *Rana* (*n*=1). The number of samples per site ranged from one to 15 (mean=7.3, SD=5.2). Of the 12 sites, four localities had amphibians that tested positive

for *Bd*: Kupusina (locality 1), Apatin (locality 2), Karavukovo (locality 3), and Jarkovci (locality 5). Seven individuals among three species within the genus *Pelophylax* tested positive. The zoospore equivalents showed infection rates that ranged between 100 and 1,000 zoospores based on the standard curve generated. Overall, the intensity of infection was considered medium to low, based on the "10,000 zoospore rule" (Vredenburg et al. 2010).

We report the first positive *Bd* detection in Serbia, although no mass mortalities or clinical signs of the infection have been observed. Comprehensive research across Europe shows that *Pelophylax* is one of the most commonly infected genera (Baláž et al. 2014a). Our study confirms that *Pelophylax* remains an important disease vector (Baláž et al. 2014b). Other genera tested negative, but sample sizes were small, and additional testing is warranted. In our study, chytrid fungus has been detected directly along the Danube River, the second largest river in Europe, which runs through nine other European countries. The role of a riverine system in the spread of *Bd* is poorly understood, but some in vitro studies point out that *Bd* could be translocated by the movement of moist river sands (Johnson and Speare 2005). The Danube River could serve as a potential corridor for the spread of the disease, and assessing *Bd* along the Danube River in other countries could yield meaningful patterns. Our future goals involve not only further systematic surveys of the lowland region, but targeted surveys of the Peripannonian and the Mountain-valley regions in order to increase sample size and understand the distribution of chytrid fungus in Serbia.

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