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First detection of *Ranavirus* infection in amphibians in Hungary

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Viruses of the genus *Ranavirus* (family *Iridoviridae*) present a considerable disease risk to ectothermic vertebrates globally (Gray and Chinchar, 2015). They have a wide range of susceptible hosts and infect at least 175 species across 52 families of fish, amphibians and reptiles (Price et al., 2017). Ranaviruses are often highly virulent and cause systemic infections in amphibians. In Europe the emergence of the pathogen is dated back to the early 1990s (i.e. Fijan et al., 1991; Cunningham et al., 1996) and in the last decades it was responsible for several disease outbreaks in the continent in amphibians. The spreading of the ranaviral associated diseases across Europe may be enhanced by the international trade of the potentially infected amphibians as it was assessed in the United States of America by Schloegel et al. (2009) and other human activities (Price et al. 2016).

Hungary is situated in the Carpathian Basin, a region with high amphibian diversity due to different climatic and zoogeographical influences (Vörös et al., 2014). Previous studies reported the presence of different *Ranavirus* strains which infected fish species and resulted in mass mortality events (e.g. Juhász et al., 2013; Fehér et al., 2016); however, our knowledge about the prevalence between amphibians in the country is missing. Here we present a study which aimed to conduct a countrywide survey in order to assess the prevalence of this emerging pathogen across amphibian species and populations in Hungary.

To screen for ranaviruses, we used DNA samples extracted from tail or toe clips belonging to 137 individuals from 8 different amphibian species, hosted by the Collection of Genetic Resources of the Hungarian Natural History Museum (Fig. 1., Table 1.). These samples were collected for phylogeographic and population genetic studies (Vörös et al., 2006; Recuero et al., 2012; Vörös et al., 2017; Herczeg et al., 2017), and were stored in -80 °C. In all cases DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extraction, following the manufacturer's protocol. Quantitative PCR (qPCR) was performed in the MNCN-CSIC lab (Madrid, Spain) following Leung et al. (2017) on a MyGo Mini qPCR machine. Negative controls and standards with known concentrations of ranaviruses were used in each plate (see Leung et al., 2017). Only the presence or absence of *Ranavirus* was considered, and samples were assigned as positive when cycle threshold (CT) was lower than 41.5 and the amplification curves presented a robust sigmoidal shape. 95% confidence intervals of *Ranavirus* prevalence were calculated with Quantitative Parasitology 3.0 (Rózsa et al., 2000). Among the 137 analysed individuals 40 tested positive for ranaviruses, and the overall prevalence was 29.2 % (95 % CI: 0.220-0.375). We found *Ranavirus* infection in 5 out of the 8 tested amphibian species (Fig. 1, Table 1). The Danube crested newt, *Triturus dobrogicus* (47.4 %; 95 % CI: 0.344-0.605) showed the highest prevalence on the species level, in contrast the lowest prevalence was experienced in the Marsh frog, *Pelophylax ridibundus* (14.3 %; 95% CI: 0.007-0.554). The Common toad, *Bufo bufo* (33.3 %; 95 % CI: 0.122-0.629) showed higher prevalence compared to the Fire

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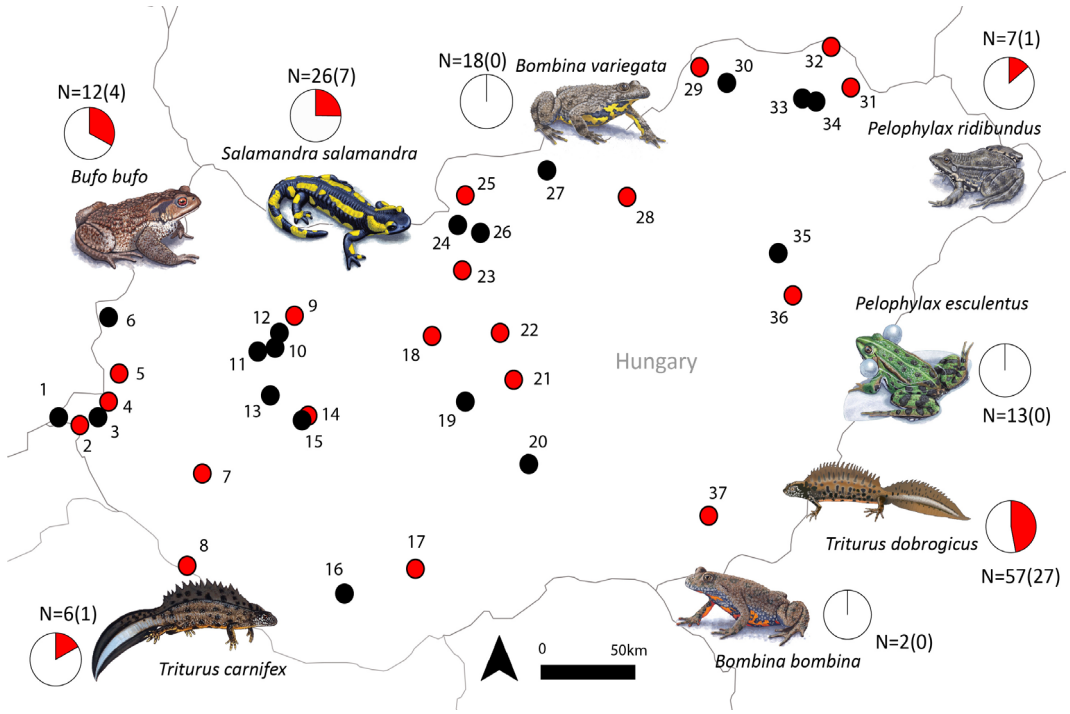

Figure 1. Spatial distribution of sampling sites in Hungary. Circles indicating the sampling locations in which *Ranavirus* presence is marked with red circles while when *Ranavirus* was not detected is indicated with black circles. N = Sample size of each species collected. Pie charts are representing the proportion of positive (red) – negative (white) samples. Illustrations courtesy of Márton Zsoldos.

salamander, *Salamandra salamandra* (26.9 %; 95 % CI: 0.128-0.465) and the Italian crested newt, *Triturus carnifex* (16.7 %; 95 % CI: 0.008-0.599). The detailed prevalence per locality is listed in Table 1.

Here we report the first detection of *Ranavirus* occurrence on amphibians in Central-Eastern Europe. While *Ranavirus* infection caused mass mortalities in several countries in Western Europe, the infected animals found in Hungary did not show any clinical signs of ranavirosis. However, ranaviral infections can be sometimes sub-clinical, and certain species may serve as natural reservoir hosts (Lesbarrères et al., 2012; Saucedo et al., 2019). The same phenomenon was experienced when studying distribution of the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) in Hungary (Vörös et al., 2018). Prevalence of *Bd* was 7.46 % on average in the country, but neither *Bd*-linked mortalities nor clinical symptoms have been associated to the presence of the fungus. Ranaviruses are multi-host pathogens that may play a crucial role in their range expansion. Accordingly, the distribution of ranaviral pathogens in Europe might be much wider than it was previously expected. Their occurrence could be rather common but outbreaks and mass mortalities are rare phenomenon and always exclusively mediated by an external cofactor such as human pollution or climate change linked to increase of temperature. Price et al. (2019) showed a positive effect of temperature on the occurrence and severity of ranavirosis in *Rana temporaria* in the United Kingdom, concluding that climate warming could have a critical impact on the survival of amphibian populations. Although, survival of the host depends on many factors, such as life history characteristics (Brunner et al., 2005), virus dosage (Brunner et al., 2005; Forzán et al., 2015) or the composition of skin microbiome (Campbell et al., 2019).

Within the frame of this study genetic origin of *Ranavirus* found in amphibians was not aimed to be determined. Nonetheless, whole genome sequencing of *Ranavirus* isolates from brown bullhead (*Ameiurus nebulosus*) captured at the northern Danube river

Table 1. Prevalence of *Ranavirus*-infected amphibian species in Hungary. Loc = locality in Fig. 1.; CT = cycle threshold; Prev = prevalence; CI = confidence intervals, Lat = latitude, Long = longitude.

Loc	Species	Sampled (infected)	CT (range)	Prev (95% CI)	Lat	Long
1	*Triturus carnifex*	2 (0)			46.9131	16.1920
2	*Triturus carnifex*	3 (1)	40.4	33.3 % (0.017-0.864)	46.8648	16.3400
3	*Bufo bufo*	1 (0)			46.8998	16.4648
4	*Bufo bufo*	1 (1)	40.9	100% (0.050-1.000)	46.9671	16.5082
5	*Triturus dobrogicus*	1 (1)	40.1	100% (0.050-1.000)	47.0996	16.6155
	Triturus carnifex	1 (0)			47.0996	16.6155
6	*Bombina variegata*	6 (0)			47.3566	16.5087
7	*Triturus dobrogicus*	2 (2)	38.3-39.3	100% (0.223-1.000)	46.6490	17.1631
8	*Triturus dobrogicus*	2 (2)	39.8-40.4	100% (0.223-1.000)	46.2252	17.0628
					46.2252	17.0628
9	*Bufo bufo*	1 (1)	41.0	100% (0.050-1.000)	47.3531	17.7791
10	*Bufo bufo*	1 (0)			47.2687	17.6948
11	*Bombina variegata*	1 (0)			47.1830	17.6826
12	*Bombina variegata*	1 (0)			47.2937	17.7520
13	*Bufo bufo*	2 (0)			47.0095	17.6303
	Triturus dobrogicus	2 (0)			47.0095	17.6303
14	*Triturus dobrogicus*	6 (2)	38.6-40.0	33.3 % (0.062-0.728)	46.9172	17.8647
15	*Bufo bufo*	1 (0)			46.9092	17.8492
16	*Bufo bufo*	1 (0)			46.0932	18.1414
17	*Bombina variegata*	2 (0)			46.2150	18.6115
	Bufo bufo	2 (2)	39.5-40.3	100% (0.223-1.000)	46.2150	18.6115
18	*Triturus dobrogicus*	1 (1)	38.1	100% (0.050-1.000)	47.2706	18.7272
19	*Bombina bombina*	2 (0)			46.9686	18.9495
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20	*Triturus dobrogicus*	2 (0)			46.7159	19.3910
					46.7159	19.3910
21	*Triturus dobrogicus*	9 (4)	38.9-40.3	44.4 % (0.168-0.748)	47.0673	19.2851
22	*Triturus dobrogicus*	9 (4)	37.3-40.9	44.4 % (0.168-0.748)	47.2760	19.2119
23	*Salamandra salamandra*	10 (3)	39.7-41.4	30% (0.087-0.619)	47.5700	18.9400
24	*Salamandra salamandra*	3 (0)			47.7600	18.9100
25	*Salamandra salamandra*	4 (1)	40.7	25% (0.012-0.751)	47.8898	18.9813
26	*Bombina variegata*	4 (0)			47.7200	19.0600
27	*Salamandra salamandra*	5 (0)			47.9900	19.5100
28	*Salamandra salamandra*	2 (2)	39.6-41.5	100%	47.9000	20.0500
29	*Triturus dobrogicus*	10 (5)	39.7-40.2	50% (0.222-0.777)	48.4731	20.5428
30	*Bufo bufo*	1 (0)			48.3958	20.7411
31	*Salamandra salamandra*	1 (1)	39.3	100% (0.050-1.000)	48.5528	21.4527
32	*Triturus dobrogicus*	9 (4)	39.2-40.1	44.4 % (0.168-0.748)	48.3678	21.5758
33	*Salamandra salamandra*	1 (0)			48.3212	21.2530
34	*Bufo bufo*	1 (0)			48.3141	21.3347
35	*Pelophylax esculentus*	8 (0)			47.6208	21.0767
35	*Pelophylax ridibundus*	2 (0)			47.4453	21.1697
36	*Pelophylax esculentus*	5 (0)			47.4453	21.1697
36	*Pelophylax ridibundus*	5 (1)	41.1	20% (0.01-0.657)	46.4708	20.6235
37	*Triturus dobrogicus*	4 (2)	38.2-41.1	50% (0.089-0.902)	46.9131	16.1920
Total		**137 (40)**				

section and the southern Tisza river section in Hungary detected European sheatfish virus (ESV)-like strains (Fehér et al., 2016). However, this strain has not been detected yet among amphibians. Ranaviral pathogens infecting amphibians, e.g. Common midwife toad virus (CMTV)-like, are spreading in Europe (Price et al., 2014), furthermore the Andaran *Alytes obstetricians* virus (AAOV), and the Frog virus 3 (FV3)-like virus also infect amphibians in the continent and cause mass mortality events (Price et al., 2014; Stöhr et al., 2015). The World Organization for Animal Health (OIE) lists ranaviruses that infect amphibians as notifiable pathogens (Schloegel et al., 2010). Constant monitoring actions are needed to screen prevalence and population demographics (Campbell et al., 2018). However, direct effects of the disease (e.g. population declines) might be hard to detect in the absence of observed mass mortality events. In our study, low infection intensity (results not presented here) was detected and these findings are corresponding with a *Ranavirus* survey of Australian endemic amphibians, in which high prevalence and low detectability was experienced (Wynne, 2019).

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