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First screening for *Batrachochytrium dendrobatidis*, *B. salamandrivorans* and *Ranavirus* infections in wild and captive amphibians in Slovenia

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In the last ten years, emerging pathogens have become one of the main causes of the global decline in amphibian populations. In particular, skin infections by two fungal species, *Batrachochytrium dendrobatidis* (hereafter *Bd*) and *B. salamandrivorans* (hereafter *Bsal*), as well as viral disease caused by the genus *Ranavirus* (family Iridoviridae) were identified as primary pathogens associated with the globally increased mortality in amphibians (PRICE et al. 2014, ALLAIN & DUFFUS 2019, SCHEELE et al. 2019, FISHER & GARNER 2020). The ability of *Ranavirus* to infect at least 175 species in 52 families of poikilothermic vertebrates (i.e., not only amphibians) and its being responsible for more than 50% of amphibian mortality processes in North America clearly demonstrate the devastating effect of ranaviruses (DUFFUS et al. 2015). At the same time, chytridiomycosis (due to *Bd*, *Bsal*) has contributed to the declines of more than 500 amphibian species worldwide, being the largest recorded loss of biodiversity due to a single disease (FISHER et al. 2012, SCHEELE et al. 2019). In addition to their broad host spectra and high virulence, the devastating effects of chytridiomycosis and ranaviruses panzootics on amphibians are mainly due to the rapid intercontinental spread of these diseases via international transport, the introduction of invasive species, and other man-made activities (PRICE et al. 2016, FITZPATRICK et al. 2018, O'HANLON et al. 2018).

In Europe, infections with *Bd* are primarily asymptomatic (ALLAIN & DUFFUS 2019), nevertheless they are widespread and afflict a wide range of amphibian hosts (ALLAIN & DUFFUS 2019). While *Bd* had never before been recorded in Slovenia, it was known from all neighbouring countries (SZTATECSNY & GLASER 2011, TESSA et al. 2013, VÖRÖS et al. 2018) except Croatia (VÖRÖS & JELIĆ 2011). *Bsal*, on the other hand, appears to occur in a more localized manner, causing severe mortalities in natural popula-

tions of salamanders in The Netherlands, Belgium, Germany and Spain (SPITZEN-VAN DER SLUIJS et al. 2016, MARTEL et al. 2020).

Like *Bd*, ranaviruses appear to be present across Western and Central Europe (DUFFUS et al. 2015, ALLAIN & DUFFUS 2019). Although the outcome of an infection varies depending on the host, season and virus strain, ranaviruses can lead to severe population declines in amphibians, fish and reptiles (PRICE et al. 2014, DUFFUS et al. 2015). In countries bordering Slovenia, *Ranavirus* has been confirmed in *Pelophylax* kl. *esculentus* in Italy and Croatia (FIJAN et al. 1991, ARIEL et al. 2010).

With 20 reported species of native amphibians (STANKOVIĆ et al. 2015) within an area of approximately 20,000 km², Slovenia is one of the richest Palaearctic countries in terms of relative amphibian diversity (ANTHONY et al. 2008). Besides amphibians inhabiting surface habitats, both subspecies of the European cave salamander or olm (*Proteus anguinus*) are present in the underground waters of the Slovenian Karst. The white subspecies (*Proteus anguinus anguinus*) is endemic in the Dinaric Karst, with the largest known populations living at localities in Slovenia, while the habitat of the black subspecies (*P. anguinus parkelj*) is limited to an area of only a few square kilometres in the southeast of Slovenia (SKET 1997, GORIČKI et al. 2017).

Despite their devastating potentials, increasing geographical distributions, and widening host spectra (ALLAIN & DUFFUS 2019), data on chytridiomycosis and ranaviruses in Slovenia are still scarce. They are in fact limited to a *Bd* test on 29 *Rana latastei* individuals from western Slovenia (GARNER et al. 2005, BALÁŽ et al. 2014) and *Bd* and ranavirus tests on a single specimen of *Lithobates catesbeianus* from the Slovenian coast (KIRBIŠ et al. 2016), all of which tested negative.

In view of Slovenia's geographical position and the diversity of its amphibian fauna, data on the occurrence of chytridiomycosis and ranaviruses in Slovenia are of utmost importance. To fill this knowledge gap we conducted the first comprehensive study on the presence of these emerging amphibian pathogens in Slovenia. As geographically isolated species and captive animals appear to be particularly susceptible to infectious diseases (HEARD et al. 2013, KOSTANJŠEK et al. 2017) and therefore most at risk, we focused on the endemic olm (*Proteus anguinus*) and amphibians in captivity.

Between 2015 and 2019, we collected swab samples from 173 live amphibians of 22 species. These included 132 samples of 17 native and one introduced amphibian species (*Lithobates catesbeianus*) from 53 natural sites across Slovenia (Fig. 1, Table 1, Supplement 1). In addition, 41 samples of captive amphibians comprising four exotic and six native species were included in the study (Table 1). The sampling set comprised 70 olm individuals from five wild populations in Slovenia and 18 captive specimens of both subspecies.

We performed sampling and DNA extractions in accordance with the procedures commonly used in amphibian chytridiomycosis research (HYATT et al. 2007). The samples were analyzed for the presence of *Bd* and *Bsal* DNA by TaqMan real-time quantitative polymerase chain reaction

(qPCR) assays, targeting specific regions of rRNA genes (BLOOI et al. 2013). All samples were tested in duplicates against a set of standards of known concentrations (0.1, 1, 10 and 100 genomic equivalents (GEs) of zoospores per qPCR). Presumably positive samples were additionally subjected to quantifications by droplet digital PCRs (ddPCR) as described previously (GUTIÉRREZ-AGUIRRE et al. 2015), using the same set of primers and known *Bd* or *Bsal* GEs as positive controls. We scored samples as positive only if both replicates clearly amplified the target sequence (more than three positive droplets above the threshold set from negative controls) and then estimated the infection burden from mean the GE. The *Bd* genomic standards were obtained from the Institute of Zoology, Zoological Society of London, while the standards for *Bsal* were provided by the University of Ghent.

A qPCR protocol specific to amphibian-like ranaviruses targeting the MCP gene (LEUNG et al. 2017) was used for detecting ranaviruses in the samples. An additional qPCR assay targeting the constitutive amphibian gene EBF₃N was included as control of the DNA extraction procedure. We used the AgPath ID OneStep RT-qPCR Mastermix (ThermoFisher) for detecting DNA as well as any RNA intermediates. All samples were tested in duplicates against positive controls consisting of diluted solutions of a plasmid containing known concentrations of MCP target sequences.

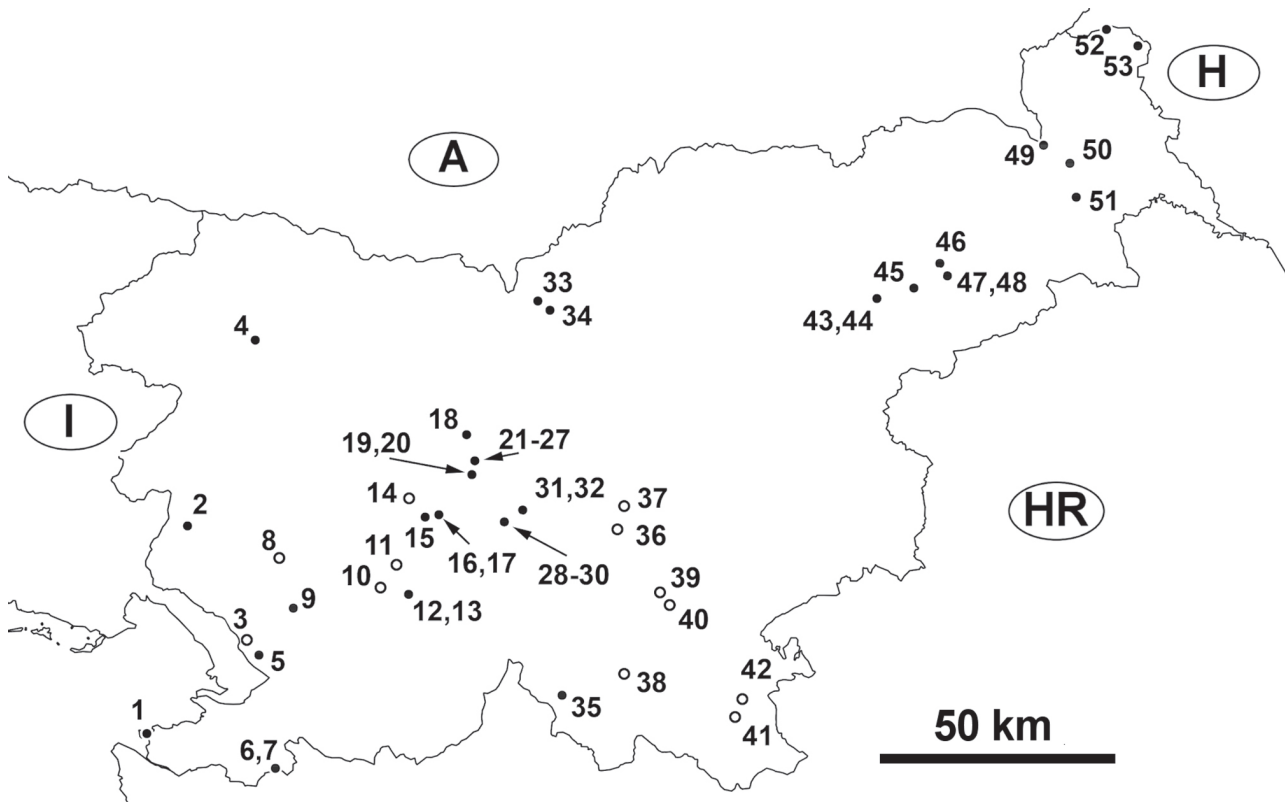


Figure 1. Map of the Republic of Slovenia, showing 53 sampling sites of amphibians screened for chytrid fungi and ranavirus infections. Subterranean sampling sites of olms (*Proteus anguinus*) are shown as white dots, epigeal sampling sites of other amphibian species are shown as black dots. Detailed information on the sampling sites is given in Supplement 1.

Table 1. List of wild and captive amphibians sampled for chytrid fungi and ranavirus including numbers of sampled specimens (N) and location numbers corresponding to Fig. 1. * species and location of *Bd*-positive individual.

Species	N	Location No.
Wild specimens		
<i>Bombina bombina</i>	5	50
<i>Bombina variegata</i>	6	6, 7, 43, 44, 45
<i>Bufo bufo</i>	9	4, 9, 12, 23-26, 32
<i>Bufo viridis</i>	1	46
<i>Hyla arborea</i>	3	18, 27, 47
<i>Ichthyosaura alpestris</i>	4	29, 34, 35,
<i>Lissotriton vulgaris</i>	5	15, 17, 30, 47
<i>Lithobates catesbeianus</i>	1	1
<i>Pelobates fuscus</i>	1	48
<i>Pelophylax</i> kl. <i>esculentus</i> *	5	5, 13, 47, 53*
<i>Proteus anguinus anguinus</i>	64	3, 8, 10, 11, 14, 36-42
<i>Proteus anguinus parkelj</i>	6	41
<i>Rana arvalis</i>	10	20, 31, 49, 51
<i>Rana dalmatina</i>	2	25, 47
<i>Rana latastei</i>	1	2
<i>Rana temporaria</i>	6	19, 22, 25, 28, 52
<i>Salamandra atra</i>	1	33
<i>Salamandra salamandra</i>	1	21
<i>Triturus carnifex</i>	1	16
Captive specimens		
<i>Ambystoma mexicanum</i>	4	Keeper 1
<i>Bufo viridis</i>	1	Keeper 2
<i>Lissotriton vulgaris</i>	2	Keeper 3
<i>Proteus anguinus</i>	17	Keepers 1, 5
<i>Proteus anguinus parkelj</i>	1	Keeper 5
<i>Pyxicephalus adspersus</i>	2	Keeper 6
<i>Salamandra atra</i>	3	Keeper 7
<i>Salamandra salamandra</i>	3	Keeper 7
<i>Trachycephalus resinifictrix</i>	1	Keeper 6
<i>Triturus carnifex</i>	3	Keeper 3
<i>Xenopus laevis</i>	4	Keeper 1

None of the 173 samples analyzed tested positive for ranavirus (MCP gene). Similarly, all samples were negative for *Bsal*, while a single individual of edible frog (*Pelophylax* kl. *esculentus*) sampled close to the northeastern border of Slovenia tested positive for *Bd* by qPCR. Quantification for that sample by ddPCR showed a zoospore load in the range of 4.4–5.2 GE. The amplification standards with a known quantity of *Bd*, *Bsal* and *Ranavirus* targets amplified as expected, indicating that the tests had run successfully. None of the captive or wild animals sampled in the study exhibited obvious signs of chytridiomycosis or ranaviriosis.

Our study covers 17 of the 20 native Slovenian amphibian species (STANKOVIĆ et al. 2015) and all five genetic lineages of both olm subspecies identified so far. Due

to the increased probability of infection via vector hosts (SCHLOEGEL et al. 2010) and captive amphibians (BECKER et al. 2014, KOSTANJŠEK et al. 2017), the invasive American bullfrog (*Lithobates catesbeianus*) and several species kept under seminatural or artificial conditions in laboratories and public and private collections, as well as a commercial breeding facility were included in the survey as well. Nevertheless, the only sample that tested positive for *Bd* was a single edible frog (*Pelophylax* kl. *esculentus*) from a natural habitat in northeastern Slovenia. Although based on a single infected specimen with a low *Bd* infection load, the presence of *Bd* in an edible frog underscores the previously indicates importance of the genus *Pelophylax* as a *Bd* reservoir (BALÁŽ et al. 2014). The proximity of the Slovenian sampling site to a location in Hungary with previously identified *Bd*-positive yellow-bellied toads, *Bombina variegata* (VÖRÖS et al. 2018), and the absence of additional positive samples in northeastern Slovenia suggest the presence of *Bd* to be limited to this region at present. A larger number of samples and identification of the *Bd* lineage would be required to confirm the origin, infection rates and potential impacts of this localized infection of *Bd* on adjacent amphibian populations.

Being threatened by pollution and loss of its underground habitats (VÖRÖS et al. 2017), the olm features in both the IUCN Red (ARNTZEN et al. 2009) and EDGE list (SAFI et al. 2013) (<http://www.edgeofexistence.org/species/olm/>) and is protected by national legislation in the countries of its range. As an endemic species with a narrow ecological niche, the olm is also very susceptible to infections (HEARD et al. 2013), especially to pathogens with a high mortality potential for urodelans, such as *Bsal* and ranaviruses (SPITZEN-VAN DER SLUIJS et al. 2016, PRICE et al. 2016). Although the negative results of the tests for chytrid fungi and ranaviruses in wild and captive olm individuals in our study are encouraging, a more comprehensive estimate of the occurrence of these pathogens in olm populations would require a larger sample set and a systematic survey over a longer period. The same is true for other captive amphibians tested in our study, including specimens from private collections, which may contribute significantly to the spread of chytrid fungi (WOMBWELL et al. 2016) and, as observed in a recent case of *Bsal* infection in Spain (MARTEL et al. 2020), may pose a serious threat to natural populations of endemic salamanders.

Our study complements the data on the current range of *Bd*, *Bsal* and ranavirus in Europe as obtained by means of standardized screening protocols (LEUNG et al. 2017, THOMAS et al. 2018) and provides the first record of *Bd* in Slovenia. This fact is not surprising, given the confirmed occurrence of *Bd* in neighbouring countries (ALLAIN & DUFFUS 2019). Nevertheless, the presence of *Bd*, categorized as a 'Notifiable Pathogen' by the OIE (World Organization for Animal Health) (SCHLOEGEL et al. 2010), requires the attention of the larger scientific community, herpetologists, and governmental institutions in charge. Being responsible for the most prominent loss of vertebrate diversity ever recorded (SCHEELE et al. 2019), we strongly believe that the

confirmation of the presence of this pathogen in Slovenia and the establishment of diagnostic protocols in the country should provide sufficient incentive for the initiation of a monitoring program for amphibian pathogens and the development of strategies to protect the Slovenian amphibian fauna at national level.

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Supplementary data

The following data are available online:

Supplementary Table S1. List of sampling sites including location numbers (corresponding to Fig. 1), site names, regions, and geodata of the sites.