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First detection of ranavirus in a wild population of Dybowski's brown frog (*Rana dybowskii*) in South Korea

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Abstract

Background: Ranavirus is an emerging infectious disease which has been linked to mass mortality events in various amphibian species. In this study, we document the first mass mortality event of an adult population of Dybowski's brown frogs (*Rana dybowskii*), in 2017, within a mountain valley in South Korea.

Results: We confirmed the presence of ranavirus from all collected frogs ($n = 22$) via PCR and obtained the 500 bp major capsid protein (MCP) sequence from 13 individuals. The identified MCP sequence highly resembled Frog virus 3 (FV3) and was the same haplotype of a previously identified viral sequence collected from Huanren brown frog (*R. huanrenensis*) tadpoles in South Korea. Human habitat alteration, by recent erosion control works, may be partially responsible for this mass mortality event.

Conclusion: We document the first mass mortality event in a wild Korean population of *R. dybowskii*. We also suggest, to determine if ranavirus infection is a threat to amphibians, government officials and researchers should develop continuous, country-wide, ranavirus monitoring programs of Korean amphibian populations.

Keywords: Ranavirus, *Rana dybowskii*, Mass mortality, Wild population, Major capsid protein

Background

Emerging infectious diseases are one of the key factors causing rapid global biodiversity declines in this century (Fey et al. 2015). Amphibians are particularly vulnerable to infectious diseases due to their permeable skin and metamorphic life cycle (Daszak et al. 1999). Fungal infections by *Batrachochytrium dendrobatidis* (Bd) and *B. salamandriborans*, causing chytridiomycosis, have been implicated as a primary cause of rapid amphibian population declines (Daszak et al. 1999; Scheele et al. 2019). In addition, ranavirus, a double-stranded DNA virus, has also been identified as a major emerging infectious disease and is associated with global amphibian declines (Green et al. 2002; Carey et al. 2003). In Northeast Asia, across China, Japan, and Korea, ranavirus infections have

caused mortality in 17, native and invasive, amphibian species (4 urodelan and 13 anuran species) as well as amphibians in the pet trade (Zhang et al. 1996; Weng et al. 2002; Kim et al. 2009; Une et al. 2009a; Kolby et al. 2014; Une et al. 2014; Duffus et al. 2015; Kwon et al. 2017; Park et al. 2017). Out of the 21 reported ranavirus infection cases, 18 have been linked to mass mortality events (Table 1). Amphibian ranavirus susceptibility and mortality are often correlated with low environmental quality, such as habitat destruction and pollution (Carey et al. 1999; Gray et al. 2009; Warne et al. 2011). Additionally, distinct ranavirus strains may have varying virulence and infection capabilities (Miller et al. 2011). Thus, it is imperative to maintain and update infection cases, as well as develop country-level screening protocols, to successfully conserve amphibians at national and global scales (Gray et al. 2009; Miller et al. 2015; García-Díaz et al. 2017).

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Table 1 List of amphibian ranavirus infection or mortality cases reported in Northeast Asia. Asterisks represent mass mortality event

Nation	Host species	Captivity	References
China	<i>Rana grylio</i>	Captive	Zhang et al.(1996)*, Zhang et al.(2001)*
	<i>Hoplobatrachus tigerinus</i>	Captive	Weng et al. (2002)*, He et al. (2002)
	<i>Rana dybowskii</i>	Wild	Xu et al. (2010)*, Zhu and Wang (2016)*
	<i>Andrias davidianus</i>	Captive	Geng et al. (2010), Geng et al. (2011)*, Zhou et al. (2012)*, Zhou et al. (2013)*, Chen et al. (2013)*, Meng et al. (2014)*
		Wild	Chen et al. (2013)*
	<i>Bombina orientalis</i>	Captive	Kolby et al. (2014)*
	<i>Cynops orientalis</i>	Captive	Kolby et al. (2014)*
	<i>Paramesotriton hongkongensis</i>	Captive	Kolby et al. (2014)*
<i>Rana nigromaculata</i>	Captive	Mu et al. (2018)*, Yu et al. (2020)*	
Japan	<i>Hynobius nebulosus</i>	Captive	Une et al. (2009a)*
	<i>Rana catesbeiana</i>	Wild	Une et al. (2009b)*
	<i>Dendrobates</i> spp.	Captive	Une et al. (2014)*
	<i>Phyllobates terribilis</i>	Captive	Une et al. (2014)*
Korea	<i>Pelophylax chosenicus</i>	Captive	Kim et al. (2009)*
	<i>Rana huanrenensis</i>	Wild	Kwon et al. (2017)*
	<i>Kaloula borealis</i>	Wild	Park et al. (2017)
	<i>Hyla japonica</i>	Wild	Park et al. (2017)

Within Korea, studies on amphibian infectious diseases have focused on *B. dendrobatidis*, the fungal agent causing chytridiomycosis (Yang et al. 2009; O'hanlon et al. 2018). In contrast, studies of ranavirus infections are limited to a handful of reported mortality events between four anuran species including Gold-spotted pond frog (*Pelophylax chosenicus*) tadpoles (Kim et al. 2009), Huanren brown frog (*R. huanrenensis*) tadpoles (Kwon et al. 2017), adult Boreal digging frogs (*Kaloula borealis*), and Japanese tree frog (*Hyla japonica*) tadpoles (Park et al. 2017). To date, all reported ranavirus strains detected in Korean amphibians share the major capsid protein (MCP) DNA sequence, similar to frog virus 3 (FV3), originally identified from *Lithobates pipiens* (formerly *R. pipiens*; Granoff et al. 1965) and *Atelognathus patagonicus* samples (Fox et al. 2006). To understand the characteristics of ranavirus infections and spread in Korea and across Northeast Asia, it is necessary to determine which viral strains are involved in such mortalities.

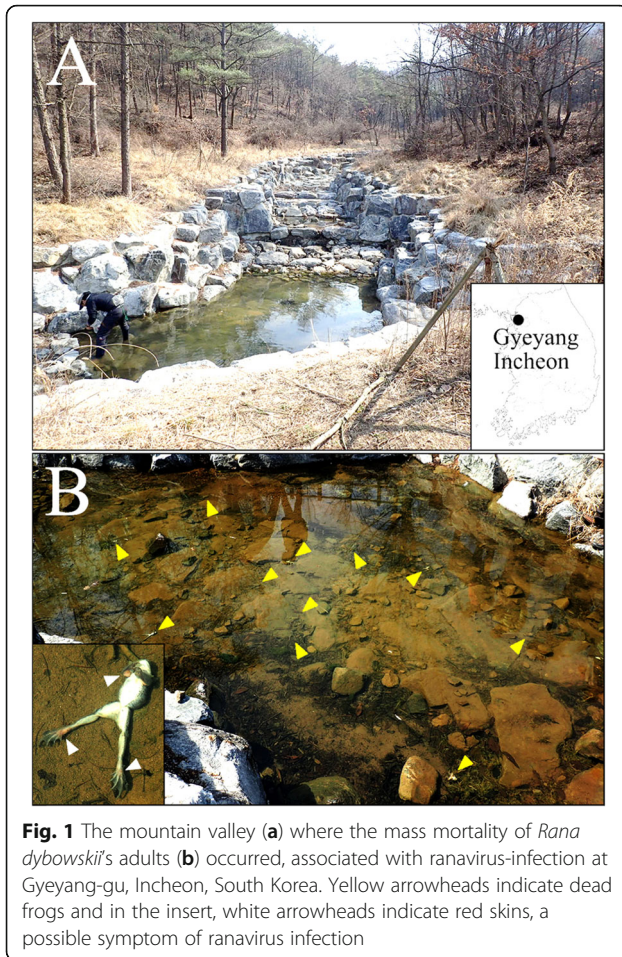
In this study, we described a mass mortality event, which occurred in 2017, in a wild population of Dybowskii's brown frogs (*R. dybowskii*) in South Korea. All sampled *R. dybowskii* were PCR-positive for ranavirus. Additionally, we determined the strain of ranavirus collected from *R. dybowskii* samples. This is the first known case of a ranavirus-associated mortality event of adult *R. dybowskii* in South Korea.

Materials and methods

On March 16, 2017, we found 22 dead adult Dybowskii's brown frogs (*R. dybowskii*) during a field survey at the upper region of a stream in Moksang-dong, Gyeyang-gu, Incheon, South Korea (37° 33' 34.05" N, 126° 42' 12.79" E). We collected 22 less decayed dead frogs in individual bags, transported the specimens to the laboratory, and preserved them at -20 °C until future use. In 2014, the stream where the frogs were collected, was heavily modified for erosion control purposes. While collecting dead frog specimens in 2017, we documented that the collection site stream was heavily modified and was lined with stones and concrete along the banks and bottom, and was planted with trees on either side (Fig. 1). Some live adult *R. dybowskii* individuals were observed within the stream where we collected the dead individuals; however, we were unable to collect any live *R. dybowskii* due to permitting. At this site, we did not observe any distinctive external symptoms or erratic behaviors, such as loss of buoyancy that were described by previous study (Miller et al. 2015), from individual live frogs.

Ranavirus detection

Prior to analysis, samples were slowly defrosted in 10 °C water. Once thawed, we examined any external physical abnormalities under a dissecting microscope (Sunny Optical Technology, China). Liver tissues, which have often been used for ranavirus detection (St-Amour and Lesbarrères 2007), were collected from each frog individual.



We extracted whole genomic DNA from 3 to 5 mg of liver tissue using the Qiagen DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). For ranavirus strain identification, the partial sequence of major capsid protein gene (MCP) was amplified using the specific primer pairs (MCP4 and MCP5; Mao et al. 1997). We ran polymerase chain reactions (PCR) following Mao et al. (1997) with a negative control using nuclease-free water and confirmed PCR products on 1% agarose gel by electrophoresis (Mao et al. 1997; Kwon et al. 2017). PCR was run on each sample at least twice to minimize viral false-positive detection.

Finally, PCR products were purified using an Accu-Prep® PCR Purification Kit (Bioneer, Daejeon, Korea) and sequenced using the same primer set (Macrogen, Seoul, Korea). Sequences were edited and assembled using Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand), and aligned using ClustalW (Thompson et al. 2003) for sequence comparison. For genetic relationship with other iridoviruses, we performed a custom nested BLAST using Geneious 9.1.8 and Bayesian Inference (BI) analysis with 18 ranavirus MCP genes, obtained from GenBank. The TIMef model was selected as the best

Akaike information criterion (AIC) scored model after testing 56 nucleotide substitution models in MOTELT-EST v3.7 (Posada and Crandall 1998). We analyzed the phylogenetic relationships among the iridoviruses by applying both maximum likelihood (ML) and Bayesian inference (BI) methods in PAUP v4.0 (Swofford 2001) and MrBayes v3.2.47 (Ronquist et al. 2012), respectively. For ML analysis and phylogenetic branching, we applied Bootstrap/Jackknife method, with 1000 bootstraps, and used the tree-bisection-reconnection (TBR) method. The BI analysis with Markov Chain Monte Carlo (MCMC) method was executed using the MrBayes (v3.2.47) software. With four random starting trees, we ran 1,000,000 generations, while sampling every 100 tree generations and discarding the first 5% of the sampled generations as burn-ins. Therefore, 500 of the 10,000 trees sampled were discarded.

Results

We found abdominal inflammation and erythema on the legs of seven collected frog specimens (Fig. 1). All 22 collected frogs were confirmed infected with ranavirus by PCR. Out of 22 PCR products, we obtained 13 partial MCP DNA sequences (> 500 bp) due to low sample quality. The obtained MCP DNA sequences (505 bp) were identical and had 100% sequence similarity to the haplotype (accession number KY264205) collected from a ranavirus-infected Huanren frog (*R. huanrenensis*; Kwon et al. 2017). In addition, the MCP sequence showed 99.8% similarity with ranavirus KRV-1 (HM133594) from South Korea, *Rana catesbeiana* virus (AB474588) from Japan, and 99.6% similarity for FV3 (FJ459783) a soft-shelled turtle iridovirus (DQ335253; Table 2). The Bayesian inference (BI) phylogenetic analysis revealed that our sequenced ranavirus grouped with a FV3-like virus including *Rana grylio* virus (RGV), FV3, and Bohle iridovirus. The nested BLAST analysis was consistent with our phylogenetic results.

Discussion

Diagnosing the physical symptoms of ranavirus infection was often difficult due to the decomposition status of the collected frog samples. Nevertheless, we found abdominal inflammation and erythema on the legs of seven frog specimens. Considering that erythema and skin ulcerations on the legs and ventrum, in amphibians, are known external characteristics of ranavirus infection (Gray et al. 2009; Park et al. 2017), we suspected our specimens might be infected with ranavirus.

Results of our molecular analyses corroborated our suspicions, as all frog specimens were confirmed infected with ranavirus. These results suggest that FV3-like ranavirus infections may be correlated with mass mortality events in populations of adult *R. dybowskii*. This fact is

Table 2 Results of custom BLAST using partial major capsid protein (MCP) DNA sequence (500 bp) of the ranavirus from *Rana dybowskii* in this study. The accession numbers of the sequences used for custom BLAST analysis are shown in Fig. 2

Sequence (host species)	Identical sites (%)
<i>Rana grylio</i> iridovirus isolate AD177LH (<i>Rana huanrenensis</i>)	100.0
Ranavirus KRV-1 (<i>Pelophylax chosonicus</i>)	99.8
<i>Rana grylio</i> iridovirus (<i>Rana grylio</i>)	99.8
<i>Rana catesbeiana</i> virus JP (<i>Lithobates catesbeianus</i>)	99.8
<i>Rana grylio</i> iridovirus isolate AD183OH (<i>Rana huanrenensis</i>)	99.8
Soft-shelled turtle iridovirus (<i>Pelodiscus sinensis</i>)	99.6
Frog virus 3 (<i>Rana pipiens</i>)	99.6
Bohle iridovirus (<i>Limnodynastes ornatus</i>)	99.2
Tiger frog virus (<i>Rana tigrina rugulosa</i>)	98.6
Pike perch iridovirus (<i>Stizostedion lucioperca</i>)	98.4
Chinese giant salamander virus (<i>Andrias davidianus</i>)	98.4
<i>Rana esculenta</i> virus (<i>Rana esculenta</i>)	98.2
Epizootic hematopoietic necrosis virus (<i>Perca fluviatilis</i>)	97.6
European sheatfish virus (<i>Silurus glanis</i>)	96.8
Ranavirus maxima (<i>Psetta maxima</i>)	96.2
Cod iridovirus (<i>Gadus morhua</i>)	96.2
<i>Ambystoma tigrinum stebbensi</i> virus (<i>Ambystoma tigrinum stebbensi</i>)	96.0
Short-finned eel ranavirus (<i>Anguilla australis</i>)	94.6

nothing new, as recent amphibian mass mortality events have been correlated with ranavirus infections across several studies (Weng et al. 2002; Une et al. 2009a; Kwon et al. 2017; Yu et al. 2020). Within the pet trade, ranavirus has been detected in a large number of amphibians, including cases in Hong Kong and Japan (Kolby et al. 2014; Une et al. 2014). Ranavirus strains, detected in the pet trade, were similar to the common midwife toad virus (CMTV) and FV3-like viruses, like the virus detected here. FV3-like viruses have been documented worldwide including Northeast Asia (Kim et al. 2009; Xu et al. 2010), Europe (de Matos et al. 2011), North and South America (Granoff et al. 1965; Fox et al. 2006), and Australia (Hengstberger et al. 1993). To date, at least 11 mass mortality events have been documented in Northeast Asia and were caused by FV3-like viruses (Zhang et al. 1996; Zhou et al. 2012). To this regard, distribution patterns of specific ranavirus strains across Northeast Asia are still under investigation (Duffus et al. 2015). FV3-like viruses have also been found in other taxa such as turtles (Chen et al. 1999) and fish (Ahne et al. 1989), highlighting the importance of ranavirus screening across taxa.

Although the majority of ranavirus-associated amphibian mortalities have occurred in captivity (Table 1; Meng et al. 2014; Mu et al. 2018), there have also been confirmed cases in wild populations. Wild ranavirus-associated mortality events have occurred across Asia, including the Heilongjiang, Jiangxi, and Henan provinces in China (Xu et al. 2010; Chen et al. 2013; Zhu and Wang 2016), the western part of Japan (Une et al. 2009b), and in Gangwon-do, Gyeongsangnam-do, and Daejeon in South Korea (Kwon et al. 2017; Park et al. 2017). Various environmental factors are known to increase ranavirus susceptibility and virulence in amphibians and facilitate mortality (Brunner et al. 2015). In this study, two environmental factors may have contributed to the mortality of adult *R. dybowskii*. First, the discovery site was heavily altered with concrete for erosion protection 3 years prior, causing water stagnation. Stagnant water during early spring drought periods may contain high concentrations of various ions and pollutants (Kang et al. 2016), possibly increasing stress hormones and making *R. dybowskii* individuals more susceptible to infection (Gahl and Calhoun 2010; Leduc 2014). In a previous study, dead adult boreal digging frogs were discovered in concrete walled, low circulation waterways (Park et al. 2017), similar to the environment observed in this study. Future studies should determine if surface alterations may influence amphibian-ranavirus susceptibility.

Second, amphibian mortality due to ranavirus has often been correlated with elevated stress hormone levels. Distinct life-history stages, including metamorphosis and reproduction, may be periods where frogs have elevated stress, thus increasing their susceptibility to infection (Green et al. 2002; Duffus et al. 2008; Gray et al. 2009). For example, ranavirus-linked mortality events occurred during the metamorphosis stage of *P. chosonicus* and during the breeding season of *K. borealis* (Kim et al. 2009; Park et al. 2017). *Rana dybowskii* is an explosive breeding species that communally spawns (Yoo and Jang 2012), possibly resulting in elevated stress hormones (Norris and Jones 2012). Thus, highlighting a need to understand how amphibian life history patterns influence viral susceptibility and virulence.

Conclusion

Here, we document the first mass mortality event of *R. dybowskii* in the wild. All collected individuals were PCR positive for ranavirus, possibly indicating that these individuals died due to viral infection. Elevated stress levels by erosion control works and/or from natural life-history stages may have contributed to ranavirus infection and mortality. To understand if ranavirus infection is a threat to Korean amphibians there are three conservation strategies, which should be implemented. First, there is a need for continuous, country-wide, monitoring

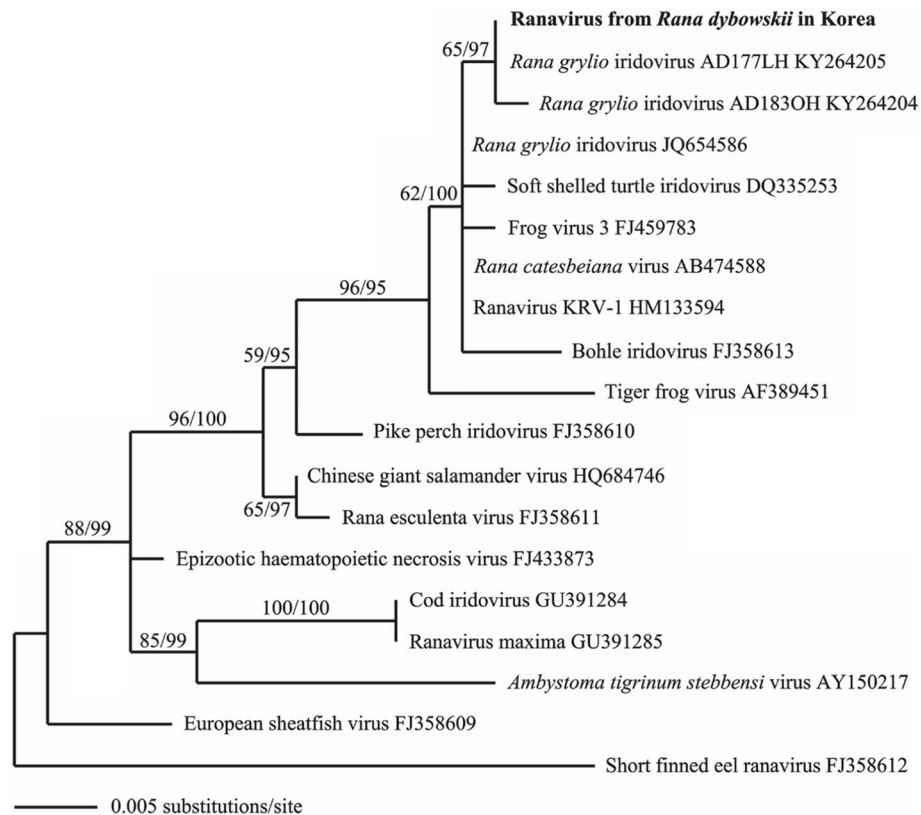


Fig. 2 Bayesian inference (BI) tree based on the 19 partial major capsid protein (MCP) DNA sequences (500 bp) of the ranavirus obtained from dead *Rana dybowskii* and from GenBank. We used short-finned eel ranavirus as the outgroup. The accession number of iridoviruses, which was obtained from GenBank, indicated after each species name. On each branch, analyzed values (ML bootstrap value/Bayesian posterior probabilities) are denoted

of amphibian populations. Second, ranavirus screening should be conducted across various taxa and not relegated to just amphibians. Third, government officials or researchers must identify which environmental factors may increase amphibian susceptibility to ranavirus. By implementing these three strategies, government officials and researchers may be able to successfully protect amphibians from ranavirus infections in Korea and perhaps globally.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s41610-020-00179-2>.

Additional file 1 PCR detection of the MCP sequence of ranavirus from the liver tissues of dead *Rana dybowskii*'s adults. The numbers on the bands represent individual frogs. P, positive MCP sequence control of ranavirus from *Rana huarenensis* tadpoles and N, negative control, which used nuclease-free water instead of extracted DNA in PCR process.

Abbreviations

AIC: Akaike information criterion; Bd: *Batrachochytrium dendrobatidis*; BI: Bayesian inference; CMTV: Common midwife toad virus; FV3: Frog virus 3; MCP: Major capsid protein; MCMC: Markov Chain Monte Carlo; ML: Maximum

likelihood; PCR: Polymerase chain reaction; RGV: *Rana grylio* virus; TBR: Tree-bisection-reconnection

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Authors' contributions

JP analyzed experimental data and wrote the manuscript draft. IK performed the molecular experiment. NY performed the field survey. NH and AGP reviewed/edited the manuscript. DP designed the study and reviewed/edited the manuscript draft. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee in Kangwon National University (KW-200618-3).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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