First case of ranavirus-associated mass mortality in a natural population of the Huanren frog (*Rana huanrenensis*) tadpoles in South Korea

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**ABSTRACT**

Globally, ranavirus is often responsible for the mass mortality of a variety of captive and wild amphibians. In Asia, several mass mortality cases of captive amphibians by ranavirus are known, but one mass mortality case in the wild has been reported in a non-endemic larval bullfrog population in Japan. In order to verify factors involved in mass mortality of *Rana huanrenensis* tadpoles (> 200 tadpoles) in a mountain stream in South Korea, we investigated possible infections by ranavirus, chytrid fungus, and lethal bacteria by conducting PCR assays of pathogens with specific primers. We found that all *R. huanrenensis* tadpoles collected (two alive and ten carcasses) showed positive PCR results for two different ranavirus primer sets targeting partial genes of a major capsid protein (MCP). The identified MCP sequence was more closely related to *Rana catesbeiana* virus JP MCP, isolated from invasive bullfrog tadpoles in Japan. We could not detect any lethal bacteria or chytrid fungus in the specimens. Our finding is the first report in Asia that ranavirus is involved in the mass mortality of endemic wild amphibians.

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**Introduction**

In recent decades, mass mortality events of amphibians have been increasing and emerging infectious diseases are recognized as one of the important factors (Fey et al. 2015). In addition to chytridiomycosis, ranavirus has played a major role in such mass mortality events (Teacher et al. 2010; Price et al. 2014; Rosa et al. 2017). *Ranavirus*, a genus of the family Iridoviridae, commonly infects fishes, amphibians, and reptiles, but host range varies along lineages and isolates (Duffus et al. 2015). Approximately 103 amphibian species in 18 families are known to have been infected (Duffus et al. 2015). Reports on ranavirus infections of amphibians are increasing in Europe, Africa, and Central and South America, in part due to increased surveillance effort, and related studies are ongoing (Miller et al. 2011; Duffus et al. 2015).

In Asia, mortality of captive or cultured amphibians by ranavirus are known from China, Japan, Thailand, and South Korea in Japanese clouded salamanders (*Hynobius nebulosus*, Une et al. 2009a), Chinese giant salamanders (*Andrias davidianus*, Geng et al. 2011), pig frogs (*Rana grylio*, Zhang et al. 1996), tiger frogs (*R. tigrina*, Kanchanakhan 1998; Weng et al. 2002), gold-spotted pond frogs (*R. plancyi chosenica*, Kim et al. 2009), and imported captive frogs (*Dendrobates* and *Phyllobates spp.*, Une et al. 2014). Also, Xu et al. (2010) reported a ranavirus infection case of wild Dybowski’s frogs (*R. dybowskii*) in China, but without any mortality. In South Korea, Park et al. (2017) reported ranavirus infections of wild narrow-mouthed toads (*Kaloula borealis*) with several mortalities and Korean tree frog tadpoles (*Hyla japonica*) without any mortality. Despite these various reports, there has only been on wild mass mortality case in introduced bullfrog (*L. catesbeiana*) tadpoles in Japan (Une et al. 2009b). Thus, further documentation of ranavirus induced mass mortality in wild populations of Asian amphibian species is of critical concern.

In this study, we report on the first Asian ranavirus-associated mass mortality event in a wild population. It occurred in endemic Huanren frog (*Rana huanrenensis*) tadpoles in a mountain stream in South Korea in June 2015.

**Materials and methods**

**Sample collection**

During a field survey on June 14, 2015, we found dozens of floating, dead Huanren frog (*R. huanrenensis*) tadpoles...
between stages 36–39 (Gosner 1960) and more than two hundred tadpole carcasses among rocks and pebbles at the edge of a mountain stream in Inje-gun, Kangwon-do, South Korea (37° 59’ 1.19’’N 128° 29’ 24.70’’E; Figure 1). We collected 10 tadpoles that were minimally decayed. Additionally, we collected two living and four dead tadpoles on June 18, 2015. During our two collection periods we could not find any carcasses of other animals, such as invertebrates and fishes, at the mortality site. Two live tadpoles were euthanized in 0.1% MSS222 for use in the study.

Detection of bacteria

In order to detect bacterial infection we incubated samples of skin, digestive canal, and other internal tissues from six tadpoles (two alive and four dead) that were collected on June 18, 2015. We swabbed each tissue several times using a swab stick (MG Scientific Inc., Wisconsin, USA) that we then rinsed by agitating in a 15 ml conical tube containing 10 ml distilled water. We inoculated this rinse onto a 3M™ Petrifilm™ Aerobic Count Plate (3M-Korea, Seoul, Korea) and incubated at 35°C for 48 hours following the manufacturer’s protocol.

After incubation, we randomly selected 54 bacterial colonies (three colonies × three tissue types × six individuals) from the plates and extracted total DNA using Qiagen DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. For bacterial identification, we targeted 16S ribosomal RNA. We amplified a 1400 bp fragment using the forward primer 27F and the reverse primer 1492R (Lane 1991, Table 1). DNA was amplified using a SimpliAmp Thermal Cycler (Life Technologies, Carlsbad, CA, USA).

Figure 1. Location where the mass-mortality of Huanren frog (Rana huanrenensis) tadpoles occurred at Inje, Kangwon, South Korea in June 2015. Yellow arrows in A, B, C indicate the areas where dead tadpoles were mainly found (A, B, C) and those in D indicate dead tadpoles among the pebbles. Inserted image in panel D shows a dead tadpole.
in 25 µl reaction volumes, consisting of 10 ng of template DNA, 0.25 µl of ELPIS rTaq DNA polymerase (ELPIS, Daejeon, South Korea, 5 U/µl), 2.5 µl of 10x PCR buffer, 2 µl of 10 mM dNTP mix (2.5 mM each), and 0.5 µl of each primer (10 pmol). The cycling conditions for PCR were as follows: for 5 min, followed by 35 cycles at 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min with a final extension step of 72°C for 10 min. We confirmed PCR products on 1% agarose gel by electrophoresis. Products were purified using an AccuPrep® PCR Purification Kit (Bioneer, Daejeon, South Korea) following its provided protocol, and sent to the Macrogen (Seoul, Korea). Prior to sequencing, samples were purified using an AccuPrep® PCR Purification Kit (Bioneer, Daejeon, Korea). Sequences were edited and assembled using Bioedit. To examine the relationship with previously known ranavirus, Mao and Marsh fragments (1099 bp) were concatenated in Geneious (ver. 9.1.7), and used for custom BLAST in NCBI. For the custom BLAST, we included 16 ranavirus MCP sequences retrieved from GenBank (for accession numbers, see Figure 2), which included those of the ranavirus previously known and often detected from amphibians, reptiles, and representative fishes (He et al. 2002; Marsh et al. 2002; Jancovich et al. 2003; Holopainen et al. 2009; Huang et al. 2009; Une et al. 2009b; Ariel et al. 2010; Jancovich et al. 2010; Geng et al. 2011; Kim et al. 2011; Lei et al. 2012). In addition, we investigated the phylogenetic position of obtained ranavirus sequences and produced a phylogenetic tree using the 18 sequences (2 from our samples, 16 from GenBank) and the sequence for short-finned eel ranavirus as an outgroup. We aligned using ClustalW (ver. 2.1), assembled all sequences in Geneious (ver.9.1.7), and then analyzed the sequence data using both maximum likelihood (ML) and Bayesian inference (BI) methods. ML analyses were run in RAxML v7.2.8. The best-scoring ML tree was inferred with 100 replicates and a nonparametric bootstrap analysis for 1000 replicates was used to evaluate node robustness. All replicates were run under the GTR + gamma model of sequence evolution. For BI analyses, Markov Chains Monte Carlo chains were run for 2.5 million generations, sampling every 1000 generations, implemented in MrBayes v3.1. Models of nucleotide substitution were chosen within MrBayes using the reversible jumping model choice (nst = mixed) with both rate variation and invariable sites (rates = invgamma).

Animal handling and experimental procedures were conducted in accordance with guidelines established by the Kangwon National University Institutional

| Gene | Primer | Nucleotide sequence | Reference |
|------|--------|---------------------|-----------|
| 16S  | 27F    | 5′-AGAGTTTGTCTGCGCTACG-3′ | Lane 1991 |
| 16S  | 1492R  | 5′-TACGGTACCTGAGCTAAGATTT-3′ | Lane 1991 |
| ITS1 and ITS2 | Bd1a | 5′-CATGTTCCATATGCACG-3′ | Annis et al. 2004 |
| ITS1 and ITS2 | Bd2a | 5′-CATGTTCACTATCGCTCCAG-3′ | Annis et al. 2004 |
| MCP  | MCP4   | 5′-GACCTGCCCACATGATG-3′ | Mao et al. 1997 |
| MCP  | MCP5   | 5′-GTTCTGGGAGGAAGAAGA-3′ | Marsh et al. 2002 |
| MCP  | M153   | 5′-ATACACGCGCCCTCTACACG-3′ | Marsh et al. 2002 |
| MCP  | M154   | 5′-GCATCGGCACGGCTACTAG-3′ | Marsh et al. 2002 |

**Detection of chytrid fungus**

In order to detect possible chytrid fungus infection from tadpole samples, we used both skin and internal tissues of 12 tadpoles (24 total samples). Because we could not separate specific internal organs due to decomposition and small body size, we used combined tissues. We extracted total DNA using the same Qiagen DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) and conducted PCR with a chytrid fungus specific Bd1a/ Bd2a primer set (Table 1) following Annis et al. (2004). The targeted 300 bp PCR product was examined on 1% agarose gel by the electrophoresis. No positive controls were used due to the lack of available chytrid-infected tissues and DNA.

**Detection of ranavirus**

In order to detect possible ranavirus from the samples, we used DNA from the 12 tadpole samples used in the chytrid fungus assay (see above). We screened for ranavirus using PCR targeting 500 bp (hereafter Mao fragment) and 600 bp (hereafter Marsh fragment) partial sequences of ranavirus major capsid protein gene (MCP) using two different primer sets of MCP4/ MCP5 (Mao et al. 1997, Table 1) and M153/ M154 (Marsh et al. 2002, Table 1), respectively. We ran the PCR following Mao et al. (1997) and Marsh et al. (2002) and confirmed PCR products on 1% agarose gel by the electrophoresis.

Among the PCR products, we selected 14 Mao fragments (skin: 3, internal tissue: 11 from 12 tadpoles) and 4 Marsh fragments (internal tissue of 4 tadpoles) of which DNA amounts were abundant for sequencing (Macrogen, Seoul, Korea). Prior to sequencing, samples were purified using an AccuPrep® PCR Purification Kit (Bioneer, Daejeon, Korea). Sequences were edited and assembled using Bioedit. To examine the relationship with previously known ranavirus, Mao and Marsh fragments (1099 bp) were concatenated in Geneious (ver. 9.1.7), and used for custom BLAST in NCBI. For the custom BLAST, we included 16 ranavirus MCP sequences retrieved from GenBank (for accession numbers, see Figure 2), which included those of the ranavirus previously known and often detected from amphibia, reptiles, and representative fishes (He et al. 2002; Marsh et al. 2002; Jancovich et al. 2003; Holopainen et al. 2009; Huang et al. 2009; Une et al. 2009b; Ariel et al. 2010; Jancovich et al. 2010; Geng et al. 2011; Kim et al. 2011; Lei et al. 2012). In addition, we investigated the phylogenetic position of obtained ranavirus sequences and produced a phylogenetic tree using the 18 sequences (2 from our samples, 16 from GenBank) and the sequence for short-finned eel ranavirus as an outgroup. We aligned using ClustalW (ver. 2.1), assembled all sequences in Geneious (ver.9.1.7), and then analyzed the sequence data using both maximum likelihood (ML) and Bayesian inference (BI) methods. ML analyses were run in RAxML v7.2.8. The best-scoring ML tree was inferred with 100 replicates and a nonparametric bootstrap analysis for 1000 replicates was used to evaluate node robustness. All replicates were run under the GTR + gamma model of sequence evolution. For BI analyses, Markov Chains Monte Carlo chains were run for 2.5 million generations, sampling every 1000 generations, implemented in MrBayes v3.1. Models of nucleotide substitution were chosen within MrBayes using the reversible jumping model choice (nst = mixed) with both rate variation and invariable sites (rates = invgamma).

Animal handling and experimental procedures were conducted in accordance with guidelines established by the Kangwon National University Institutional
Animal Care and Use Committee (Permit Number: KW-160616-1).

Results and discussion

Visual inspection of dead tadpoles revealed that the skin of some tadpoles were slightly swollen, but gross lesions, external hemorrhages, or edema on the body were not confirmed, largely due to the decomposed condition of the tadpoles. In addition, live tadpoles collected from the site did not show any erratic swimming or loss of buoyancy.

During bacterial infection assays, we identified a total of six bacterial species from 49 colonies (Ralstonia pickettii, Paraburkholderia fungorum, Phyllobacterium myrsinacearum, Sphingomonas echinoides, Caulobacter vibrioides, Bacillus thuringiensis; E value: 0.0, Query cover: 100%, Ident: 99%; Table 2). Detection rate of R. pickettii was the highest, while B. thuringiensis was the lowest. In amphibians, the most well-known lethal bacterial diseases include bacterial dermatosepticemia (red-leg syndrome), flavobacteriosis, and mycobacteriosis that are caused by Aeromonas hydrophila, Flavobacterium, and Mycobacterium, respectively (Densmore and Green 2007). If bacterial infection was mainly responsible for the mortality at our study site, we should detect such bacteria at least from some of the bacterial colonies examined. In our analyses, we could not find any of these bacteria from the tadpole samples, indicating that known bacterial diseases were less likely to cause the mass mortality of R. huanrenensis tadpoles at our study site.

For chytrid fungus assays, none of the 12 tadpole samples gave positive chytrid fungus results. Despite not including positive controls, we have confidence in our results because we had successfully detected chytrid fungus (Accession Number: AY997031).

Table 2. List of bacterial species identified from the skin, internal tissue, and digestive canal samples of two live and four dead Huanren frog (Rana huanrenensis) tadpoles collected from the mass-mortality site on June 18, 2015. We successfully analyzed 49 bacterial sequences and determined species (mean bp: 1193 bp, range: 768–1389 bp) out of total 54 colonies (each three colonies × three different organs × six tadpoles).

| Class              | Species                        | Skin | Internal tissue | Digestive canal | (%)  |
|--------------------|--------------------------------|------|-----------------|-----------------|------|
| Alphaproteobacteria| Caulobacter vibrioides         | 2    | 2               | 1               | 10.2 |
|                    | Phyllobacterium myrsinacearum  | 1    | 3               | 1               | 10.2 |
|                    | Sphingomonas echinoides        | 1    | 1               | 3               | 10.2 |
| Betaproteobacteria | Bacillus thuringiensis         | 1    | 1               |                 | 2.0  |
| Betaproteobacteria | Paraburkholderia fungorum      | 6    | 4               | 4               | 28.6 |
|                   | Ralstonia pickettii            | 7    | 6               | 6               | 38.8 |

Figure 2. Phylogenetic tree based on partial MCP DNA sequences of ranavirus. The sequences (1099 bp) obtained from Huanren frog (Rana huanrenensis) tadpoles in this study are in large bold font and showed over 99% identity with the MCP gene sequence of Rana catesbeiana virus JP, Frog virus 3, Rana grylio iridovirus, Ranavirus KRV-1, soft-shelled turtle iridovirus in a descendant identity order. Numbers at the end of the taxon name refer to GenBank accession numbers. Numbers on the branches represent support values for the major groups—ML bootstrap support, followed by Bayesian posterior probabilities.
EU779865, FJ373885) from several imported frog species using the same primer set and the same protocol. Our result implies that R. huanrenensis samples were less likely infected by chytrid fungus. Moreover, a chytrid fungus strain found in South Korea was also found not to kill endemic amphibians (Bataille et al. 2013). This also lends weight to the unlikeness that chytrid fungus is involved in the mass mortality of R. huanrenensis tadpoles at our study site.

For ranavirus assays, we confirmed Mao (S Fig. 2) and Marsh (S Fig. 3) fragments from both skin and internal tissues of all 12 R. huanrenensis tadpoles examined. For the Mao fragment, 11 tadpoles had the same sequence (skin: 2, internal tissue: 10, Accession Number: KY264205), while one individual (#6) had a 1 bp difference (skin: 1, internal tissue: 1, Accession Number: KY264204). For the Marsh fragment, four individuals sequenced (including # 6 individual) had identical sequences (Accession Number: KY496311). In a BLAST analysis, two ranavirus MCP sequences which concatenated KY264205+KY496311 and KY264204+KY496311 (both 1099 bp) had a 99.9% identity with each other and were closest to MCP sequence of Rana catesbeiana virus JP with identity of 99.5% in both cases (Table 3). The positions of the two sequences on the phylogenetic tree were consistent with BLAST results (Figure 2).

The base substitution of G with A, which observed in KY264204, was not found in the previously reported 16 ranavirus sequences from NCBI. The substitution produces amino acid change (Aspartic acid to Asparagine). Considering several studies dealt with diversity in ranavirus mutations (Huang et al. 2011; Zhu and Wang 2016), related issues should be further investigated with more cases in the future.

The foregoing result indicates that ranavirus could be involved in the mass mortality of R. huanrenensis tadpoles at our study site. In previous studies, certain anthropogenic and ecological drivers such as contaminants from fish farms, high individual density, or low-food condition might facilitate ranavirus mortality (Reeve et al. 2013; North et al. 2015). Although we cannot exclude the possibility of these factors in our study, such factors are unlikely because of the absence of other animal carcasses at study site. In the wild, amphibian mass mortality associated with ranavirus has been often reported in Europe and America (Miller et al. 2011; Duffus et al. 2015), while in Asia, only one case, in L. catesbeiana (an introduced species), was reported in Japan (Une et al. 2009b). Our result is the first report in Asia that ranavirus is associated with the mass mortality of an Asian endemic frog in the wild.

Frog virus 3 (FV3)-like virus may be responsible for mass mortality of frogs in Asia. To date, six different species of ranavirus are globally recognized and among them, FV3, Bohle iridovirus (BIV), and Ambystoma tigrinum virus (ATV) mainly infect amphibians (Jancovich et al. 2015). In Asian frogs, Rana grylio iridovirus and Rana catesbeiana virus JP, which are close to FV3, were previously reported from cultured pig frogs (Zhang et al. 1996) and wild bullfrogs (Une et al. 2009b), respectively. Also, ranavirus (KRV-1) from cultured gold-spotted pond frog tadpoles in South Korea (Kim et al. 2011) and ranavirus from imported captive Dendrobates and Phylllobates spp. in Japan (Une et al. 2014) were both identified to be close to FV3. In addition, the MCP characterized in this study share close sequence identity with Rana catesbeiana virus JP MCP. These results show that FV3-like virus mainly affect Asian frogs.

With this finding, that ranavirus is associated with mass mortality in a wild Korean frog, broad screening of ranavirus in various habitats and other amphibian species, additional mortality case reports, and studying potential anthropogenic and ecological drivers for ranavirus mortality are urgently encouraged in South Korea as well as other Asian countries.

### Table 3. Results of custom BLAST using concatenated two ranavirus sequences (Accession Number: KY264305 + KY496311, KY264–24 + KY496311) obtained from Huanren frog (Rana huanrenensis) tadpoles in this study and 16 sequences from GenBank. The sequence lengths compared were identical as 1099 bp. For the accession number of each gene from GenBank, see Figure 2.

| Sequence | KY264205 | KY264204 |
|----------|----------|----------|
| +KY496311 | +KY496311 |
| obtained Ranavirus known | 99.909 | 99.909 |
| Rana catesbeiana virus JP | 99.454 | 99.454 |
| Frog virus 3 | 99.454 | 99.363 |
| Rana grylio iridovirus | 99.454 | 99.363 |
| Ranavirus KRV-1 | 99.454 | 99.363 |
| Soft-shelled turtle iridovirus | 99.181 | 99.181 |
| Bohle iridovirus | 98.726 | 98.726 |
| Tiger frog virus | 98.089 | 98.089 |
| Chinese giant salamander virus | 97.998 | 97.998 |
| Rana esculenta virus | 97.998 | 97.998 |
| Pike perch iridovirus | 97.907 | 97.907 |
| Epizootic haematopoietic necrosis virus | 97.270 | 97.270 |
| European catfish virus | 96.815 | 96.815 |
| Cod iridovirus | 96.724 | 96.724 |
| Ranavirus maxima | 96.633 | 96.633 |
| Ambystoma tigrinum stebbensi virus | 96.087 | 96.087 |
| Short-finned eel ranavirus | 93.722 | 93.722 |

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Disclosure statement
No potential conflict of interest was reported by the authors.

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