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PCR Detection of *Ranavirus* in Gold-spotted Pond Frogs (*Rana plancyi chosenica*) from Korea

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Abstract - In recent years, there has been a rapid global decline in amphibian populations, and infectious diseases have been associated with this decline. Diseased Gold-spotted pond frogs (*Rana plancyi chosenica*) were collected from a frog farm in Korea and identified using morphological and molecular analysis to identify the disease. The typical symptoms of ranaviral infection were observed in the tadpoles and adults frogs. The nucleotide sequence analysis revealed that the sequences showed the closest similarity with sequences to Frog virus 3, which belongs to the genus *Ranavirus*.

Key words : amphibian, infectious disease, *Rana plancyi chosenica*, frog virus 3, ranavirus, iridovirus

INTRODUCTION

There has been a global decline in amphibian populations (Alford *et al.* 2001), and many factors are known to be closely associated with the decline; these factors include destructions and alterations of the physical habitats of the animals, emergence of infectious diseases, introduction of predatory invasive species, and changes in environmental conditions, such as increased exposure to ultraviolet radiation and acid precipitation (Alford and Richards 1999; Gardner 2001; Beebee and Griffiths 2005). Blaustein and Kiesecker (2002) suggested that the decline could be attributed to complex interactions among multiple factors and that the causative factors differed among regions. Recently, there have been a number of reports that have implicated infectious diseases as the cause of the global decline in amphibian populations (Daszak *et al.* 1999; Longcore *et al.* 1999). It is generally accepted that emerging infectious diseases, including chytridiomycosis, ranavirus disease, and saprolegniasis, play a major role in the high mortality rates in amphibian populations worldwide (Berger and Speare 1998; Daszak *et al.* 1999; Pessier *et al.* 1999; Daszak *et al.* 2003).

A number of studies on frog diseases associated with the decline in amphibian populations have reported that several viral agents of the family *Iridoviridae* (genus *Ranavirus*) are responsible for the disease outbreaks (Cunningham *et al.* 1996; Hyatt *et al.* 2000). Ranaviruses belong to the family *Iridoviridae*; they are icosahedral cytoplasmic DNA viruses that cause systemic disease in infected animals and often result in high morbidity and mortality (Williams 1996). Vertebrate iridoviruses that cause systemic infections characterized by hematopoietic necrosis are being increasingly identified in cultured fish and amphibians, and these viruses cause economic losses in many countries. In Korea, ranavirus infections were reported in a wide variety of cultured fishes (Do *et al.* 2005; Jeong *et al.* 2006); however, there was no report on ranavirus infection in wild or cultured frogs

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in Korea. In this study, diseased Gold-spotted pond frogs (*Rana plancyi chosenica*) was collected from a frog farm in Korea, and the disease was identified as ranavirus infection using morphological and molecular analyses.

MATERIALS AND METHODS

The diseased *R. plancyi chosenica* tadpoles and adults were collected from a frog farm located at SinBuk-myeon, Chuncheon-si, Gangwon-do between 2006 and 2007. The diseased frogs were placed into a bag containing pieces of ice, transported to the laboratory, and stored in a freezer (−20°C) until use. The healthy tadpoles were stored in 2 containers (300 × 150 × 200 cm), each containing 50 tadpoles; two diseased tadpoles were added to 1 of the containers to investigate the transmission of the infection via exposure to infected tadpoles under controlled conditions. The tadpoles in both containers were observed for infection, and the mortalities were recorded every day over 10 days.

The diseased fogs were observed under light microscopes and their characteristics were recorded. The diseases were identified on the basis of morphological characteristics (Green *et al.* 2002). In addition, the skin tissue or infected intestinal parts were removed for molecular identification of the pathogens, and DNA was extracted by using the protocol provided in the DNeasy Tissue Kit (Qiagen Science, USA). The DNA was amplified using Prime Taq Premix (G-2000) obtained from GENET (mixing solution [pH 9.0] in a 1 mL microtube, 1 unit/10 μL Prime *Taq* DNA polymerase, 2 × reaction buffer, 4 mM $MgCl_2$, enzyme stabilizer, loading dye, and 2 mM dNTPs). The gene for the major capsid protein was amplified by using the specific primer pairs for detection of iridoviruses, i.e., M153 (5′-ATGACC-GTCGCCCTCATCAC-3′) and M154 (5′-CCATCGAGCC-GTTCATGATG-3′) (Marsh *et al.* 2002). The total volume of the Prime *Taq* Premix was adjusted to 20 μL with 2 μL of each primer (10 pM), 2 μL of template DNA, and 4 μL of pure water, and PCR was performed using a thermal cycler (Applied Biosystems, USA). The PCR protocol was as follows: 1 cycle at 94°C for 3 min; 30 cycles at 94°C for 30 sec, 64°C for 30 sec, and 72°C for 30 sec; and 1 cycle at 72°C for 5 min. The PCR products were sequenced using the AMI PRISM 377 automated sequencer (Perkin-Elmer, USA). A sequence-similarity search of the National Center for Biotechnology Information (NCBI) database was conducted using the Basic Local Alignment Search Tool (BLAST) algorithm.

RESULTS AND DISCUSSION

Diseased Gold-spotted pond frog (*R. plancyi chosenica*) tadpoles were found at the study sites in both 2006 and 2007. In July 2006, there were 10 infected tadpoles in the cultivation ponds during the metamorphosing period of the frogs. In July 2007, more than 1,000 tadpoles and 25 adult frogs further died in the cultivation sites. The metamorphos-

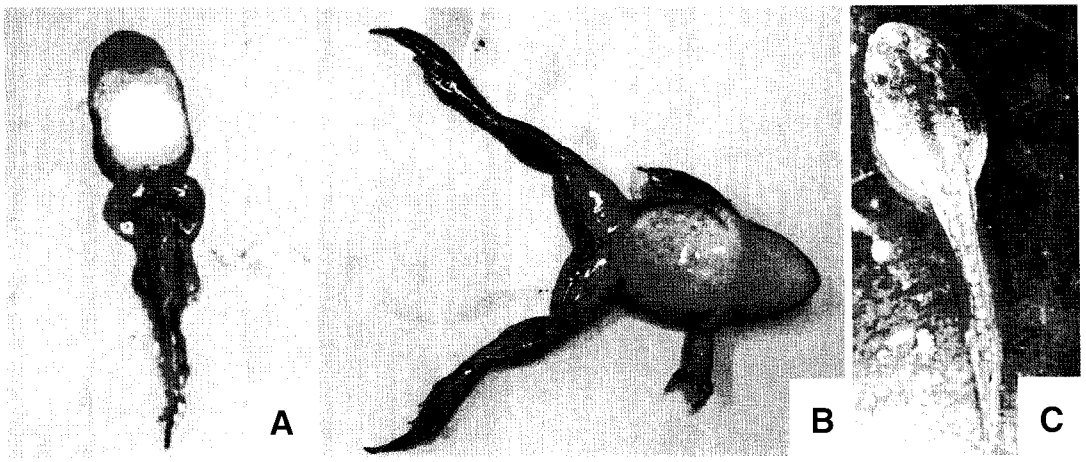

Fig. 1. Morphological features showing symptoms of diseased frogs found in Korea. A tadpole (A), an adult (B) and a tadpole from the transmission test of Gold-spotted pond frogs (*Rana plancyi chosenica*) infected by *Ranavirus* (C). Abscission of leg during metamorphosis was observed. The incomplete leg was red and had the symptom of edema.

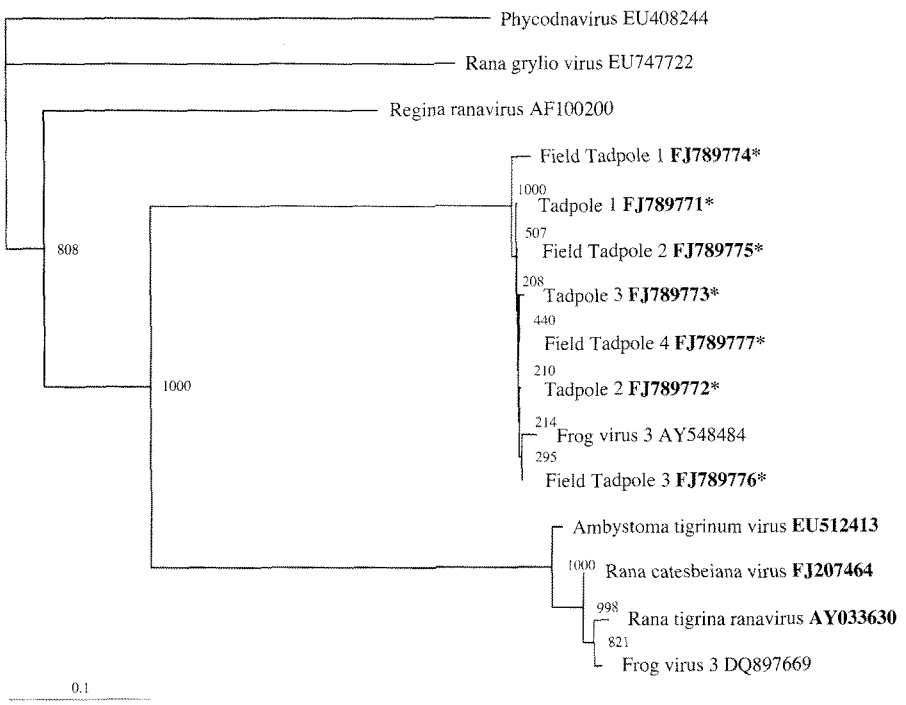

Fig. 2. Neighbor joining tree illustrating the phylogenetic relationship of the sequences obtained from diseased Gold-spotted pond frogs (*Rana plancyi chosenica*) in Korea and other ranavirus sequences. Asterisks indicate the sequences form this study and Numbers at nodes represent percentage of bootstrap analysis based on 1,000 replicates. Phycodnavirus is used as an outgroup.

ing tadpoles had abscised legs, and the incomplete legs showed symptoms of hemorrhage and edema, which are typically observed in ranaviral infections (Fig. 1A, Green *et al.* 2002). Hemorrhage and edema were also observed in the legs of the infected tadpoles. In adult frogs, the skin of the abdomen and legs was red in color, which is a symptom of hemorrhage (Fig. 1B). The PCR products obtained after amplification with the specific primers M153 and M154 were 625 bp in length, and the nucleotide sequence analysis of this product revealed that the MCP-amplified sequences showed the closest similarity with sequences to frog virus 3 (Fig. 2). These sequences showed 99% similarities to the *FV3 MCP* gene, which was isolated from infected tadpoles of *R. catesbeiana* (DQ897669) in Brazil and *R. pipiens* in the USA (AY548484). The sequences were deposited in the GenBank under accession numbers FJ789774, FJ789775, FJ789776 and FJ789777.

All the tadpoles showed symptoms of ranaviral infection in the transmission test, and they died 10 days after the infected frogs were added to the container (Fig. 1C). However, the tadpoles in the control container showed no symptoms of the infection. DNA was extracted from the infected tadpoles that had been stored in the container, and the sequence data of the DNA was analyzed. The sequences (GenBank accession numbers FJ789771, FJ789772 and FJ789773) showed 100% similarity to the ranavirus sequences isolated from infected tadpoles of *R. catesbeiana* (FJ207464) in Taiwan and 99% similarity to DQ897669 and AY548484 (Fig. 2). Sequence analysis revealed that the sequences of the tadpole viruses used for inoculations were identical to those obtained from the viruses that infected the healthy tadpoles in the container, thereby confirming that the viruses from the infected frogs were transmitted to the healthy tadpoles in the container.

Ranaviruses are broadly distributed and infect a wide range of reptilian, piscine, and anuran species, including farmed frogs; they are also being reported as emerging pathogens of aquatic organisms all over the world (Mao *et al.* 1999; Hyatt *et al.* 2000). Clinical signs of infection in tadpoles and frogs can include evidence of cutaneous erythema, petechiation, and subcutaneous edema, or even death without external manifestations of disease. In this study, the infected adults and tadpoles of the Gold-spotted pond frog showed the symptoms of hemorrhage and edema, which are typically observed in ranaviral infections (Green *et al.* 2002). Results from morphological and molecular analyses as well

as those from the transmission tests suggest that the virus infecting the *R. plancyi chosenica* tadpoles and adults was frog virus 3, which belongs to the genus *Ranavirus*.

Ranaviruses are highly virulent, with reported mortality rates often greater than 90% (Green *et al.* 2002). In addition, ranaviruses are capable of infecting animals from more than 2 different taxonomic classes under natural conditions, implying that fishes serve as a reservoir for the amphibian viruses, or vice versa (Mao *et al.* 1999). However, treatments for the control of ranavirus infections have not been previously described in detail. In cases of colony outbreaks, isolation of affected individuals, thorough disinfection of contaminated enclosures, and quarantine-type animal-handling practices could be advisable treatments. Studies have suggested that the disease outbreaks are often caused by complex interactions among many biotic and abiotic factors, and these outbreaks are responsible for the decline in some local amphibian populations (Blaustein and Kiesecker 2002). Therefore, in addition to the efforts that are being adopted to control environmental factors, programs for conservation and reintroduction of amphibians should include measures for disease control.

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