Mycobiology 36(1) : 10-12 (2008)

Detection of Infectious Fungal Diseases of Frogs Inhabiting in Korea

Suk Kim[1], Ahn-Heum Eom[1]*, Daesik Park[2] and Nam-Yong Ra[3]

[1]*Department of Biology, Korea National University of Education, Chungbuk 363-791, Korea*
[2]*Division of Science Education, Kangwon National University, Chuncheon, Kangwon 363-791, Korea*
[3]*Department of Biology, Kangwon National University, Chuncheon, Kangwon 363-791, Korea*

(Received January 25, 2008. Accepted March 11, 2008)

In recent years, there has been a rapid decrease in amphibian populations worldwide, and infectious diseases have been associated with this decline. Diseased frogs inhabiting Korea were collected from fields, and the diseases were identified by morphological and molecular analyses. Two fungal diseases-saprolegniasis and chromomycosis-were detected in the frogs. Saprolegniasis caused by *Saprolegnia* spp. was found in *Rana plancyi chosenica* from Gangwon-do and *Rana huanrenensis* from Chungbuk. Chromomycosis, which is caused by infection with *Cladosporium cladosporioides*, was detected in *Rana catesbeiana* from Busan.

KEYWORDS : Amphibian, Chromomycosis, Fungal, Infectious disease, Saprolegniasis

Many factors are known to be related with the worldwide decline in amphibian populations (Alford and Richards, 1999; Beebee and Griffiths, 2005). Although the precise causes for the declines remain to be determined yet, modifications in the physical habitat, infectious diseases, predation by exotics, and changes in environmental conditions such as increased exposure to ultraviolet radiation and acid precipitation are known to be closely associated with the decline in the amphibian populations (Gardner, 2001). Blaustein and Kiesecker (2002) suggested that the declines were the result of complex interactions among multiple factors and that the causative factors differed among regions.

Recently, there are increasing reports of infectious diseases as the cause of the global decline in amphibian populations (Daszak *et al.*, 1999; Longcore *et al.*, 1999). A variety of diseases, including chytridiomycosis, ranavirus disease, and saprolegniasis, have been generally accepted to play a major role in the high mortality in amphibian populations worldwide (Berger and Speare, 1998; Daszak *et al.*, 1999; Pessier *et al.*, 1999). Infectious fungal diseases affecting frogs include chytridiomycosis, chromomycosis, mucormycosis, and saprolegniasis. In particular, chytridiomycosis that is caused by *Batrachochytrium dendrobatidis* has received recent attention with high mortality of amphibian populations around the world. The other fungal disease saprolegniasis that is caused by pathogenic oomycetes, namely, *Saprolegnia* spp., has also been considered responsible for the high mortality in amphibians. Infection is due to the complex interactions of pathogens with environmental factors (Kiesecker and Blaustein, 1995; Kiesecker *et al.*, 2001).

*Corresponding author <E-mail : eomah@knue.ac.kr>

Studies have suggested that disease outbreaks are often the result of complex interactions among many biotic and abiotic factors and are responsible for the decline in the amphibian populations (Blaustein and Kiesecker, 2002). Therefore, along with the increasing efforts that are recently being adopted to control environmental factors, disease control should also be implemented in conservation and reintroduction of amphibians. However, despite the significance of infectious fungal diseases among amphibian diseases, there has been no systematic study regarding them in Korea. In this study, diseased frogs from Korea were collected from field sites and a frog farm, and their diseases were identified using morphological and molecular analyses.

Materials and Methods

Diseased frogs were collected between 2006 and 2007 from various regions, including farms. Three diseased species of frogs were found from fields and a frog farm in Korea: Gold-spotted frog (*Rana plancyi chosenica*) tadpoles from a farm in Chuncheon, Gangwon-do; American Bullfrog (*Rana catesbeiana*) specimens from a reservoir in Gijang-gun, Busan; and adult Korean Stream Brown frogs (*Rana huanrenensis*) from a ditch in Chun Taesan, Yeongdong-gun, Chungbuk. A landing net was used to catch the frogs. The dead frogs were put into a bag containing pieces of ice, transported to the laboratory, and stored in a freezer until use.

The diseased fogs were observed, and their characteristics were recorded using light microscopes. Diseases were identified based on morphological characteristics. For molecular identification of the pathogens, skin tissue or infected intestinal parts were removed, and DNA was

extracted following the protocol of the DNeasy Plant Mini Kit (Qiagen Science, USA). The DNA was amplified using Prime Taq Premix (G-2000) obtained from GENET (mixing solution [pH 9.0] with a 1 *ml* microtube, 1 units/10 μl Prime Taq DNA polymerase, 2× reaction buffer, 4 mM $MgCl_2$, enzyme stabilizer, loading dye, and 2 mM dNTPs). Using the universal fungal primer pairs ITS1 (5'-TCCGCAGGTTCACCTACGGA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), the internal transcribed spacer1 (ITS1) and internal transcribed spacer4 (ITS4) regions were amplified (Bruns and Gardes, 1993). After adjusting the total volume to 20 μl with 10 pM of ITS1 of Prime Taq Premix and 2 μl ITS4 primer, 2 μl template DNA (20 ng), and 4 μl pure water, PCR was performed using a thermal cycler (Applied Biosystems). The PCR protocol was as follows: 1 cycle at 94°C for 4 min; followed by 35 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 4 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

Symptoms of two infectious diseases, namely, saprolegniasis and chromomycosis, were observed in the three species of frogs. Diseased Korean Golden frog (*R. plancyi choseni*) tadpoles were found in a frog farm in 2006. A fuss-like mold was observed on most parts of the tadpole tail or head (Fig. 1A). The PCR products that were amplified from the diseased tissue were 750 bp and nucleotide sequence analysis showed that the sequence had the closest similarity with *Saprolegnia australis* (Genebank accession no. AM228837.1, Table 1). It is highly possible that the disease affecting the *R. plancyi choseni* individuals in this study was saprolegniasis. Saprolegniasis was also detected in wild frogs and Korean Stream Brown frogs (*R. huanrenensis*). The diseased frogs presented with an eye infected by the pathogen (Fig. 1B). One eye of the diseased frogs was covered with white mold, and their legs were swollen. The frogs moved slowly and died after being transported to the lab. It has been reported that the skin of frogs with saprolegniasis is covered with a characteristic matted white cotton-like material (Pessier, 2002). The *R. plancyi choseni* and *R. huanrenensis* specimens that we studied presented with the same symptoms. The PCR product from the infected tissue was 700 bp, and the sequence was closest to *Saprolegnia diclina* (Table 1). Saprolegniasis is caused by infectious oomycetous water molds such as *Saprolegnia*, *Aphanomyces*, and *Achlya*. It is known as a common disease affecting frog eggs and tadpoles in frog farms (Anver and Pond, 1984).

Diseased American Bullfrogs (*R. catesbeiana*) were found around a reservoir in Busan. Black molds and red spots on the flesh on the inside of the thigh and the flank were observed in these frogs (Fig. 1C). The other seven frogs found at the site showed the same symptoms. The morphological characteristics of this disease were identical to those described by Gonzalez-Mendoza (1988). The

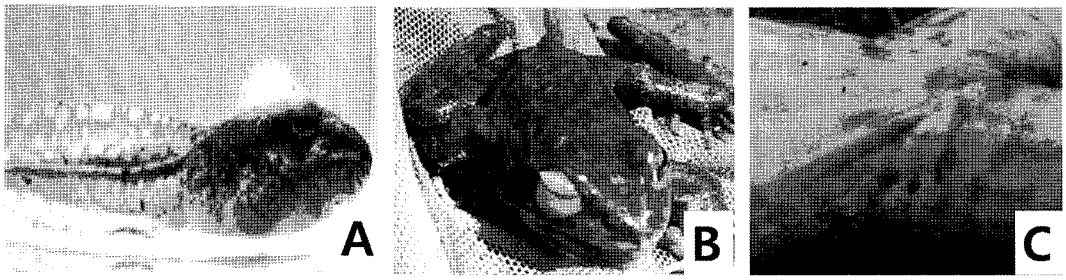

Fig. 1. Morphological feature showing symptoms of diseased frogs found in Korea. A. a tadpole of Gold-spotted pond frog (*Rana plancyi chosenica*) infected by *Saprolegnia australis*. Mold like fuss was observed in in the head. B. Korean stream brown frog (*Rana huanrenensis*) infected by *Saprolegnia diclina*. The symptom was observed in one eye. C. American Bullfrog (*Rana catesbeiana*) infected by *Cladosporium cladosporioides*. Red spots in the thigh and abdomen, gray-black pigmentation in the side were observed.

Table 1. Diseases of frogs identified by sequence analysis

Frogs	Results of BLAST search on NCBI		Species	Disease
	Similarity (%)	Accession No.		
Rana plancyi chosenica	514/565 (90%)	AM228837.1	*Saprolegnia australis*	Saprolegniasis
	582/618 (94%)	AM228848	*Saprolegnia diclina*	Saprolegniasis
Rana catesbeiana	489/501 (97%)	EU030342	*Cladosporium cladosporioides*	Chromomycosis

PCR product that was amplified from the DNA extracted tissue of the diseased frog was 600 bp, and its nucleotide sequence was closest to *Cladosporium cladosporioides* (Table 1). *C. cladosporioides* has been known to be the representative pathogen causing chromomycosis in amphibians (Roberts, 1986). Molecular analysis showed that the disease infecting *R. catesbeiana* in this study was likely to be chromomycosis. In this study, two infectious diseases were found to affect frogs collected from a frog farm and field sites in Korea. Our result can be used as the framework for studying infectious amphibian diseases in Korea. Further research will help in protecting against the extinction of amphibian species due to infectious diseases in Korea.

Acknowledgements

This study was supported by Korea Ministry of Environment as a part of the Eco-Technopia 21 project (#052-081-044).

References

Alford, R. A. and Richards, S. J. 1999. Global amphibian declines: a problem in applied ecology. *Ann. Rev. Ecol. Syst.* 30:133-165.

Anver, M. R. and Pond, C. L. 1984. Biology and diseases of amphibians. In: Laboratory Animal Medicine, pp. 427-447. Eds. J. G. Fox, B. J. Cohen and F. M. Loew. Academic Press, New York.

Beebee, T. J. C. and Griffiths, R. A. 2005. The amphibian decline crisis: a watershed for conservation biology? *Biol. Conservation* 125:271-285.

Berger, L. and Speare, R. 1998 Chytridiomycosis: a new disease of wild and captive amphibians. *ANZCCART Newsletter* 11:1-3.

Blaustein, A. R. and Kiesecker, J. M. 2002. Complexity in conservation: lessons from the global decline of amphibian populations. *Ecol. Letters* 5:597-608.

Daszak, P., Berger, L., Cunningham, A. A., Hyatt, A. D., Green, D. E. and Speare, R. 1999. Emerging infectious diseases and amphibian population declines. *Emerg. Infect. Diseases* 5:735-748.

Gardner, T. 2001. Declining amphibian populations: a global phenomenon in conservation biology. *Animal Biodiversity and Conservation* 24:25-44.

Gonzalez-Mendoza, A. 1988. Chomoblastomycosis. In: Laboratory Diagnosis of Infectious Diseases-Principles and Practice, pp. 590-599. Eds. A. Balows, W. J. Hausler and E. H. Lennette. Springer-Verlag, New York, NY.

Kiesecker, J. M. and Blaustein, A. R. 1995. Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. *Proc. Nat. Acad. Science, USA* 91:11049-11052.

Kiesecker, J. M., Blaustein, A. R. and Belden, L. K. 2001. Complex causes of amphibian population declines. *Nature* 410: 681-684.

Longcore, J. E., Pessier, A. P. and Nichols, D. K. 1999. Batrachochytrium dendrobatidis gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91:219-227.

Pessier, A. 2002. An overview of amphibian skin disease. *Seminars in Avian and Exotic Pet Medicine* 11:162-174.

Pessier, A. P., Nichols, D. K., Longcore, J. E. and Fuller, M. S. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). *J. Veterin. Diagnostic Investigation* 11:194-199.

Roberts, G. 1986. Laboratory methods in basic mycology. In: Bailey and Scott's Diagnostic Microbiology, pp. 678-774. Eds. S. M. Finegold and E. J. Baron. The C.V. Mosby Company, St. Louis, MO.