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First detection of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in free-ranging populations of amphibians on mainland Asia: survey in South Korea

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ABSTRACT: Chytridiomycosis, a disease that has caused amphibian population declines globally and elevated many species of anurans to endangered or threatened status, has recently been declared an internationally notifiable disease. *Batrachochytrium dendrobatidis* (*Bd*), the amphibian chytrid fungus causing this disease, has not been previously reported in Korea or on mainland Asia. Thirty-six frog specimens representing 7 species were collected from the wild in South Korea and examined for *Bd* using standard PCR. *Bd* was detected in 14 (38.8%) samples from 3 species (*Bufo gargarizans*, *Hyla japonica*, and *Rana catesbiana*). Skin sections from all 14 PCR-positive frogs were examined using 2 staining techniques: haematoxylin and eosin (H&E) and *Bd* immunoperoxidase (IPX). In histological sections, zoosporangia were found in 6 frogs, with lower sensitivity for H&E (21%) than for IPX (46%). Intensity of infection, based on histopathology, was low in all frogs. These results confirm that *Bd* is present in South Korea and, hence, on the Asian mainland. Studies are urgently required to determine the impact of chytridiomycosis on Korean amphibians, and to map the distribution of *Bd* in Korea and other Asian mainland countries.

KEY WORDS: Chytridiomycosis · *Batrachochytrium dendrobatidis* · Amphibian decline · Korea · Fungus

INTRODUCTION

The fungus *Batrachochytrium dendrobatidis* (*Bd*), which causes chytridiomycosis, is one of the most serious amphibian pathogens that have caused amphibian declines and extinctions worldwide (Berger et al. 1998, Skerratt et al. 2007). International and domestic amphibian trade appears to be responsible for the dispersal and transport of this pathogen between countries (Daszak et al. 1999, Weldon et al. 2004, Fisher & Garner 2007, Skerratt et al. 2007). Chytridiomycosis has not been previously reported in Korea or on the Asian mainland.

South Korea has 12 native and 1 introduced species of free-ranging anurans and 5 species of salamanders (Yang et al. 2001, Min et al. 2005). Of these, *Pelophylax nigromaculatus* is classified as near threatened (Kuzmin et al. 2004), *P. chosenicus* is classified as vul-

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nerable (Matsui 2004), and *Hynobius yangi* is classified as endangered (Stuart 2008) on the IUCN Red List. Amphibians imported into South Korea do not currently have to meet any specific disease standards. The World Organisation for Animal Health (OIE) recently declared 2 diseases of amphibians — chytridiomycosis and ranaviral disease — as internationally notifiable diseases (World Organisation for Animal Health 2008). One of the requirements that have to be met by OIE member countries is the determination of their *Bd* status by undertaking a survey using an appropriate diagnostic technique. Since the *Bd* status of South Korea was unknown, we conducted the first survey for *Bd* in Korea.

MATERIALS AND METHODS

Frogs were collected opportunistically from the northern part of South Korea between June 11 and December 13, 2007 (see Table 1). During nocturnal and occasional diurnal surveys, frogs were captured individually by hand, with a new pair of disposable latex gloves being used for each frog in order to avoid cross-contamination between individuals (Skerratt et al. 2008). The animals were sacrificed by soaking them in a bath of 0.05% aqueous tricaine methane sulfonate (MS-222). We aseptically cut small pieces of skin from the abdomen, inguinal region, and web between toes; we then divided this into 2, and placed 1 in 10% formalin for histological analysis and the other in a 1.5 ml microtube for DNA extraction. Instruments were flamed after dissection of each specimen to prevent cross-contamination. DNA was immediately extracted with Gene Releaser (Bio Ventures). The PCR assay used species-specific primers (*Bd*1a *and Bd*2a) located within internal transcribed spacer ITS1 and ITS2 to amplify the 5.8S region of nuclear rDNA (Annis et al. 2004). The PCR products were examined using 1.0% agarose gel and some of the positive bands were cut and sequenced to confirm the *Bd* sequence. Each sample was tested in triplicate, and was only recorded as positive if all 3 replicates indicated the presence of *Bd*. For PCR positive amphibians, histological sections of formalin fixed skin were stained using 2 techniques: haematoxylin and eosin (H&E) and *Bd* immunoperoxidase (IPX), the latter technique using polyclonal antibodies specific to *Bd* (Berger et al. 2002). The epithelium was scanned for zoosporangia or lesions using a compound light microscope (Pessier et al. 1999, Berger et al. 2000).

RESULTS

Thirty-six individuals from 7 amphibian species were collected from Gyeonggi-Do, Gangwon-Do, Chungcheongbuk-Do and Incheon (Fig. 1), of which 14 (38.8%; 95% CI, 23 to 57%) were found to be positive based on PCR (Table 1). Positive PCR reactions were confirmed by sequencing. Positive cases came from 3 species, *Bufo gargarizans*, *Hyla japonica*, and *Rana catesbiana*, the latter being an introduced species now naturalized in Korea. Prevalences had wide CIs, particularly for *B. gargarizans*, owing to small numbers of specimens (Table 1). Histological examination of epidermis from all PCR-positive frogs revealed that 3/14 (21%) were positive on H&E and 6/13 (46%) were positive on IPX (see Fig. 2). In the latter group, one frog was excluded since no epidermis was present in the IPX sections. All H&E-positive frogs were also positive based on IPX. The zoosporangia were typical of *Bd*, with some zoosporangia showing colonial morphology with a dividing septum that was clearly visible in the zoosporangium (Fig. 2), and all were located in the *stratum corneum* or *stratum granulosum*. As is typical for IPX, red-brown stain also appeared as amorphous, poorly demarcated material in the cytoplasm of epidermal cells containing zoosporangia (Fig. 2). IPX staining also occurred in epidermal cells adjacent to the visible zoosporangia. The intensity of infection was low and lesions were minor in all cases, with a minimal degree of local hyperplasia associated with zoosporangia.

DISCUSSION

This study has demonstrated that the amphibian chytrid fungus *Bd* is present in South Korea. This is

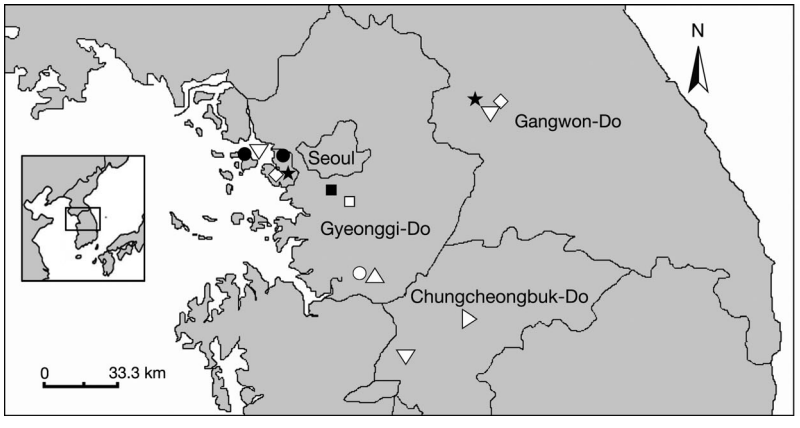

Fig. 1. Map of South Korea showing survey locations for frogs. Rectangle in inset map demarcates area shown in larger map. Filled symbols: frogs positive for chytridiomycosis; open symbols: frogs negative for the disease. (○,●) American bullfrog; (★) Japanese tree frog; (◇) Dybowski's brown frog; (□,■) Asian toad; (△) gold-spotted pond frog; (▽) black-spotted pond frog; (▷) wrinkled frog

Table 1. *Batrachochytrium dendrobatidis*. Prevalence of infection in free-ranging native and introduced species of amphibians in South Korea based on standard PCR. IPX: immunoperoxidase; CI: confidence interval

Common name	Species	Native or introduced	No. of samples tested	No. of positives			Prevalence (%) mean (95% CI)
				PCR	H&E staining	IPX test	
Asian toad	*Bufo gargarizans*	Native	2	1	0	0	50 (1–99)
Japanese tree frog	*Hyla japonica*	Native	10	8	0	1	80 (44–97)
Black-spotted pond frog	*Pelophylax nigromaculata*	Native	5	0	–	–	0 (0–52)
Gold-spotted pond frog	*Pelophylax chosenicus*	Native	5	0	–	–	0 (0–52)
Wrinkled frog	*Glandirana emeljanovi*	Native	1	0	–	–	0 (0–98)
Dybowski's brown frog	*Rana dybowskii*	Native	4	0	–	–	0 (0–60)
American bullfrog	*Rana catesbiana*	Introduced	9	5	3	5	55.6 (21–86)
Total			36	14	3	6	38.8 (23–57)

also the first report of *Bd* on the Asian mainland. The search effort for *Bd* in Asia appears to have been low, with only 3 reports in the literature. The first survey found no *Bd* in Hong Kong, whether in free-ranging or captive amphibians (Rowley et al. 2007). Chytridiomycosis in Asia was first found in Japan in captive amphibians (Une et al. 2008) and subsequently in free-ranging *Rana catesbiana* (ProMED 2007). The other positive report is of chytridiomycosis in free-ranging amphibians in Indonesia on the island of Java (Kusrini et al. 2008). The geographic separation of these positive records may be more an artifact of search effort than a true absence in Asia. Nonetheless, the detection of chytridiomycosis on the Asian mainland means that *Bd*, which can spread as a front progressing from 15–43 km yr^{-1} (Lips et al. 2008) and possibly 100 km yr^{-1} (Laurance et al. 1996) across the landscape, now has the Asian mainland open to its spread. Surveys need to be done urgently further north on the Korean Peninsula and in China as well as in other Asian mainland countries.

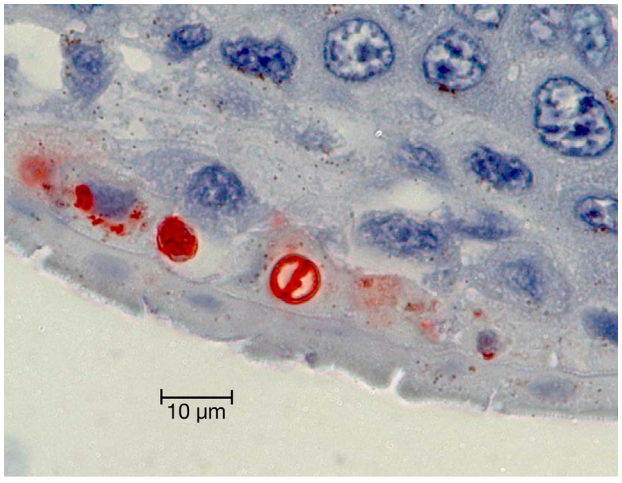

Fig. 2. *Batrachochytrium dendrobatidis* infecting *Rana catesbiana*. Immunoperoxidase (IPX) staining of epidermis showing 2 reddish-brown zoosporangia of *Bd*, one with colonial morphology as shown by a septum dividing the zoosporangium (right). With IPX, staining often occurs in the cytoplasm of epithelial cells containing zoosporangia and adjacent to zoosporangia as shown here (R. Speare pers. obs.)

Although the number of amphibians surveyed was small (36), *Bd* was detected at a high prevalence (38.9%) by standard PCR. Although 3 of the 7 species were positive, numbers sampled for the negative species were too low for any meaningful interpretation (Table 1). Of the positive species, *Hyla japonica* and *Bufo gargarizans* are new host records for *Bd*. Prevalence in the 3 positive species had wide CIs owing to small sample sizes. Fifty-six percent of the free-ranging American bullfrogs carried *Bd*, which is consistent with prevalence reported in surveys in other countries where they have been introduced (Mazzoni et al. 2003, Hanselmann et al. 2004, Cunningham et al. 2005, Garner et al. 2005, 2006). Since American bullfrogs can be infected with *Bd*, but appear to be relatively resistant to clinical chytridiomycosis (Daszak et al. 2004), they are ideal carriers of the disease. In this survey, infected native frogs (Japanese tree frog) were found sympatrically with infected American bullfrogs in Incheon. American bullfrogs spread extensively when introduced and occupy many habitats. In South Korea, American bullfrogs are widespread, but their distribution further north in the Democratic People's Republic of Korea is unknown. If *Bd* is widespread in American bullfrogs in Korea, endemic frogs may be exposed to a constant force of infection with the fungus.

Japanese tree frogs also had a high prevalence of *Bd*. When amphibian populations appear stable, species with high prevalences of *Bd* are more likely to have a lower level of susceptibility to clinical chytridiomycosis (Retallick et al. 2004), making the duration of infection longer and prevalence higher since prevalence is proportional to incidence by duration of infection (Nelson et al. 2001). These species may play important roles as reservoirs or carriers of *Bd* (Retallick et al. 2004). Thus, both the American bullfrog and the

Japanese tree frog appear to be important carriers of *Bd* in Korea. This survey has demonstrated the occurrence of *Bd* in Korean frogs, but larger numbers of animals of each species need to be sampled to obtain more accurate data on prevalences.

When PCR positive skin samples were examined by histology, zoosporangia of *Bd* were confirmed. Confirmation using 2 diagnostic techniques increases confidence in the diagnosis. The intensity of infection based on histological analysis was low, but provided additional data that was not available using standard PCR. Our study also demonstrated that histology had a lower sensitivity than PCR (21.4 and 46% for H&E and IPX, respectively) in this PCR positive group. This result is consistent with previous studies on low intensity infections of chytridiomycosis (Boyle et al. 2004, Speare et al. 2005, Kriger et al. 2006, Hyatt et al. 2007). The staining of material with IPX in the cytoplasm of epidermal cells containing zoosporangia and in adjacent epidermal cells probably indicates that antigens of *Bd* are excreted or secreted by the zoosporangia into the epidermis. Staining of the cytoplasm of cells adjacent to those containing zoosporangia is common (R. Speare pers. obs.) and may be due to the adjacent cell containing a zoosporangium which may be out of the plane of the section, or possibly due to uninfected cells taking up antigens secreted by *Bd* in an adjacent cell. This has not been studied.

Although we did not observe clinical signs of chytridiomycosis in the frogs tested or find dead frogs in the populations from which the samples were collected, detection of *Bd* in Korea is a major concern. Because infection with *Bd* may increase the vulnerability of amphibians, threatened Korean species will need to be particularly protected against infection. Although we have shown that *Bd* is present in South Korean amphibians, additional mapping is required to determine the distribution of *Bd*. Mapping studies are also urgently required to determine the distribution of *Bd* further north on the Korean Peninsula and in China and also in other Asian mainland countries. Experimental laboratory infections are also needed to determine the susceptibility of native Korean amphibians to chytridiomycosis, particularly those species that are currently threatened. To reduce the consequences of chytridiomycosis in South Korea, strategies to decrease the transmission of *Bd* and the impact of chytridiomycosis on Korean amphibians should be implemented.

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