

Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians

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Abstract

Population declines and extinctions of amphibians have been attributed to the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), especially one globally emerging recombinant lineage ('Bd-GPL'). We used PCR assays that target the ribosomal internal transcribed spacer region (ITS) of Bd to determine the prevalence and genetic diversity of Bd in South Korea, where Bd is widely distributed but is not known to cause morbidity or mortality in wild populations. We isolated Korean Bd strains from native amphibians with low infection loads and compared them to known worldwide Bd strains using 19 polymorphic SNP and microsatellite loci. Bd prevalence ranged between 12.5 and 48.0%, in 11 of 17 native Korean species, and 24.7% in the introduced bullfrog *Lithobates catesbeianus*. Based on ITS sequence variation, 47 of the 50 identified Korean haplotypes formed a group closely associated with a native Brazilian Bd lineage, separated from the Bd-GPL lineage. However, multilocus genotyping of three Korean Bd isolates revealed strong divergence from both Bd-GPL and the native Brazilian Bd lineages. Thus, the ITS region resolves genotypes that diverge from Bd-GPL but otherwise generates ambiguous phylogenies. Our results point to the presence of highly diversified endemic strains of Bd across Asian amphibian species. The rarity of Bd-GPL-associated haplotypes suggests that either this lineage was introduced into Korea only recently or Bd-GPL has been outcompeted by native Bd strains. Our results highlight the need to consider possible complex interactions among native Bd lineages, Bd-GPL and their associated amphibian hosts when assessing the spread and impact of Bd-GPL on worldwide amphibian populations.

Keywords: chytrid fungus, conservation, emerging infectious disease, endemism, frogs, host–parasite co-evolution, invasive species, population declines, prevalence, salamanders

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Introduction

Populations of amphibian species around the world have declined, sometimes to extinction, with the spread of the emerging infectious disease chytridiomycosis,

caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) (Berger *et al.* 1998; Waldman *et al.* 2001; Skerratt *et al.* 2007; Fisher *et al.* 2009a). The pressing need to mitigate the potentially devastating effects of this fungus has spawned research into the mechanisms by which Bd causes morbidity and mortality (Voyles *et al.* 2009; Carver *et al.* 2010), how it became virulent (Fisher *et al.* 2009b) and its historical and current distribution (Weldon *et al.* 2004; Goka *et al.* 2009; Cheng *et al.*

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2011). The global Bd panzootic may be associated with the emergence of one highly virulent Bd lineage, the 'global panzootic Bd lineage' denoted Bd-GPL (Farrer *et al.* 2011), but Bd-GPL predates recent amphibian population declines (Rosenblum *et al.* 2013). In contrast, amphibian hosts may be less affected by local, less virulent Bd lineages as observed in Spain, South Africa, Switzerland (Farrer *et al.* 2011), Japan (Goka *et al.* 2009), China (Bai *et al.* 2012) and Brazil (Schloegel *et al.* 2012). These studies suggest that Bd lineages are widely divergent genetically and in some cases phenotypically (Fisher *et al.* 2009b). Undoubtedly many more lineages remain to be characterized.

The distribution and diversity of endemic Bd lineages, and their relationship to Bd-GPL, need to be resolved so that we can begin to understand the origin, mechanisms of spread and impact of chytridiomycosis on global amphibian populations. Research to date has mainly focused on rapidly declining amphibian populations (Stuart *et al.* 2004; Skerratt *et al.* 2007; Fisher *et al.* 2009b), which may have resulted in oversampling of the virulent Bd strains associated with epizootics. Bd lineages carried by invasive, introduced and commercially traded species have been among the first sampled and characterized (Mazzoni *et al.* 2003; Fisher & Garner 2007). In addition, culturing Bd, especially from animals bearing low infection loads, presents serious challenges; thus, less virulent lineages are less likely to be sequenced and may be underreported. To compile a fair representation of the worldwide distribution and diversity of Bd lineages, systematic sampling biases need to be eliminated. This is especially important now as studies begin in earnest in important centres of amphibian biodiversity, such as Asia and Africa, where Bd lineages are largely unknown (Fisher *et al.* 2009a).

Recent studies have begun to describe the epidemiology of chytridiomycosis in Asia. A survey of Bd infection across 15 Asian countries revealed that Bd prevalence was low (2.35% overall) (Swei *et al.* 2011). Moreover, among infected animals, Bd loads generally were low, considerably below thresholds necessary to cause disease; thus, infected animals demonstrated no clinical signs of chytridiomycosis (Swei *et al.* 2011). Such low prevalence and infection loads in Asia differ markedly from those noted in areas of Bd epizootic outbreaks associated with amphibian population declines (Berger *et al.* 1998; Bosch *et al.* 2001; Lips *et al.* 2006; Skerratt *et al.* 2007). Global models predict that the range of environments in Asia suits the spread of Bd (Rödder *et al.* 2010; Swei *et al.* 2011), so other factors must explain differences in its epidemiology. This suggests that either Bd is an emerging but potentially benign pathogen of Asian amphibians or Bd is endemic to Asia, allowing sufficient time for amphibians to have

evolved mechanisms of resistance to or tolerance of it. However, any tentative conclusions must be tempered by the limited sampling efforts that have been made to date in Asia. Moreover, we do not know how Bd virulence might be modulated by biotic or abiotic environmental factors, nor how virulence varies by lineage.

Substantial diversity of Bd haplotypes previously had been demonstrated in Japan, based on sequencing the 5.8S rDNA and ITS regions of Bd (Goka *et al.* 2009). Three haplotypes (JP-B, JP-K and JP-J), found infecting the endemic Japanese giant salamander *Andrias japonicus*, form a Japan-specific clade ancestral to the lineages associated with the global pandemic. Using the same methods, a wide diversity of Bd haplotypes has recently been detected in China, including an endemic, basal haplotype (CN30) related to the Japanese clade that was isolated from the endemic ranid frog *Babina pleuraden*. These haplotypes may comprise a Bd lineage that is widespread across Asia and well differentiated from the Bd lineage associated with the global pandemic (Bai *et al.* 2012). As this endemic lineage has been recovered so far from only two species, confirmation awaits wider sampling and analyses of additional molecular markers.

Commercial trade in amphibians may have been important in the introduction and spread of Bd strains in Asia (Gilbert *et al.* 2012), as appears to be the case worldwide (Fisher & Garner 2007; Schloegel *et al.* 2012). The American bullfrog *Lithobates catesbeianus*, in particular, has been identified as a key vector for the spread of Bd among frog farms, food markets and wild populations in Asia. Recently, Bd samples isolated from *L. catesbeianus* in Japan were assigned to a Bd lineage endemic to Brazil, suggesting that some Bd strains were introduced from Brazil on bullfrogs (Schloegel *et al.* 2012). Curiously, native Japanese amphibians were found to be infected by global Bd-GPL but not by Brazilian Bd strains. This observation raises the possibility that Bd-GPL outcompetes Bd-Brazil on the skin of native Japanese frogs. However, Bd-GPL appears unable to prevail over endemic Bd when infecting *L. catesbeianus*. Competition between endemic and introduced Bd strains thus may be determined by properties of the host immune system, environmental factors or host/environmental interactions that remain to be elucidated.

South Korea has a diverse but understudied amphibian fauna comprising 18 species: four hynobiid salamander species, the only known Asian plethodontid salamander *Karsenia koreana* (Min *et al.* 2005), 12 native frog species and the introduced American bullfrog (*L. catesbeianus*). Only two frog species (*Pelophylax nigromaculatus* and *P. chosonicus*) currently are listed as threatened on the IUCN Red List, but two other species (*Hyla suweonensis* and *Kaloula borealis*) are accorded special protection in Korea because of their restricted

ranges and small population sizes. In Asia, Bd first was found in Korea, infecting both native and exotic species, mostly near the capital Seoul (Yang *et al.* 2009). Since then, Bd infection has been consistently monitored in amphibian populations across the Korean peninsula, infecting at least seven native species (Jeong *et al.* 2010), yet without any observations of morbidity or mortality attributable to the disease.

In this study, we determine the prevalence, infection intensity and genetic diversity of Bd in amphibians of South Korea using PCR assays targeting the ribosomal internal transcribed spacer region (ITS). We also describe a new method to efficiently and reliably isolate Bd from amphibians with low levels of infection and subsequently compare three newly isolated Korean strains with worldwide strains using 19 polymorphic loci. We compare our results with those obtained in earlier studies that focused on Bd in Asia and suggest that endemic Bd strains in Asian amphibians are more widely distributed and genetically variable than previously thought. We also investigate the role of native Korean amphibians as vectors of Bd in Korea.

Material and methods

Sampling of amphibians in South Korea

Frogs and salamanders were sampled across South Korea, including lowland and mountainous areas both in agricultural and in natural landscapes, from spring 2010 to autumn 2011. Exact GPS coordinates were recorded for 42 sites, while the GPS positions of remaining sites were determined based on map localities. Each subject caught was handled individually using a new pair of disposable, nonpowdered latex gloves to avoid cross-contamination of samples. Immediately after capture, the ventral surface, hind legs and feet of each subject were swabbed following a standardized protocol (Hyatt *et al.* 2007) using sterile cotton swabs (MW100 and MW113, MWE Medical Wire). Subjects were released at the site of collection immediately after sampling. Each sample was kept in a sterile vial, placed in ice during transit and stored at -20°C until processed in the laboratory.

DNA extraction and PCR assays from swab samples

DNA was extracted from swabs using 50 μL of PrepMan Ultra (Applied Biosystems) as described by Hyatt *et al.* (2007). All samples were tested for Bd infection using a highly sensitive nested PCR method targeting 5.8S rDNA and the ribosomal internal transcribed spacer regions (ITS) of Bd (Goka *et al.* 2009). Samples were run in duplicate, and DNA from Bd culture (AbercrombieR-Lbooroo-

longensis-09-LB1) obtained from Dr. Lee Berger (Centre for Public Health and Tropical Medicine, James Cook University, Townsville, Australia) was used as a positive control to guard against false-negative results. Samples were recorded as positive for Bd infection if an amplified band of approximately 300 bp could be visualized by ethidium bromide staining under UV light for at least one of the duplicate runs.

Samples that were determined to be positive for Bd infection were submitted to a new round of nested PCR (Goka *et al.* 2009) in October 2011. Not all samples previously identified as Bd positive amplified, presumably owing to DNA degradation especially when little Bd DNA had been extracted from swabs. All successfully amplified products were purified and sequenced at the Genome Research Facility in the School of Biological Sciences, Seoul National University. Amplicons that resulted in ambiguous sequences were cloned using the RBC A&T cloning kit (RC013, Real Biotech) following the manufacturer's protocol. For each cloned sample, 10 clones with the correct size insert were sequenced. Bd infection intensity was estimated as Bd zoospore genomic equivalents (ZGE) in the swab samples using a qPCR assay (Hyatt *et al.* 2007) and standard samples with known Bd quantity. Obtained ZGE values were corrected for dilutions following extraction and PCR procedures.

Bd prevalence in South Korea

Spatial variation in Bd infection in South Korea was visualized by interpolating the prevalence of Bd among sample sites using the inverse distance weighting (IDW) method (Shepard 1968). IDW uses the distance between sampled points to infer the expected Bd prevalence, giving higher weight to geographically nearby points and down weighting more geographically distant points. Twelve points, separated by variable distances, were used to interpolate Bd prevalence for each $\sim 1\text{ km}^2$ grid cell. The interpolated surface was then clipped to the South Korean administrative boundaries. IDW was implemented in ArcMap version 9.3.

Mountain ranges naturally separate the South Korean peninsula into four regions (northwest, northeast, southwest and southeast), with Jeju Island representing a fifth region separated from the mainland by the Korean South Sea (Fig. 1). Phylogenetic work on the Japanese tree frog (*Hyla japonica*) has shown that these mountain ranges can limit gene flow among frog populations (Jang *et al.* 2011). We therefore tested whether infection rates varied among regions, using a generalized linear model with logit-link function and binomial error distribution, including region as a factor. We examined differences in prevalence between specific

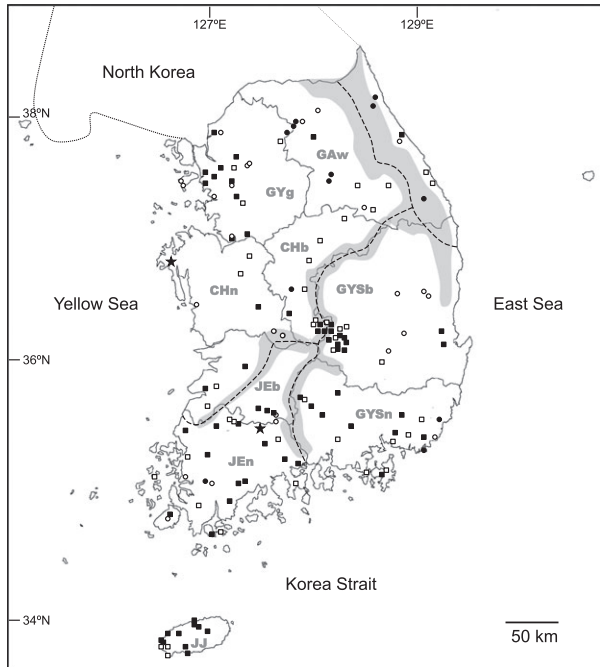


Fig. 1 Location of sampling sites for *Batrachochytrium dendrobatidis* in South Korea. Square symbols indicate sites with GPS location based on verbatim locality information, while circle symbols indicate sites with exact GPS location. Black symbols denote Bd-positive sites, and white symbols denote Bd-negative sites. Black stars represent sites where ITS haplotypes associated with the Bd-GPL lineage were detected. Grey shading represents the mountain ranges, with dotted lines indicating the division of the four regions (northwest, northeast, southwest and southeast). Solid lines within South Korea delimit the Korean provinces: Chungcheongbuk (CHb), Chungcheongnam (CHn), Gangwon (GAw), Gyeonggi (GYg), Gyeongsangbuk (GYSb), Gyeongsangnam (GYSn), Jeju Island (JJ), Jeollabuk (JEB), Jeollanam (JEn).

regions using pairwise comparisons by Bonferroni post hoc tests. Analyses were conducted with R v2.7.1.

Phylogenetic analyses of ITS gene

The ITS sequences obtained in this study were aligned with worldwide sequences from previous studies (Goka *et al.* 2009; Bai *et al.* 2012; Schloegel *et al.* 2012) (Table S1, Supporting information), using Clustal_W (Thompson *et al.* 1994). Sequences from two *Terramyces* sp. (AUS3, ITA2590), four *Boothomyces* sp. (AUS8, AUS9, AUS12, ITA2633) and one *Kappamyces* sp. (ITA2582) were used as the outgroup, following Goka *et al.* (2009). The alignment was manually refined using Goka *et al.* (2009) as a template with the software BioEdit (Hall 1999). Phylogenetic relationships among sequences were inferred using maximum parsimony, maximum-likelihood, Bayesian inference and median-joining network methods. As the maximum parsimony and maximum-likelihood methods

do not retain gap data, we created a new alignment for these analyses by first coding the gaps as binary data (0/1) at the end of the alignment using the program GapCoder (Young & Healy 2003) and then replacing the 0s and the 1s in the alignment by As and Ts, respectively.

Maximum parsimony analysis was conducted in MEGA v.5 (Kumar *et al.* 2008) using heuristic close-neighbour-interchange search (Nei & Kumar 2000) and 100 random sequence additions to produce the initial trees. All characters were weighted equally, and support for each node was estimated using bootstrap analysis of 10 000 replicates. Maximum-likelihood analysis was performed in RAXML v.7.3 (Stamatakis 2006) using the GTR GAMMA model of evolution for tree inference and 10 000 bootstraps (Stamatakis *et al.* 2008). Bayesian inference was performed using MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003) with four chains for 10 million generations under a GTR+G model of evolution determined using jModelTest v.0.1.1 (Posada 2008). Burn-in of MCMC chains was evaluated using the online program AWTY, examining cumulative plots of posterior probabilities of the 20 most variable splits (Wilgenbusch *et al.* 2004).

A phylogenetic network was constructed using the median-joining method (Bandelt *et al.* 1999) implemented in the program Network v.4.6.1 (<http://www.fluxus-engineering.com>) to further assess the relationships among worldwide Bd ITS haplotypes. This model-free method uses a parsimony approach, based on pairwise differences, to connect each sequence to its closest neighbour, and allows the creation of internal nodes ('median vectors'), which are interpreted as unsampled or extinct ancestral genotypes to link the existing genotypes in the most parsimonious way. The parameter *epsilon*, which controls the level of homoplasy, was set at the same value as the weight of characters used to calculate the genetic distances (weight value = 10).

Culture isolation

Bd is most readily isolated from heavily infected subjects or from the mouthparts of tadpoles (Longcore *et al.* 1999). The very low Bd loads typical of infected Asian amphibians have hindered efforts to isolate endemic Asian Bd strains (Swei *et al.* 2011). Our attempts to culture Bd from wild-caught tadpoles similarly were frustrated by failure to find tadpoles with conspicuous dekeratinization in the mouthparts, typical signs of Bd infection (Knapp & Morgan 2006). A recent study suggests that tadpoles can be infected by sharing the same body of water as infected adults (Greenspan *et al.* 2012). We thus designed a method to isolate Bd from adult frogs with low Bd infection levels by fostering the transmission of Bd to tadpoles

in laboratory culture and then isolating the fungus from infected tadpoles' mouthparts.

We reared groups of 10 *Bombina orientalis* tadpoles to developmental stages between 25 and 36 (Gosner 1960) within polypropylene containers (41.6 × 33.7 × 29.5 cm). Each tadpole container was immersed within a similar but larger container (84.1 × 51.4 × 37.5 cm) that held one naturally Bd-infected adult conspecific. Containers were filled with carbon-filtered, UV-sterilized water to a depth of 10 cm, with gravel and rocks provided as dry refuge. Water exchange between the adult and tadpole containers occurred through small holes (<0.3 mm) drilled into the bottom of the walls of the smaller containers. Adults and tadpoles were held in these conditions for between 2 and 4 weeks at 20 °C under ambient photoperiod. Tadpoles were fed boiled spinach every other day, and adults were fed live mealworms (*Tenebrio molitor*) every third day. Water was changed every third day. After 2 weeks, tadpoles were examined every fourth day under a low power stereoscopic microscope for the presence of dekeratinized areas in the jaw sheaths.

Six tadpoles with signs of Bd infection from each container were euthanized by immersion in a 5 g/L solution of MS-222 (Torreilles *et al.* 2009), and jaw sheaths were dissected in aseptic conditions. Isolation of Bd was attempted from lower and upper jaw sheaths separately, 12 times per infected adult. Each piece of jaw was aseptically wiped on an agar plate with nutrients and antibiotics to remove bacteria, yeast and fungal spores (Longcore *et al.* 1999). The cleaned jaws then were placed into sterile 12-well culture plates with 15 mL mTGH liquid media, 200 mg/L penicillin-G and 400 mg/L streptomycin sulphate. After 1–3 weeks of incubation at 20 °C, cultures were examined for the presence of active zoospores using an inverted microscope. A volume of 1 mL of culture containing zoospores was transferred by pipette to a new 12-well plate with liquid media and no antibiotics, and incubated at 20 °C. All successfully cultured isolates were spun at 1700 g for 10 min. A portion of the pellets was used for DNA extraction, while the remaining was resuspended in 1 mL 10% DMSO 10% FCS in liquid media and transferred into a 2-mL cryotube for cryopreservation at –80 °C (Boyle *et al.* 2003).

Multilocus genotyping of cultured Korean Bd strains

DNA was extracted from Korean Bd cultures with the Qiagen DNeasy kit (Qiagen, Ltd.) following the manufacturer's instructions. Multilocus sequence typing (MLST) was carried out using a selection of the 19 most polymorphic loci chosen over a panel of 36 microsatellite or single nucleotide polymorphism markers described in previous studies (6873X2, 8392X2, 8009X2, b7-10c, BDC5, BdSC1.2, BdSC1.2.4, BDC24, BdSC2.02, BdSC3.1, BdSC4.3, BdSC4.16,

BdSC5.1, BdSC6.2, BdSC6.15, BdSC8.10, BdSC9.7, APRT13, HMG17) (Morgan *et al.* 2007; James *et al.* 2009; Schloegel *et al.* 2012). PCRs were conducted as described in the study by Schloegel *et al.* (2012), but using proofreading AmpliTaq Gold DNA polymerase (Applied Biosystems) for amplification. Amplicons were purified and sequenced in both forward and reverse directions at the Genome Research Facility in the School of Biological Sciences, Seoul National University. SNPs in the loci were detected by direct observation of forward and reverse reads using BioEdit (Hall 1999). For markers with a variable short tandem repeat region (b7-10c) or with single-point deletions (BdSC1.2.4), amplicons were cloned using the RBC A&T cloning kit (RC013, Real Biotech) following the manufacturer's protocol. For each cloned sample, 10 clones with the correct size insert were sequenced.

The Korean genotypes obtained were analysed alongside the worldwide samples genotyped by Schloegel *et al.* (2012). The proportion of shared alleles (Bowcock *et al.* 1994) and Cavalli-Sforza genetic distances (Cavalli-Sforza & Edwards 1967) among individuals were calculated with MSA v.4.05 (Dieringer & Schlötterer 2003), using a bootstrap analysis of 50 000 replicates. The distance matrices obtained were used to construct 50% consensus trees with the neighbour-joining method implemented in PHYLIP v.3.68 (Felsenstein 2004).

Results

Prevalence and infection intensity of Bd in South Korea

From March to September of 2010 and 2011, 1863 individuals, comprising all 18 extant South Korean amphibian species, from 149 field sites across the country, were swabbed. In all, 330 individuals (17.7%) of 13 native and one introduced (*L. catesbeianus*) species tested positive for Bd infection (Tables 1 and S2, Supporting information). Bd was detected in 78 of 149 sites sampled (52.4%, Fig. 1). None of the individuals that we found exhibited clinical signs typically associated with heavy chytridiomycosis infection, and neither morbidity nor mortality attributable to disease has been reported to date in South Korea.

Two salamander species (*Hynobius leechii* and *H. queletensis*) showed the highest prevalence of Bd (48.0% and 39.7%, respectively), followed by the introduced American bullfrog (*L. catesbeianus*; 24.7%). Seven other native amphibian species showed prevalence above 10%, including the endangered golden-spotted pond frog (*P. chosonicus*). Three of six samples obtained from captive *Karsenia koreana*, the only known lungless salamander (family Plethodontidae) in Asia (Min *et al.* 2005), also were found infected by Bd (Table 1).

Table 1 Summary of *Batrachochytrium dendrobatidis* prevalence in amphibian species sampled in South Korea during 2010 and 2011

Species	Number of Samples	Bd Positives	Prevalence (%) (95% CI)	Haplotype	Bd Load
<i>Bombina orientalis</i>	365	61 (30)	16.7 (13.9–20.3)	1, 4(2), 7(8), 8, 9(10), 10–13, 14(3), 15(3), 16, 17, 18(2), 21(3), 22, 23(2), 24–27, 28(2), 29–36, 37(2), 38, 39, 50	13.85 (±4.79)
<i>Bufo gargarizans</i>	90	2 (1)	2.2 (0.9–7.00)	4	20.00
<i>Bufo stejnegeri</i>	35	2	5.7 (2.4–17.4)	—	—
<i>Glandirana rugosa</i>	97	19 (4)	19.6 (14.3–27.7)	4(3), 47	14.12 (±13.09)
<i>Hyla japonica</i>	406	53 (7)	13.1 (10.6–16.2)	4, 9(3), 19(2), 32, 40–46	28.07 (±14.95)
<i>Hyla suweonensis</i>	2	0	0 (0–95.0)	—	—
<i>Hynobius leechii</i>	123	59 (1)	48.0 (41.4–56.2)	20	1.50
<i>Hynobius quelpaertensis</i>	116	46	39.7 (33.1–48.1)	—	—
<i>Hynobius yangi</i>	3	0	0 (0–77.6)	—	—
<i>Kaloula borealis</i>	20	0	0 (0–16.0)	—	—
<i>Karsenia koreana</i> *	6	3 (1)	50.0 (34.3–92.4)	14	2.67
<i>Lithobates catesbeianus</i>	93	23 (14)	24.7 (18.7–33.5)	2(2), 3(11), 4, 6, 7, 20, 48, 49	305.36 (±130.72)
<i>Onychodactylus fischeri</i>	20	0	0 (0–16.0)	—	—
<i>Pelophylax chosonicus</i>	8	1	12.5 (5.3–52.1)	—	—
<i>Pelophylax nigromaculatus</i>	163	5 (1)	3.1 (1.6–6.4)	4, 5	—
<i>Rana coreana</i>	59	11	18.6 (12.4–19.4)	—	—
<i>Rana dybowskii</i>	249	46 (4)	18.5 (15.0–23.1)	4 (4)	5.17 (±1.92)
<i>Rana huanrenensis</i>	14	2	14.3 (6.6–41.0)	—	—
Total	1863	330 (63)	17.7 (16.3–19.2)	112	61.18 (±22.43)

Bd positives, number of individuals infected with Bd (numbers of positive samples successfully sequenced in brackets); Prevalence, percentage of Bd-infected animals (with associated 95% confidence interval); Haplotype, identity of Korean Bd haplotype (number of sequences of named haplotype); Bd load, mean Bd infection intensity in zoospore genomic equivalents and associated standard errors.

*Samples were obtained from captive individuals kept in Kangwon University that were collected in Gyeryong Mountain (Chungcheongnam Province).

Two main areas of high interpolated Bd prevalence, one around Seoul in the northwest and the other in the southwest, emerged from the IDW analysis (Fig. 2; red denotes prevalence between 51 and 100%). Prevalence varied significantly among regions (likelihood ratio test, $LRT_3 = 97.01$, $P < 0.0001$), lower in the northeast (4.6%) and northwest (11.3%) than in the southeast (30.4%) and southwest (24.5%; Table 2). The southwest and southeast regions did not significantly differ in prevalence ($P = 0.65$) but all other pairwise comparisons between regions yielded significant differences ($P < 0.05$) by Bonferroni post hoc tests.

The geographical differences in Bd prevalence in South Korea might be influenced by the disproportionate sampling of *H. leechii*, *H. quelpaertensis* and *L. catesbeianus*, the three species with highest Bd prevalence (Tables 2 and S2, Supporting information), in the southern parts of the country. When results from these three species were excluded from the data set, lowering the sample size to 1537 individuals, prevalence in three of the five southern provinces decreased to less than 20%, whereas prevalence in Jeju Island and Gyeongsangbuk Province remained above 20% (Table 2). Prevalence in southwest and southeast regions decreased to 14.2% and 20.0%,

respectively, after exclusion of the data from the three species, but no change in prevalence was noted in northern regions (Table 2, Fig. S1, Supporting information). Regions significantly varied in prevalence (likelihood ratio test, $LRT_3 = 55.77$, $P < 0.0001$). Pairwise comparisons between the northeast and other regions, and between the northwest and the southwest, showed significant differences ($P < 0.05$) by Bonferroni post hoc tests.

ITS haplotype diversity and Bd infection intensity

We examined genetic diversity of Bd infecting amphibians by submitting all samples that tested positive for Bd infection to a new round of nested PCR assay (Goka *et al.* 2009). PCR amplifications were successful for 63 of 330 samples (Tables 1 and S3, Supporting information). Of the 63 samples, 37 were successfully genotyped by direct sequencing. The remaining 26 PCR amplicons exhibited multiple peaks in initial sequencing reactions, indicating the presence of more than one ITS haplotype. These samples were cloned and 10 clones were sequenced per amplicon.

Of these samples, one haplotype was obtained from three of the 26 samples, two haplotypes from eight sam-

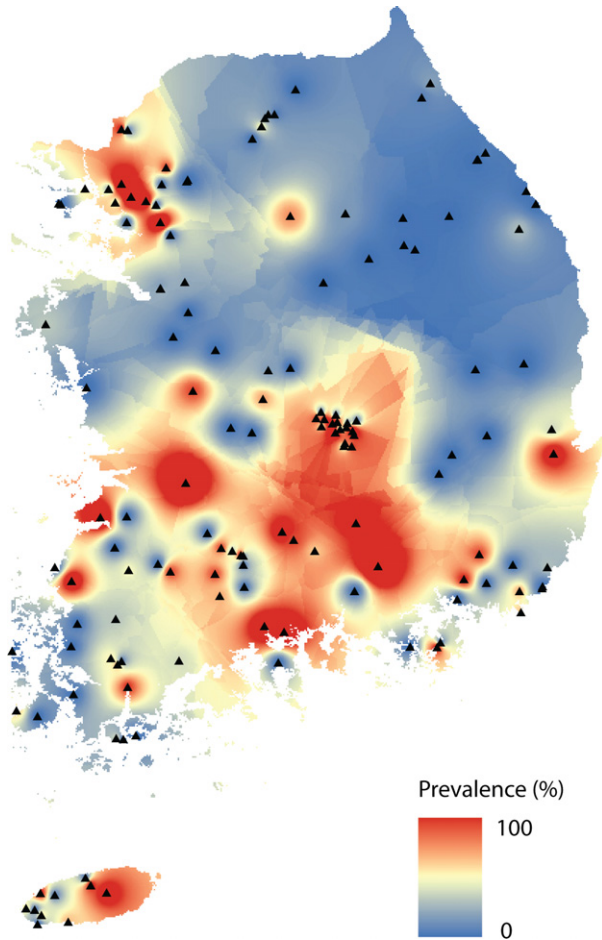


Fig. 2 Spatial variation in prevalence of *Batrachochytrium dendrobatidis* in South Korea. This heatmap depicts the interpolated prevalence of Bd across the South Korean landscape, constructed with an inverse distance weighting method, based on amphibian population monitoring data obtained during 2010 and 2011. Prevalence values are colour-coded following a gradient from blue (0–10%) to light blue (10–22%), yellow (22–35%), orange (35–50%) and red (50–100%). Collection localities are indicated by triangles.

ples, three haplotypes from 10 samples, four haplotypes from two samples and five haplotypes from three samples (Table S3, Supporting information). In total, 50 different haplotypes were identified (KR1 to KR50; GenBank accession nos JX983045–JX983089). Of the 50 haplotypes, 45 are unique to Korea, three (KR1, KR3 and KR7) are identical to haplotypes isolated in Japan (JP-U, JP-P and JP-O, respectively) (Goka *et al.* 2009) and two (KR6 and KR18) are identical to haplotypes from a native Brazilian Bd strain isolated in a USA food market [UM142 clone D and J (Schloegel *et al.* 2012), herein renamed BR1 and BR2; Fig. 3, Table S1, Supporting information]. The most common Korean haplotypes were KR4 (13 of 63 samples), KR9 (12 of 63 samples) and KR3 (11 of 63 samples; Table 1). KR4 was the most widely distrib-

uted across species (seven species), while KR3 was found exclusively on the introduced bullfrog (*L. catesbeianus*). Thirty-one haplotypes, the largest number found in any species in our study, were recovered from the oriental fire-bellied toad (*Bombina orientalis*) (30 of 63 samples), but this may be an artefact of our having had more success sequencing Bd isolated from this species.

We successfully estimated Bd infection intensity by qPCR assay in 59 of the 330 positive samples. All Bd intensity values were lower than the recognized mortality threshold of 10 000 zoospore genomic equivalents (ZGE; Table 1) (Kinney *et al.* 2011). The highest Bd loads were observed in *L. catesbeianus* (305.36 ± 130.72 ZGE), while mean Bd loads in other species were much lower (range: 1.50–28.07 ZGE; Tables 1 and S3, Supporting information).

Phylogenetic relationships among ITS haplotypes

Maximum parsimony (MP), maximum-likelihood (ML) and Bayesian inference (BI) phylogenies yielded similar trees, but the BI tree was the best resolved, albeit with poor node support (Figs 3 and S2, Supporting information). In this phylogeny, the most basal cluster consisted of 18 Korean haplotypes, including the most common KR4 haplotype, nine Brazilian haplotypes and two Japanese haplotypes. Two other small clusters were separated from this basal clade: one grouping Korean and Brazilian haplotypes (posterior probability = 0.51) and another comprising Korean, Japanese and Chinese haplotypes, including the second most common Korean haplotype KR9 (posterior probability = 0.69). A group of worldwide haplotypes and three Korean haplotypes (KR1, KR2 and KR11) formed a separate cluster corresponding to the Bd-GPL clade with a posterior probability of 0.55. A set of Brazilian, Japanese and Chinese haplotypes were positioned at the base of this Bd-GPL clade (Figs 3 and S2, Supporting information).

None of the clusters of haplotypes recovered in the BI tree were supported by the MP and ML phylogenies, but similar clusters were observed in the median-joining phylogenetic network (Fig. 4). A notable exception was the clustering of KR1 and KR11 (within the global lineage in the BI tree) with native Brazilian haplotypes (Fig. 4). Overall, the results of the phylogenetic analyses of the ITS sequences show that the majority of the Korean ITS haplotypes clustered with Brazilian and Asian haplotypes but were separated from the Bd-GPL clade.

Culture isolation from native Korean amphibians

Three fire-bellied toads (*B. orientalis*) (subjects BO319, BO327 and BO331), collected in the same watershed in Hwacheon (Gangwon Province, GPS coordinates

	Data set	Number of sites	Number of samples	Bd positive	Prevalence (%) (95% CI)
Province					
Chungcheongbuk	Full	11	164	29	17.7 (13.6–23.5)
	Reduced	11	153	27	17.7 (13.4–23.7)
Chungcheongnam	Full	7	56	1	1.8 (0.7–8.0)
	Reduced	7	52	0	0 (0.0–6.0)
Gangwon	Full	19	536	43	8.0 (6.4–10.2)
	Reduced	19	532	43	8.1 (6.4–10.3)
Gyeonggi	Full	25	274	32	11.7 (9.0–15.4)
	Reduced	24	261	28	10.7 (8.1–14.5)
Gyeongsangbuk	Full	19	175	49	28.0 (23.1–34.3)
	Reduced	17	134	34	25.4 (20.1–32.5)
Gyeongsangnam	Full	19	169	55	32.5 (27.3–39.2)
	Reduced	17	102	17	16.7 (11.9–24.2)
Jeju	Full	13	218	55	25.2 (21.0–30.7)
	Reduced	7	139	30	21.6 (16.8–28.3)
Jeollabuk	Full	11	54	17	31.5 (23.2–43.1)
	Reduced	8	27	3	11.1 (5.4–27.2)
Jeollanam	Full	25	217	49	22.6 (18.5–27.9)
	Reduced	19	137	20	14.6 (10.6–20.7)
Region					
Region NW	Full	54	831	94	11.3 (9.7–13.3)
	Reduced	50	793	87	11.0 (9.3–13)
Region NE	Full	8	196	9	4.6 (2.8–7.9)
	Reduced	8	196	9	4.6 (2.8–7.9)
Region SW	Full	35	269	66	24.5 (20.7–29.4)
	Reduced	25	162	23	14.2 (10.5–19.6)
Region SE	Full	40	349	106	30.4 (26.7–34.8)
	Reduced	34	241	53	22.0 (18.1–26.9)

Calculations made using the complete data set on Bd infection in Korean amphibian species ('Full') or using a reduced data set with results from *Hynobius leechii*, *Hynobius quelpaertensis*, *Lithobates catesbeianus* excluded ('Reduced'); see text.

38.128N, 127.762E), were swabbed and found to be infected with low loads of Bd (range: 2.98–114.23 ZGE). We genotyped the ITS region to characterize the Bd strains infecting these subjects: BO319 – haplotypes KR09 and KR18BR2; BO327 – haplotype KR09; and BO331 – haplotypes KR09, KR10 and KR46 (Fig. 4). To culture the Bd, the three subjects were held individually in enclosures with 10 conspecific tadpoles. After 2–4 weeks of experimental inoculation, dekeratinized areas were observed in the jaw sheaths of 8 to 10 tadpoles per container (80–100% infection rate). Isolation of Bd from infected jaw sheaths was successful from at least two tadpoles per container.

Multilocus sequence typing of cultured Korean Bd strains

Three Korean Bd cultures (KBO319T9, KBO327T6 and KBO331T3) were genotyped using a multilocus sequence typing (MLST) method based on 19 polymorphic loci. New alleles unique to the Korean strains were identified in 15 of the 19 analysed loci. No more than two alleles

per sample were identified for the three Korean Bd strains after sequencing of 10 clones per amplicon in two loci, suggesting that only one strain was present in each culture. Phylogenetic analysis of the MLST data set showed that the Korean genotypes formed a cluster well separated from the Bd-Brazil and the Bd-GPL lineages (bootstrap support $\geq 95\%$; Fig. 5). This divergence between Korean and Brazilian lineages was not observed in the ITS phylogeny (Figs 3 and 4). The three Korean genotypes, especially KBO319T9, diverged from one another with significant bootstrap support.

Discussion

Prevalence and infection intensity of Bd in South Korea

As found in previous surveys throughout Asia (Goka *et al.* 2009; Swei *et al.* 2011; Bai *et al.* 2012), Bd infection intensity was low in Korean amphibians. Infected individuals showed no clinical signs of chytridiomycosis, and we observed no morbidity or mortality in the field.

Table 2 Prevalence of *Batrachochytrium dendrobatidis* infection in amphibians by region

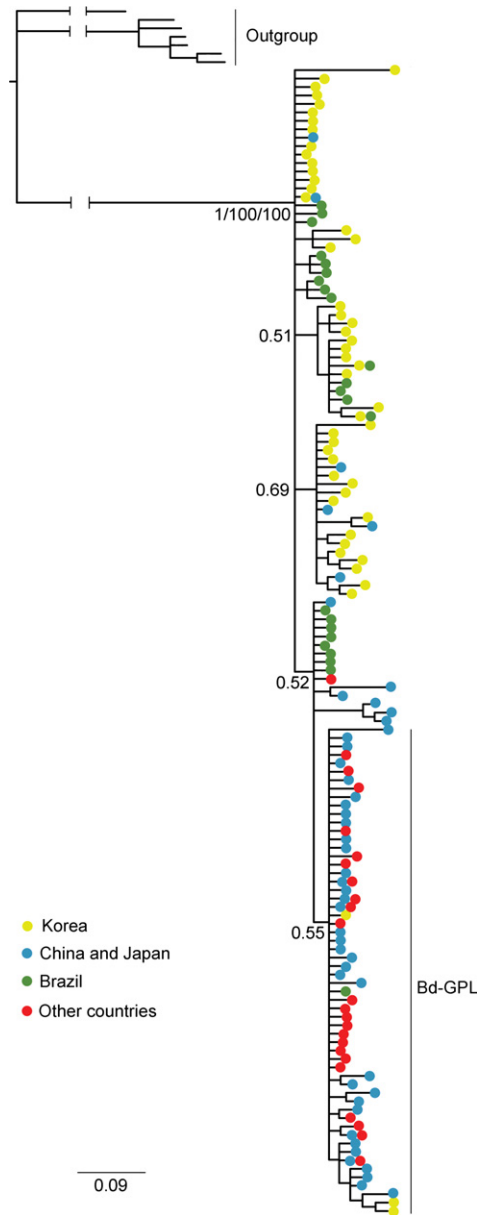


Fig. 3 Phylogenetic tree of ITS rRNA haplotypes from worldwide *Batrachochytrium dendrobatidis* samples. The strict consensus tree from phylogenetic analysis was constructed using a Bayesian inference method. The numbers above the branches indicate for each node the posterior probability (PP) support from Bayesian inference, bootstrap support (10 000 replicates) for maximum-likelihood and for maximum parsimony methods, respectively (nodes with PP >0.5 and/or bootstrap value >50% are shown). Only the support values discussed in the text are indicated. The length of the basal branch was cropped owing to space limitations. Samples are colour-coded by their geographical origin. Sequences from two *Terramycetes* sp., four *Boothiomycetes* sp. and one *Kappamycetes* sp. were used as outgroup, following Goka *et al.* (2009). A detailed version of this tree, including sample and outgroup names and all node support values, is available in the supplementary materials (Figure S2, Supporting information).

Nonetheless, our results point to relatively high prevalence of Bd in most Korean amphibians. Indeed, two salamander species demonstrated higher Bd prevalence (40–48%) than the non-native bullfrog (25%), and seven other native frog species were recorded with Bd prevalence above 10%. These results contrast with the low prevalence found previously in wild Asian amphibians (2.35% overall) (Swei *et al.* 2011).

The high Bd prevalence and low infection intensity found in Korean amphibians suggest a historical presence of Bd in Korea, consistent with the hypothesis that Bd may be endemic to Asia. In contrast, Swei *et al.* (2011) interpreted low Bd prevalence as an indication that Bd only recently may have been introduced and appears not to be self-sustaining in Asia. However, that study, based on opportunistic sampling with small sample sizes, may not truly reflect the extent of Bd distribution. We conducted planned, intensive surveys of all amphibian species throughout South Korea and found surprisingly high prevalence but low infection intensity. Detailed surveys throughout Asia will be useful in assessing the generality of our results.

Bd prevalence was significantly lower in the northeast, separated by mountain ranges from other regions of the country. Because of its mountainous terrain, the northeast of the country lacks flat plains, which typically are converted to rice fields. Many Korean amphibians, including those showing high Bd prevalence (e.g. *L. catesbeianus*, *B. orientalis* and *H. japonica*), mate in rice fields, often in dense breeding aggregations. This may facilitate opportunities for Bd transmission both within and among species above those typical of less anthropogenically disturbed habitats. Rice fields dominate much of the landscape in Asia, so this potential mode of transmission might be especially important. More detailed comparative studies of Bd prevalence between natural and rice field environments should be undertaken to evaluate this hypothesis.

Endemic Bd lineages in South Korea and Asia

The phylogenetic analyses of ITS and MLST data provide conflicting results on the origin of Bd in South Korea and Asia. Analysis of ITS sequences suggests that between 47 and 50 ITS haplotypes from Korea occupy a basal position in the tree, forming various clusters separated from the global Bd-GPL clade. None of these Korean haplotypes appear to be closely related to the four Japanese and Chinese endemic haplotypes (JP-B, JP-K, JP-J and CN30) identified in other studies (Goka *et al.* 2009; Bai *et al.* 2012). Rather, our phylogenetic network shows that many Korean haplotypes are identical or closely related to haplotypes isolated from Brazil. On their own, these results would suggest an ongoing

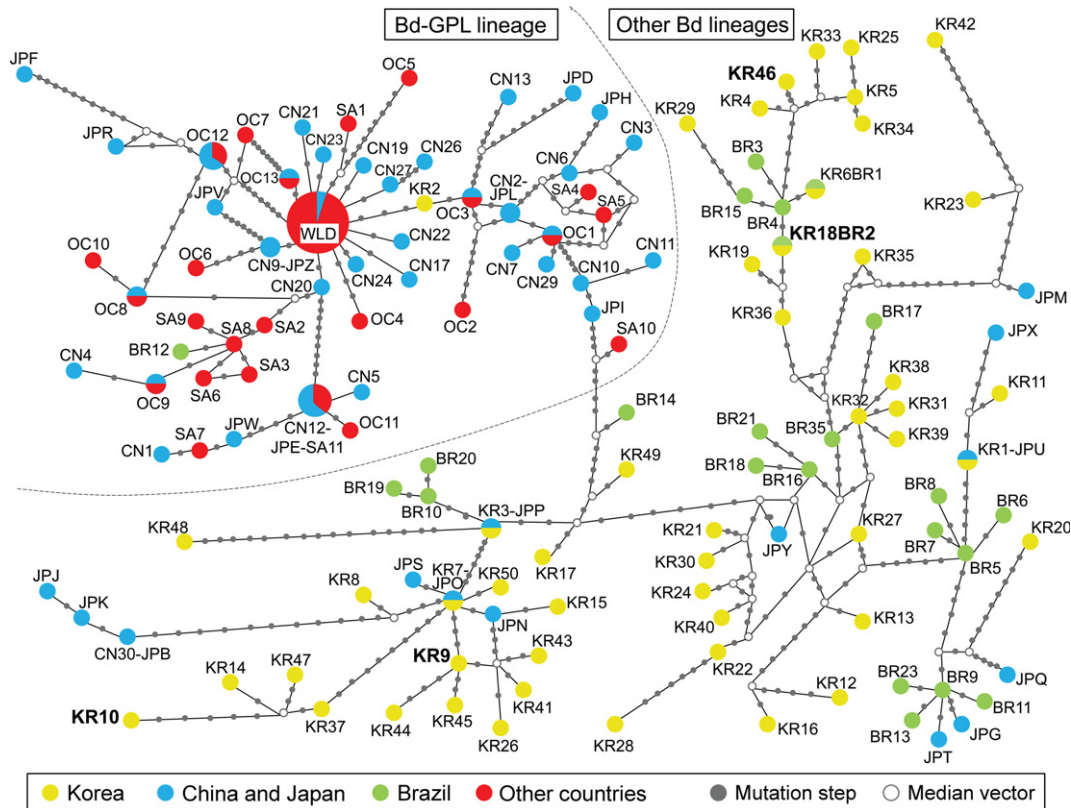


Fig. 4 Median-joining phylogenetic network of *Batrachochytrium dendrobatidis* ITS rRNA haplotypes. This network includes all the most parsimonious trees linking the isolates. Each unique haplotype is represented by a circle with its size relative to its frequency in the data set. Each node is separated by a specific number of mutations represented by grey dots and in some cases by internal nodes termed median vectors (grey circles). Blue circles denote haplotypes from China (CN), Japan (JP) or both; yellow, haplotypes from Korea (KR); green, haplotypes from Brazil (BR); and red, haplotypes from South Africa (SA) and other countries (OC). Nodes with multiple colours indicate haplotypes shared by more than one country. WLD (the largest, blue/red) indicates the most common global haplotype found in the USA, Ecuador, Italy, China and Japan (total = 42 sequences). Haplotypes identified in cultured Korean Bd strains are in bold. Owing to space limitations, the figure does not indicate equivalency of these haplotypes: OC1 = CN8, OC3 = CN28, OC8 = CN15, OC9 = CN16, OC12 = CN14 = JPC, OC13 = CN25.

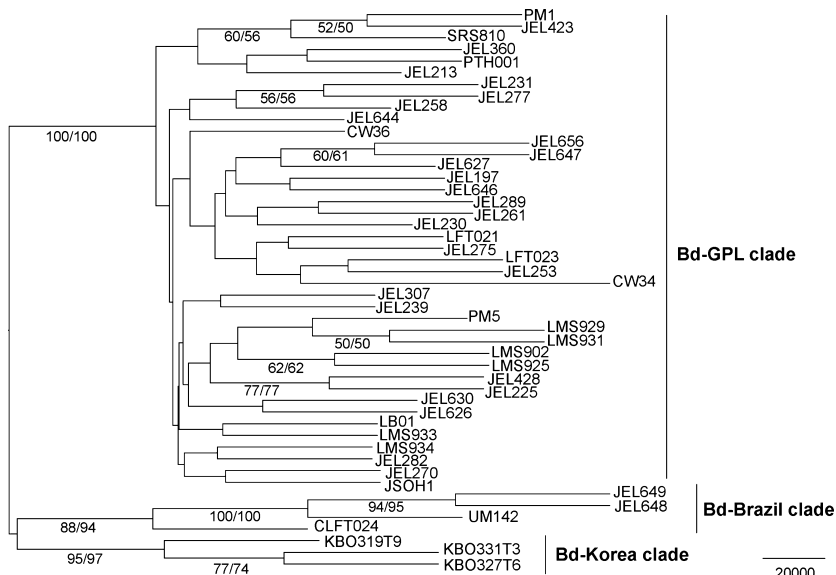


Fig. 5 Phylogenetic tree based on multilocus genotyping data from worldwide *Batrachochytrium dendrobatidis* strains. Neighbour-joining distance tree based on the proportion of shared alleles calculated from multilocus sequence typing of 19 loci showing the relationships among global Bd strains. Numbers along branches indicate nodal support: bootstrap values (>50%) from proportion of shared alleles and Cavalli-Sforza distance calculations (both with 50 000 replicates).

movement of native Bd strains between Korea and Brazil, perhaps associated with the pet trade (Schloegel *et al.* 2012). However, the MLST analysis of three Korean Bd strains isolated from *B. orientalis* reveals that the Korean Bd isolates represent a new clade, well separated from the Bd-Brazil lineage. This clade consists of one of the most common Korean ITS haplotypes (KR9), as well as other haplotypes identical or similar to haplotypes identified in native Brazilian strains (KR18 and KR46, Fig. 4). Thus, the results of ITS and MLST analyses differ in their support for the presence of endemic Bd lineages in Korea.

Caution needs to be exercised when using ITS sequences to assess Bd diversity or to determine phylogenetic relationships among strains (Schloegel *et al.* 2012). Until now, the ITS region has served as a useful marker to detect and genotype Bd because of its high copy number and short length, which promote amplification even of samples with low DNA quality. However, Schloegel *et al.* (2012) recently identified up to 22 distinct ITS sequences within particular Bd strains, so clearly ITS sequences are not strain specific and other markers must be used to resolve ambiguities. Here, we identified Bd ITS sequences that matched those associated with Bd-Brazil; yet, other markers that we used suggested to the contrary that these Bd strains were unique and presumably endemic to Korea. Thus, Japanese samples identified as originating from Brazil using ITS data (Schloegel *et al.* 2012) need to be genotyped with other markers to confirm the presence of Bd-Brazil in Japan. More generally, these conflicting results highlight the limitations of the ITS region as a means to identify Bd strains.

Our phylogenetic analyses reveal nonetheless that ITS sequences of Korean and Brazilian Bd strains consistently differ from those of Bd-GPL. Thus, despite its limitations, ITS may be a useful marker for distinguishing native Bd lineages from the Bd-GPL lineage. Thus we suggest that ITS should be mainly used as a first rapid tool to identify individuals or populations harbouring potentially novel Bd genotypes. The isolated strains then should be identified using a suite of markers.

Importantly, almost all ITS haplotypes that we identified in Korean amphibians, as well as the 11 Japanese haplotypes identified to date, form clusters that diverge from the Bd-GPL lineage. If, as argued above, we consider that ITS can reliably identify genotypes that differ from the Bd-GPL lineage, these results suggest that many of the Bd strains that infect Korean and Japanese amphibians are endemic to these regions. The high allelic diversity of Bd lineages, revealed by MLST analyses of Bd isolated from just three frogs collected in one watershed, probably does not begin to estimate total Bd strain diversity in the region. As two other Bd lineages isolated in Switzerland, Spain and South Africa

(BdCH and BdCAPE) (Farrer *et al.* 2011) were not included in our genetic analyses, we cannot rule out the possibility that Asian strains are related to these lineages, but this seems very unlikely. Many Bd strains appear endemic to Asia. Rather than being restricted to particular host species, they are highly diversified, widely distributed and associated with a large number of Asian amphibian species. This provides further support for the hypothesis that Bd lineages around the world are much more diverse than previously thought (Farrer *et al.* 2011; Schloegel *et al.* 2012; Rosenblum *et al.* 2013).

To fully evaluate the diversity of endemic Bd strains in Asia, and worldwide, and to ascertain the origin of the emergent hypervirulent Bd-GPL lineage, additional Bd strains native to each area need to be isolated and characterized using comparative genomic methods (Farrer *et al.* 2011; Rosenblum *et al.* 2013). The new isolation method developed in our study should serve as a useful tool to culture Bd from individuals infected with low Bd load, as is generally observed in Asian amphibians as well as possibly those elsewhere infected by native Bd strains. The method especially lends itself to amphibian species that are closely associated with aquatic habitats, but might be used with terrestrial species by exposing larvae to wash from infected adults. Studying how Bd affects common amphibian host species that are infected at high prevalence (such as *B. orientalis* or *H. japonica* in Korea) may provide insight into how Bd diversity and distribution impact rarer species of high conservation value.

Spread of native and introduced Bd lineages in South Korea

The American bullfrog (*L. catesbeianus*), introduced from eastern North America to many countries including Korea to establish frog farms, has been suggested as a primary vector for the spread of Bd worldwide. Bullfrogs infected with Bd have been readily found in frog farms, food markets and wild populations in Asia (Goka *et al.* 2009; Bai *et al.* 2012; Gilbert *et al.* 2012) and elsewhere (Schloegel *et al.* 2009, 2012). We found that Bd diversity, prevalence and infection intensity of *L. catesbeianus* were among the highest of all species that we surveyed, supporting the hypothesis that *L. catesbeianus* serves as a vector of Bd in Korea. Further, we identified Bd from this species with an ITS haplotype that is associated with the Bd-GPL lineage (KR2, Fig. 4), which suggests that *L. catesbeianus* may be spreading introduced Bd strains.

However, our results also show that wide-ranging native species such as the oriental fire-bellied toad *B. orientalis* can be infected by multiple Bd strains with

high prevalence. ITS sequence analyses, although equivocal, suggest that some of the Bd strains isolated from *B. orientalis* represent introduced strains (KR1 and KR11). *Bombina orientalis* and other wide-ranging native species like the Japanese tree frog *Hyla japonica* are found in larger numbers than *L. catesbeianus* in natural and agricultural landscapes across Korea. This suggests that, once new Bd strains are introduced into a region, native species might play an important role in their spread and proliferation. The lower prevalence observed in the mountainous northeast region of South Korea suggests that the spread of Bd by native amphibians also may be dependent on habitat and land use.

We detected just three haplotypes (KR1, KR2 and KR11; Figs 3 and 4), in four individuals, that may be associated with the Bd-GPL lineage. Only the clustering of KR2 with Bd-GPL was unambiguously supported by the phylogenetic analyses. Although our sample size for ITS sequences was limited to 63 individuals, we still would expect to observe more haplotypes associated with introduced Bd-GPL if they were present in the same frequency as they have been found in Japan (12 of 25 ITS haplotypes) and China (29 of 30 haplotypes, according to our phylogenetic network). This suggests that the presence of Bd-GPL is still very limited in wild Korean amphibian populations. Possibly the Bd-GPL lineage only was introduced very recently into Korea. We consider this explanation unlikely, however, first because the Bd-GPL-associated haplotypes detected in Korea were observed in sites distant from major ports of entry into the country such as Seoul or Busan (Fig. 1) and second because high levels of Bd introduction have been found in neighbouring China and Japan (Goka *et al.* 2009; Bai *et al.* 2012).

Another intriguing possibility is that endemic Korean Bd strains outcompete introduced Bd-GPL, owing to the driving force of host–pathogen co-evolution. Experimental infection studies of amphibians with both native and introduced Bd strains should be undertaken to elucidate the role of endemic pathogen–host interactions in limiting the spread of emerging pathogenic Bd. Such studies also would help increase our understanding of the mechanisms involved in effecting resistance to or tolerance of Bd-GPL among amphibian populations. We suggest that future studies on Bd in Asia will need to consider the interactions between introduced global panzootic Bd strains, native Bd strains and their associated amphibian hosts when assessing the spread and virulence of Bd-GPL in amphibian populations. Likewise, elsewhere in the world where Bd is found but without associated amphibian population declines, potential interactions between local and global Bd strains on host species need to be considered.

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A.B., J.J.F., M.S.M. and B.W. conceived of and designed the study. A.B., M.C. and H.J.B. conducted the laboratory benchwork. A.B., M.C., J.J.F., H.J.B., M.S.M. and B.W. conducted the field surveys. A.B., J.J.F., M.C. and G.W. analyzed the data. B.W., H.L. and M.S.M. contributed reagents, materials, and analysis tools. A.B., J.J.F. and B.W. wrote the paper.

Data accessibility

ITS rRNA sequences: GenBank Accession nos JX983045–JX983089.

Alignment of ITS rRNA haplotypes from *Batrachochytrium dendrobatidis* samples collected worldwide and

outgroup species, in Fasta format, has been provided as supplementary information (Data S1, Supporting information). The same alignment with the gaps at the end coded as A/T characters, used for maximum parsimony and maximum-likelihood analyses, has been provided as supplementary information in Nexus format (Data S2, Supporting information). Genotype data of *Batrachochytrium dendrobatidis* based on 19 loci, including representative allele sequences for each locus, in Genepop format, have been provided as supplementary information (Data S3, Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 ITS rRNA sequences of *Batrachochytrium dendrobatidis* included in our phylogenetic analyses.

Table S2 Prevalence of *Batrachochytrium dendrobatidis* in wild Korean amphibians in the nine Korean provinces.

Table S3 ITS rRNA haplotypes of *Batrachochytrium dendrobatidis* identified in wild amphibians collected in South Korea.

Fig. S1 Heatmap of the prevalence of *Batrachochytrium dendrobatidis* in South Korea using reduced data set.

Fig. S2 Detailed version of the phylogenetic tree of ITS rRNA haplotypes from worldwide *Batrachochytrium dendrobatidis* samples.

Data S1 Alignment of ITS rRNA haplotypes from *Batrachochytrium dendrobatidis* samples collected worldwide and outgroup species (Fasta format).

Data S2 Alignment of ITS rRNA haplotypes from *Batrachochytrium dendrobatidis* samples collected worldwide and outgroup species with gaps coded as A/T characters at the end of the alignment (Nexus format).

Data S3 Multilocus genotype data of *Batrachochytrium dendrobatidis* based on 19 loci (Genepop format) and representative allele sequences for each locus.