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Novel chytrid pathogen variants and the global amphibian pet trade

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Article Impact Statement: Pathogen variants dispersed by global wildlife trade threaten endemic species, necessitating enhanced border surveillance and containment.

Abstract

Global wildlife trade spreads emerging infectious diseases that threaten biodiversity. The amphibian chytrid pathogen *Batrachochytrium dendrobatidis* (Bd) has caused population declines and species extinctions worldwide except in Asia. Fire-bellied toads (*Bombina orientalis*), exported in large numbers from Asia, are tolerant of Bd and carry hypervirulent ancestral chytrid BdAsia-1 variants. We assayed the virulence of a new isolate of BdAsia-1 on the model Australasian frog host *Litoria caerulea*. Infected individuals ($n = 15$) all showed rapid disease progression culminating in death, whereas sham-inoculated individuals ($n = 10$) presented no clinical signs of disease and all survived (log rank test, $\chi^2 = 15.6$, $df = 1$, $p < 0.0001$). The virulence of the new isolate of BdAsia-1 is comparable to the one we assayed previously ($\chi^2 = 0.0$, $df = 1$, $p = 0.91$). Internationally traded wildlife, even when they appear healthy, can carry hypervirulent variants of pathogens. Once new pathogen variants escape into the environment, native species that have had no opportunity to evolve resistance to them may perish. Our study suggests that hypervirulent pathogens are being spread by the international pet trade. Notifiable wildlife diseases attributable to locally endemic pathogens often fail to generate conservation concern so are rarely subject to border surveillance or import controls. Because of the danger novel variants pose, national border control agencies need to implement disease screening and quarantine protocols to ensure the safety of their endemic fauna.

KEYWORDS
amphibian chytridiomycosis, *Batrachochytrium dendrobatidis*, global wildlife trade, pathogen pollution, pathogen virulence

Variantes Patógenas Nuevas de Quitridios y el Mercado Mundial de Anfibios Mascota

Resumen: El mercado mundial de fauna dispersa enfermedades infecciosas emergentes que amenazan a la biodiversidad. El quitridio patógeno de anfibios *Batrachochytrium dendrobatidis* (Bd) ha causado declinaciones poblacionales y la extinción de especies en todo el mundo excepto Asia. El sapo *Bombina orientalis*, exportado en grandes cantidades desde Asia, es tolerante al Bd y carga genéticamente las variantes ancestrales hipervirulentas de quitridio BdAsia-1. Analizamos la virulencia de una nueva cepa de BdAsia-1 con el modelo de la rana australo-asiática hospedera *Litoria caerulea*. Todos los individuos infectados ($n = 15$) mostraron una progresión acelerada de la enfermedad que culminaba con la muerte, mientras que los individuos con inoculación simulada ($n = 10$) no presentaron señales clínicas de la enfermedad y todos sobrevivieron (prueba log de rango, $\chi^2 = 15.6$, $df = 1$, $p < 0.0001$). La virulencia de la nueva cepa de BdAsia-1 es comparable a la que analizamos previamente ($\chi^2 = 0.0$, $df = 1$, $p = 0.91$). La fauna comercializada internacionalmente, incluso cuando parece estar saludable, puede portar variantes hipervirulentas de los patógenos. Una vez que un patógeno nuevo se introduce al ambiente, pueden perecer las especies nativas que no han tenido la oportunidad de evolucionar la resistencia a estos patógenos. Nuestro estudio sugiere que los patógenos hipervirulentos

se están dispersando mediante el mercado internacional de mascotas. Con frecuencia las enfermedades silvestres notificables que pueden atribuirse a los patógenos endémicos no generan interés para la conservación, así que rara vez están sujetas a la vigilancia fronteriza o el control de importación. Debido al riesgo que representan las variantes nuevas, las agencias nacionales de control fronterizo necesitan implementar evaluaciones patológicas y protocolos de cuarentena para asegurar la seguridad de su fauna endémica.

PALABRAS CLAVE
contaminación por patógenos, mercado mundial de fauna, quitridiomicosis anfibia, virulencia patógena, *Batrachochytrium dendrobatidis*

【摘要】
全球野生动物贸易引起的新发传染病传播对生物多样性产生了威胁。两栖动物壶菌病原体蛙壶菌 *Batrachochytrium dendrobatidis* (Bd) 已导致全球除亚洲以外地区的两栖动物种群数量下降及物种灭绝。从亚洲大量出口的火腹蟾蜍 (*Bombina orientalis*) 对 Bd 有耐受性, 且携带高致病性的祖先型壶菌变体 BdAsia-1。本研究检测了一个新分离的 BdAsia-1菌株对澳大利西亚模式青蛙宿主澳洲绿树蛙 (*Litoria caerulea*) 的毒力。研究发现, 被感染的个体 (n=15) 均表现出快速的疾病进展且最终死亡, 而假接种的个体 (n=10) 则没有出现疾病临床症状且全部存活(时序检验, $\chi^2 = 15.6$, df=1, $p < 0.0001$)。新分离的BdAsia-1变体毒力与我们之前检测的菌株毒力一样强 ($\chi^2 = 0.0$, df=1, $p = 0.91$) 。国际贸易中的野生动物即使看起来很健康, 也可能携带高致病性的病原体变体。一旦新型病原体变体逃逸到环境中, 没有机会进化出抵抗力的原生物种就可能会灭亡。我们的研究表明, 高致病性的病原体正在通过国际宠物贸易传播。由地方特有的病原体引起的野生动物应报告疾病往往没有引起保护关注, 因此很少受到边境监察或进口管制。考虑到新型病原体变体所带来的危险, 国家边境管制机构应实施疾病筛查和检疫条款, 以保障地方特有动物群的安全。**【翻译:胡怡思;审校:聂永刚】**

关键词: 两栖动物壶菌病, 蛙壶菌, *Batrachochytrium dendrobatidis*, 全球野生动物贸易, 病原体毒力, 病原体污染

INTRODUCTION

Emerging infectious diseases (EIDs) increasingly threaten global biodiversity, which, in turn, can negatively affect public health and economic prosperity (Daszak et al., 2000). Translocation of wildlife by global trade not only incurs the risk of disruption to ecosystems by the accidental introduction of exotic species, but also spreads pathogens that may exploit naive endemic flora and fauna (Ogden et al., 2019; Pyšek & Richardson, 2010). Global trade of wildlife driven by food and medicine markets (Gratwicke et al., 2010; Scheffers et al., 2019; Smith et al., 2009), the exotic pet trade (Hughes et al., 2021; Moorhouse et al., 2017; Sung & Fong, 2018; Tingley et al., 2017), and medical testing (Vredenburg et al., 2013) has expanded the range of EIDs and accelerated the rate of biodiversity loss (Rosen & Smith, 2010). The protection of global biodiversity thus demands more effective, comprehensive regulation of the international animal trade to prevent pathogen spillover (Daszak et al., 2000; Voyles et al., 2015).

Global declines of amphibian populations and the extinction of as many as 90 species have been attributed to chytridiomycosis, the EID caused by the chytrid fungi *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal) (Fisher & Garner, 2020; Scheele et al., 2019). Despite the catastrophic effect Bd infection has had on amphibians around the world, some species appear resilient to infection (Fu & Waldman, 2017; Voyles et al., 2018). Notably, outbreaks of chytridiomycosis have not been reported in Asia, where amphibians may have evolved resistance to the disease (Bataille et al., 2015; Fu & Waldman, 2019). Some globally traded species, including African clawed frogs (*Xenopus laevis*) and American bullfrogs (*Rana catesbeiana*), also survive infection without presenting clinical signs of chytridiomycosis (Fisher & Garner, 2007, 2020; Weldon et al., 2004). Nonetheless, they can transmit the pathogen to other species (Fisher & Garner, 2020; Reeder et al., 2012).

Oriental fire-bellied toads (*Bombina orientalis*) often are infected with Bd in the wild, typically appearing healthy and bearing subclinical pathogen loads (Bataille et al., 2013). These frogs are exported in large numbers from Asia to North America (Herrel & van der Meijden, 2014) and Europe (Kopecký et al., 2016; Wombwell et al., 2016) for the pet trade. In transit and on delivery, many *B. orientalis* have been found to be infected with Bd (Kolby et al., 2014; Wombwell, 2014). They thus can serve as reservoirs of chytrid fungi (Brannelly et al., 2018; Briggs et al., 2010), which may spill over into the natural environment and infect other amphibian species. We previously demonstrated that *B. orientalis*, after inoculation with a known

virulent Bd isolate (BdGPL) (the global pandemic lineage), was both resistant to and tolerant of the pathogen (Fu & Waldman, 2019). Even frogs that test negative for infection can release infective zoospores into the environment (Shin et al., 2014).

Whole genome sequencing reveals that Bd originated in Asia and spread worldwide over the past several decades with the rise of global trade in amphibians (O'Hanlon et al., 2018). To date, six major Bd lineages have been identified (Byrne et al., 2019; O'Hanlon et al., 2018). *Bombina orientalis* usually is infected by BdAsia-1, an endemic Asian lineage (Figure 1) (Bataille et al., 2013; O'Hanlon et al., 2018). The Bd lineages vary considerably in virulence and pathogenicity (Berger et al., 2005; Retallick & Miera, 2007), modulated by host susceptibility and by genetic and environmental factors (James et al., 2015; Olson et al., 2021). For example, although BdGPL has been associated with most disease outbreaks (Fisher & Garner, 2020), some BdGPL variants seem harmless to hosts (Greener et al., 2020).

Evaluation of the risk posed by pathogens carried by globally traded animals requires robust assessments of their likely impact. Virulence is defined within the context of the interaction of a pathogen and its hosts (Fisher et al., 2021). For BdAsia-1, virulence may vary among susceptible hosts. More generally, virulence can vary among isolates of the same lineage. In adult Australasian green tree frogs (*Litoria caerulea*), isolate KBO347 causes more rapid disease progression and higher rates of mortality than BdGPL (Fu & Waldman, 2019). However, this same isolate appears less virulent than BdGPL in juvenile common toads (*Bufo bufo*) in some but not all treatment conditions (O'Hanlon et al., 2018). To determine the risk that BdAsia-1 may pose as an invasive pathogen, we tested a second BdAsia-1 isolate (KBO327) on *L. caerulea* to obtain measures of morbidity and mortality. The results could serve as a basis for examining border surveillance and mitigation protocols that may be needed to protect native species.

METHODS

Sample collection and maintenance

Litoria caerulea were caught in the wild in New Guinea during November and December 2016 and shipped directly to South Korea for our research. Frogs were housed individually in closed plastic containers (290 × 90 × 200 mm) appropriate to their size. We provided them moistened unbleached autoclaved paper towels with small open water reservoirs, which were changed under sterile conditions three times weekly. The water was carbon filtered and UV treated to ensure it was pathogen-free. Frogs were fed mealworms (*Tenebrio molitor* larvae) and nutrient powder supplements (Superworm, Seoul, South Korea) (20% calcium, 25% crude protein, vitamin D3, and minerals) ad libitum. Frogs were held in a containment room at 21–22°C on a 12 h light, 12 h dark photoperiod. Relative humidity was 40%. Frogs were hand captured by collectors registered with the Natural Resources Conservation Agency (BKSDA). Animal collection and maintenance protocols were approved by the Institute of Laboratory Animal Resources (ILAR-17-04-118) of Seoul National University.

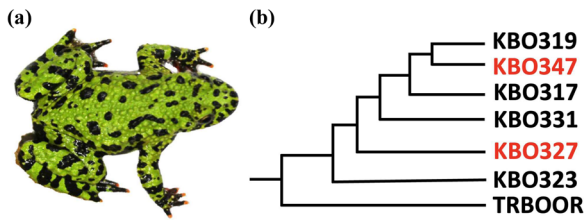

FIGURE 1 (a) Oriental fire-bellied toad (*Bombina orientalis*) from South Korea and (b) the phylogeny of BdAsia-1 isolates, showing KBO327 to be an early-branching lineage (modified from O'Hanlon et al., 2018). The virulence of KBO327 was compared with that of KB0347, which was previously studied (Fu & Waldman, 2019)

Infection experiments

We previously cultured several isolates of Bd from Korean *B. orientalis* (Bataille et al., 2013), all of which fall into the basal Bd lineage described as BdAsia-1 (O'Hanlon et al., 2018). In this study, we infected frogs with isolate KBO327 (passage 9). We cultured the isolate in TGhL broth (6.4 g tryptone, 1.6 g gelatin hydrolysate, 3.2 g lactose monohydrate, and 800 ml distilled water) for 4 or 5 days and then transferred 1 ml of culture to each TGhL plate (1% agar) (Longcore et al., 1999). One week later, we flooded each plate with 1–2 ml sterilized water and let the plate stand for 20–30 min before collecting zoospores from it. The Bd zoospore activity was counted under a microscope using a hemocytometer.

Each individual in the infection group (n = 15; mean mass [SD] = 44.79 g [15.97]) was inoculated with 500,000 Bd zoospores. Control groups were treated similarly but without Bd zoospores (n = 10, mean mass 49.02 g [22.29]). Subjects were assigned at random to each treatment. Procedures were carried out as previously described (Fu & Waldman, 2019). Beginning 24 h after treatment, we monitored subjects at least once daily for clinical signs of chytridiomycosis, including lethargy, cutaneous erythema, inappetence, and skin sloughing (Berger et al., 2009). We recorded a subject as dead when it failed to show a righting reflex and lacked a detectable heartbeat.

The experimental protocol was approved by the Institute of Laboratory Animal Resources (ILAR-17-04-118) and the Institutional Biosafety Committee (SNUIBC-R170502-1) of Seoul National University.

Bd screening and monitoring

We swabbed all frogs with MW113 rayon swabs (Medical Wire and Equipment, Corsham, UK) along their ventral skin, including legs and feet 10 times with a standardized protocol (Hyatt et al., 2007) to test them for Bd infection. Frogs were screened upon arrival in Korea and immediately before inoculation. The DNA from each swab was extracted using 50 μl PrepMan Ultra (Applied Biosystems, Foster City, CA)

following the manufacturer's protocol. Real-time polymerase chain reaction (PCR) was used to assess Bd load, following reaction conditions and primers as previously described (Fu & Waldman, 2019). We used primers ITS1-3 Chytr (50-CCTTGATATAATACAG TGTGCCATATGTC-30) and 5.8S Chytr (50-AGCCAAGAGAT CCGTTGTCAA-30). Thermocycler conditions consisted of an initial uracil–DNA glycosylase incubation step at 50°C for 2 min, followed by polymerase activation at 95°C for 10 min, then by 50 cycles of 10 s at 95°C and 1 min at 60°C, and lastly a melting curve of 95°C for 15 s, 55°C for 15 s, and 95°C for 15 s (Boyle et al., 2004). Prior to inoculation, none of the subjects tested positive for Bd.

After inoculation, we monitored subjects regularly, measuring Bd loads of all subjects weekly. Each sample was run in duplicate, and infection intensity was estimated using a standard curve prepared by serial dilution of Bd ITS standards. PrepMan Ultra dilutions (1:5) were included on every running plate as negative controls. We transformed the infection loads by computing base-10 logarithm scores.

Histological examination

Pelvic and ventral regions of skin from Bd-infected frogs that died were dissected. The tissue was fixed in freshly made 10% neutral buffered formalin (formaldehyde 10 ml, distilled water 90 ml, sodium phosphate monobasic dehydrate 0.65 g, and sodium phosphate dibasic anhydrous 0.40 g) for 24 h at 4°C. After 2–6 h of washing with running distilled water, the fixed tissue was dehydrated in an automatic tissue processor (Leica ASP300S, Leica, Wetzlar, Germany). The tissue then was embedded in paraffin, sectioned at 5 μm (Leica RM2255, Leica, Wetzlar, Germany), and placed on silane-coated slides for hematoxylin and eosin staining. Stained samples were observed under fluorescence microscopy (Axio Observer Z1, Carl Zeiss, Göttingen, Germany).

Body condition measurements

We measured the body mass of each subject before infection and at death or the end of the experiment. We then compared body mass changes between the Bd infection and control groups.

Statistical analyses

We used Cox proportional hazards model (Cox, 1972) to compare survival of experimental and control treatments; mass measured prior to inoculation was a covariate. We analyzed differential survival among treatments with the nonparametric Kaplan–Meier procedure. Analyses were conducted with the coxph and survfit functions, respectively, in the R package survival. We compared mass change and infection loads of *L. caerulea* during the course of the experiment between treatments with one-way analysis of variance (aov function) after verifying that the data were normally distributed. All computations were made with R 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

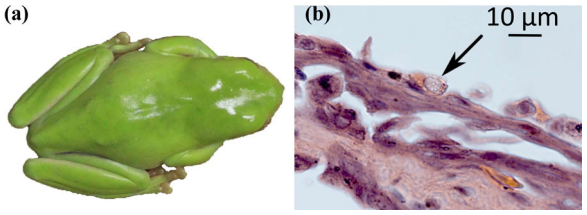

FIGURE 2 (a) Australasian *Litoria caerulea* and (b) histological section of an infected individual's epidermis after Bd infection. The arrow indicates a zoosporangium filled with zoospores in the stratum corneum.

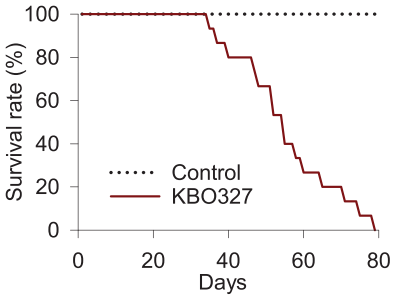

FIGURE 3 Survival rate of *L. caerulea* exposed to KBO327 and sham inoculates (controls) over time

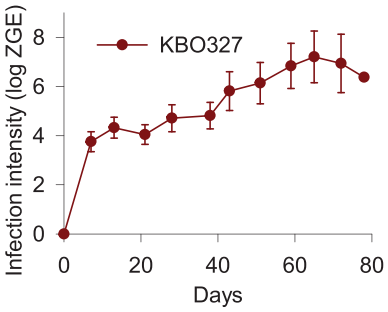

FIGURE 4 Infection intensity, given in base 10 log-transformed zoospore genomic equivalents (ZGE), over time of *L. caerulea* exposed to KBO327 (error bars, SE)

RESULTS

Thirty-four days after KBO327 infection, *L. caerulea* (Figure 2a) started to die, and we observed zoosporangia in the outer epidermal layers of their skin (Figure 2b). By day 78, all subjects in the experimental group died but all control subjects survived (Figure 3) (Kaplan–Meier survival analysis, log rank test, $\chi^2 = 15.6$, df = 1, $p < 0.0001$). Infection loads continued to increase until the subjects died (Figure 4). The KBO327-infected subjects lost body mass (mean [SE] = −7.74 g [1.58]), whereas control subjects gained mass (1.43 g [1.58]) ($F_{1,19} = 10.34$, $p < 0.005$). Initial variation in mass of the subjects did not

affect these conclusions (Cox proportional hazards model, $z = 1.122$, $p = 0.26$). We were able to compare the virulence of KBO327 with that of KBO347, which we tested previously, because experiments were run under the same conditions (Fu & Waldman, 2019). Survival analyses failed to reveal any differences in the effects of the isolates on host survival (log rank test, $\chi^2 = 0.0$, df = 1, $p = 0.91$).

DISCUSSION

The global trade of wildlife widely disperses exotic species and the pathogens they carry. These pathogens can spill over into populations of endemic species. Such "pathogen pollution" constitutes a major threat to global biodiversity and human health (Daszak et al., 2000). Although we previously demonstrated that *L. caerulea* is highly susceptible to BdAsia-1, those results were based on a single isolate. Our finding here that a second isolate of this lineage causes the same effects in *L. caerulea* demonstrates with some generality that BdAsia-1 is potentially more virulent than BdGPL. Both BdAsia-1 isolates were cultured from *B. orientalis*, which is exported in large numbers from Asia for the pet trade. These frogs thus potentially serve as vectors of BdAsia-1 variants from Asia to other continents. Because native amphibians there are immunologically naïve to those variants, they may not have evolved effective defenses against them. Extant BdAsia-1 lineages have not yet been detected in North America or Europe (O'Hanlon et al., 2018; Byrne et al., 2019), so the opportunity exists to prevent their establishment on those continents.

Hypervirulence of BdGPL has been attributed to hybridization and genetic recombination among lineages, but because BdAsia-1 is basal to BdGPL, our results suggest that ancestral Bd strains were hypervirulent in the absence of recombination events. Instead, the hypervirulence of the ancestral BdAsia-1 lineage was driven by the evolution of enhanced resistance and tolerance mechanisms in their amphibian hosts (Fu & Waldman, 2019). Our results stand in stark contrast to the hypothesis that endemic Bd strains evolve to be hypovirulent to their hosts (Belasen et al., 2022). Because *B. orientalis* does not clear itself of Bd, but is tolerant of it, carrying low infection loads without showing clinical signs of disease (Bataille et al., 2013), this host species may be especially likely to spread infection to other species. Catastrophic effects on naive species thus may occur as these Bd strains spill over into areas outside their native range (Picco & Collins, 2008; McKenzie & Peterson, 2012).

The virulence of a pathogen is determined by many factors, including environmental conditions, community dynamics, population densities, and life stage, all of which can affect host resistance or tolerance (Adams et al., 2017; Turner et al., 2021). *Litoria caerulea* has become a model species for studies on the pathogenesis of chytridiomycosis (Voyles et al., 2009). Australian populations of the species are moderately susceptible to Bd (Brannelly et al., 2021), but our study subjects, sourced from New Guinea, were highly susceptible to BdGPL and BdAsia-1 isolates. Australian populations of the species may have evolved some resistance through exposure to Bd, unlike those from New Guinea, which remains Bd-free (Bower et al., 2020). This might explain why the pathogen appears less deadly to Australian *L. caerulea* populations than those from New Guinea.

In a previous study, differential susceptibility of British common toads (*B. bufo*) to BdGPL and endemic Bd lineages, including BdAsia-1, was noted (O'Hanlon et al., 2018). The pathogen is present throughout Great Britain (Garner et al., 2005); so, like Australian *L. caerulea* populations, *B. bufo* may have evolved resistance to or tolerance of the pathogen. Disease outbreaks in these toads have not been recorded in Britain, unlike elsewhere in Europe (Bosch & Martínez-Solano, 2006). O'Hanlon et al. (2018) found that larvae inoculated with BdAsia-1 or BdGPL were significantly more infectious than those inoculated with other endemic lineages. Also, subjects infected with BdAsia-1 and BdGPL experienced similar levels of mortality in some treatments, but BdGPL caused more deaths in others (Appendices S3 & S4 and Figure 2g in O'Hanlon et al. [2018]). These results should be interpreted with caution because body size, rather than Bd infection per se, is the primary determinant of survival in this study system (Garner et al., 2009).

Large numbers of *B. orientalis* are exported from Asia each year for the pet trade. Based on U.S. Fish and Wildlife Service (USFWS) Law Enforcement Management Information System records, 3,553,738 *B. orientalis* were imported into the United States from 2001 to 2009 (ranked third among traded amphibians) (Herrel & van der Meijden, 2014). Moreover, 34,438 *B. orientalis* were exported from the United States to other countries. From 1998 to 2002, 1,016,579 wild-caught *B. orientalis* were imported and 78,606 were exported (Schlaepfer et al., 2005). Similar numbers likely were imported into Canada, the United Kingdom and the European Union (Gerson, 2012; Kopecký et al., 2016; Wombwell et al., 2016). From 1999 to 2013, at least 97,000 amphibians were exported from South Korea each year (Sinclair et al., 2021), and *B. orientalis* is also exported from North Korea (Goka et al., 2009). Like other Asian frogs, *B. orientalis* is frequently transshipped via holding facilities in other Asian hub locations, such as Hong Kong and Singapore (Lau et al., 1997; Kolby et al., 2014; Sinclair et al., 2021), where further opportunities occur for them to become cross-infected.

Internationally traded *B. orientalis* are likely to carry multiple hypervirulent Bd strains, some endemic to Asia and particularly infectious to amphibians elsewhere that are naïve to them. Risk assessment models, which rank species with respect to the likelihood that they will become established as invasive species (Kopecký et al., 2016), fail to consider the danger species pose as reservoirs of virulent pathogen variants. Eventual release of hypervirulent Asian Bd variants into the environment seems inevitable, potentially through wastewater discharge (Kolby et al., 2014) and the escape or release of pets (Tinsley et al., 2015).

Chytrid fungus infects amphibians on all inhabited continents. Some islands that are hotspots of amphibian biodiversity may still be free of the pathogen and merit special attention. Effective surveillance policies are urgently needed to prevent Bd incursions into New Guinea (Bower et al., 2020) and

Madagascar (Vredenburg et al., 2012; Kolby et al., 2015), but Bd may have already been established at high elevations in Madagascar (Bletz et al., 2015). Elsewhere, efforts toward general containment or elimination of the pathogen are not practicable and are unlikely to produce meaningful conservation outcomes. Although chytridiomycosis is classified as a notifiable disease (Schloegel et al., 2010), the costs of effective border surveillance have been difficult to justify, so disease monitoring remains minimal.

Yet, some Bd variants, presently restricted to particular geographic regions, potentially pose extreme risk to endemic species elsewhere. In light of our findings, we argue that border surveillance of animals bearing these virulent variants needs to be implemented. The international amphibian trade is poorly monitored with little administrative infrastructure in place to screen amphibians for disease. In the United States, for example, live amphibian shipments are examined by the USFWS to ensure compliance with national and international agreements on animal trade. Their activities focus on identifying animals subject to the Convention on International Trade in Endangered Species and other species subject to special protection. Responsibility for disease screening falls to the Centers for Disease Control and Prevention (CDC) or the Department of Agriculture's Animal and Plant Health Inspection Service (APHIS). In practice, CDC operations are directed toward identifying pathogens that threaten human health, whereas APHIS targets potential risks to the agricultural sector. Thus, as presently constituted, no government agency is prepared to administer regulations on border surveillance for wildlife disease. Regulatory agencies in other countries similarly are not designed to intercept pathogens on wildlife (e.g., Peel et al., 2012; Wombell et al., 2016; Woodhams et al., 2021).

The World Organisation for Animal Health (Office International des Épizooties [OIE]) has established detailed protocols to prevent spread of Bd from countries that have not been declared free of the pathogen (OIE, 2021a). Prescribed procedures apply to live amphibians intended for use in the pet trade, as foodstuffs, or for agricultural, industrial, scientific, pharmaceutical, research, or exhibition purposes. Recommendations are regularly revised by the Aquatic Animal Health Standards Commission of the OIE for consideration by competent authorities in importing countries. Yet, we know of no country that has adopted or implemented these standards to prevent the spread of Bd. International health certificates (IHCs) often are required when transporting live amphibians across borders, but certification is based on cursory examination by registered veterinarians. Because animals can carry Bd without showing clinical signs of disease, such requirements are unlikely to prevent pathogen transmission. Thus, IHCs need to include the results of PCR-based Bd screening tests, either by swabbing individuals or by pooling samples obtained, for example, by water filtering (e.g., Kolby et al., 2014, 2015; Brunner, 2020).

Upon arrival, imported amphibians, especially those known to carry virulent pathogens without showing clinical signs of disease, should be transported directly to quarantine or containment facilities for additional pathogen screening followed by at least 10 days of monitoring for disease (CADEH, 2006). Containment facilities might be operated by government agencies, such as APHIS, or licensed importers subject to inspection by government agency regulators. Facilities should be designed to ensure they meet BSL2 (PC2) biosafety standards to prevent accidental pathogen release (e.g., through packaging materials, water, and effluents) (Young et al., 2007; OIE, 2021a). More definitive recommendations on screening methods are being developed for inclusion in future editions of the Aquatic Animal Health Code (OIE, 2021b,c). Further studies may determine that strain-specific Bd and multiplex PCR methods are necessary to ensure the detection of novel variants (Byrne et al., 2017; Ghosh et al., 2021) and coinfections (Blooi et al., 2013).

The implementation of safeguards requires overcoming technical and political hurdles. Costs of routine inspections and testing, as well as the maintenance of animals in disease-free containment facilities on arrival, will need to be passed onto end users. Pathogens can spread within and among species through numerous stages of the supply chain (Sinclair et al., 2021), resulting in mass mortality events. On visiting such facilities, we have observed such disease outbreaks ourselves. By reducing the incidence of such events, implementation of OIE-recommended quarantine procedures should prove to be of net financial benefit to importers.

In some cases, researchers themselves may be culpable for inadvertently spreading wildlife pathogens. Ten years ago, a colleague visited a research colony of *B. orientalis* that had been imported from South Korea and inquired as to whether they had been screened for Bd. Hearing that they had not, permission was sought to swab them for standard analysis. The request was summarily denied, presumably because of the risk potentially positive findings would pose to the laboratory's research program. Certainly, all users of amphibians, including researchers, need to be aware of the disease risks that accompany the importation of wildlife. Whether legally mandated or not, every precaution needs to be taken to prevent the spread of dangerous variants.

Bombina orientalis is one of the most widely traded Asian amphibians internationally and its tolerance of known hypervirulent Bd variants makes it of special concern. The species may serve as a vector for other potentially lethal pathogens as well. A related species of fire-bellied toad, *B. microdeladigitora*, native to China and Southeast Asia, has been found infected by *Batrachochytrium salamandrivorans* (Bsal) in the wild, and frogs exported to Germany also tested positive for the fungus (Nguyen et al., 2017). *Bombina microdeladigitora* infected with Bsal, just like *B. orientalis* infected with Bd, present no clinical signs of disease. Potentially, these *Bombina* might spread Bsal to regions of the world, such as North America, where the pathogen presently is absent.

Extirpations of some salamander populations in Europe have been caused by Bsal (Martel et al., 2013). To prevent its introduction to North America, potential hosts were listed as injurious wildlife under provisions of the Lacey Act (USFWS, 2016), which blocked virtually all salamander imports (Grear et al., 2021). Possibly *Bombina* species should be added to the list of banned species under the Lacey Act. However, given that

Bd lineages already are widely distributed and infect amphibians on every continent, justification and implementation of such a trade ban would be difficult to achieve. We thus recommend that national agencies responsible for border biosecurity develop infrastructure and enforcement mechanisms needed to abide by OIE guidelines. Mandatory screening for potentially dangerous pathogens and quarantine before release are just as crucial to maintaining ecosystems as they are to protecting agriculturally important species and human health.

As a general principle, effective wildlife management policy needs to recognize that even when complete elimination of pathogen transfer is not possible, novel variants should be recognized and plans to mitigate their threats developed and implemented expeditiously (Auliya et al., 2016; Wombwell et al., 2016). Although our study focuses on the dangers of novel variants of chytrid fungal pathogens to amphibians, similar threats arise from the global trade of other taxa. For example, snake fungal disease (SFD or ophidiomycosis), caused by *Ophidiomyces ophiodiicola* (Oo), is rapidly spreading throughout North America (Lorch et al., 2016). Although SFD can cause mortality and population declines of native species, infected snakes do not always present clinical signs of disease. Novel Oo variants have been identified in North America and Europe (Franlinos et al., 2017; Ladner et al., 2022), and their spread probably has been facilitated by the global snake trade (Hierink et al., 2020; Marshall et al., 2020). Protocols to prevent the accidental release of dangerous pathogen variants into areas where endemic species will be most at risk must be implemented with urgency.

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