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Original Contribution

Amphibian Pathogens in Southeast Asian Frog Trade

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Abstract: Amphibian trade is known to facilitate the geographic spread of pathogens. Here we assess the health of amphibians traded in Southeast Asia for food or as pets, focusing on *Batrachochytrium dendrobatidis* (*Bd*), ranavirus and general clinical condition. Samples were collected from 2,389 individual animals at 51 sites in Lao PDR, Cambodia, Vietnam and Singapore for *Bd* screening, and 74 animals in Cambodia and Vietnam for ranavirus screening. *Bd* was found in one frog ($n = 347$) in Cambodia and 13 in Singapore ($n = 419$). No *Bd* was found in Lao PDR ($n = 1,126$) or Vietnam ($n = 497$), and no ranavirus was found in Cambodia ($n = 70$) or Vietnam ($n = 4$). Mild to severe dermatological lesions were observed in all East Asian bullfrogs *Hoplobatrachus rugolosus* ($n = 497$) sampled in farms in Vietnam. Histologic lesions consistent with sepsis were found within the lesions of three frogs and bacterial sepsis in two ($n = 4$); one had Gram-negative bacilli and one had acid-fast organisms consistent with *mycobacterium* sp. These results confirm that *Bd* is currently rare in amphibian trade in Southeast Asia. The presence of *Mycobacterium*-associated disease in farmed *H. rugolosus* is a cause for concern, as it may have public health implications and indicates the need for improved biosecurity in amphibian farming and trade.

Keywords: Amphibian trade, *Batrachochytrium dendrobatidis*, Chytrid, Ranavirus, Pathogens, Mycobacterium, Southeast Asia, Laos, Cambodia, Vietnam, Singapore

Introduction

Amphibians are the most threatened group of vertebrates, with over 40% of species in decline and one-third classified as threatened by the IUCN (Stuart et al. 2004). Factors driving population declines include overharvesting, competition with invasive species, habitat alteration, environmental contaminants, climate change, and infectious disease (Collins 2010). The situation appears to have deteriorated in the last three decades, with the majority of "rapidly declining" species experiencing so-called "enigmatic declines," in which populations dwindle in spite of adequate habitat, with drought and disease implicated in many cases (Pounds et al. 2006; Collins 2010).

Commercial trade in amphibians is a vast enterprise that operates on national and international scales (Warkentin et al. 2009; Gratwicke et al. 2010; Schloegel et al. 2010). At the local level, amphibians represent an

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important protein source for some rural communities in Asia (Neang 2010), but international trade in frog's legs for luxury markets in France, Belgium, and the United States is a multi-million dollar industry (Schloegel et al. 2010). There is also demand for amphibians as pets (Andreone et al. 2005), for use in laboratories (Weldon et al. 2007), as sources of traditional medicine (Rowley et al. 2010) and as bait for the fishing industry (Picco and Collins 2008). With such wholesale demand for amphibians, the resulting trade can exacerbate pressure on wild populations through unsustainable wild harvests, as a source of non-native introductions and as a disseminator of pathogens (Collins 2010).

The chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*) has been prominent as a cause of amphibian decline and extinction in the Neotropics, Nearctic, and Australia (Berger et al. 1998; Lips et al. 2006; Seimon et al. 2007; Skerratt et al. 2007; Fisher et al. 2009; Vredenburg et al. 2010; Catenazzi et al. 2011). As the causal agent of the disease chytridiomycosis, *Bd* interferes with normal skin function of susceptible amphibian species, leading to disruption of osmoregulation, subsequent electrolyte imbalance, and eventual death (Berger et al. 1998; Voyles et al. 2009). Among susceptible species, *Bd* appears to follow a density-independent pattern of spread, which heightens extinction risk in affected populations (Collins 2010). Low genetic diversity of *Bd* isolates obtained from disparate global locations appears to indicate that the fungus is a novel pathogen that has been disseminated rapidly over a wide geographic area (James et al. 2009). Commercial trade in amphibians has been demonstrated as a vehicle for facilitating this spread in several locations (Cunningham et al. 2005; Picco and Collins 2008; Une et al. 2008; Catenazzi et al. 2010), with *Bd* being identified in as many as 62% (306/493) of frogs imported through three ports in the United States (Schloegel et al. 2009).

Ranavirus (family Iridoviridae) is another important emerging pathogen of amphibians, and also affects reptiles and fish (Johnson et al. 2008; Jancovich et al. 2010). The virus has been implicated in mass mortalities of several wild amphibian populations in Europe and North America (Cunningham et al. 1996; Bollinger et al. 1999; Green et al. 2002). As with *Bd*, trade has been implicated in its geographic spread, with the bait trade in larval tiger salamanders (*Ambystoma tigrinum*) responsible for its wide dissemination in the western United States (Jancovich et al. 2005; Picco and Collins 2008). In addition, approximately 8.5% (50/588) of North American bullfrogs (*Rana catesbeiana*) imported into the United States tested positive for the virus (Schloegel et al. 2009).

Amphibian populations in Southeast Asia are comparatively understudied relative to those in North America and Australia, with almost a third of species known from Lao PDR, Vietnam, and Cambodia in 2005 having been described in the previous 8 years (Rowley et al. 2010). In Asia, *Bd* was first identified in a captive amphibian collection in Japan (Une et al. 2008), but has since been detected in wild populations in several Asian countries (Yang et al. 2009; Bai et al. 2010; Savage et al. 2011), and concurrent with declines in high altitude amphibians in Gede Pangrango National Park in West Java (Kusrini et al. 2008). However, not all Asian surveys have been successful in detecting *Bd*, with surveillance among traded animals in Hong Kong (Rowley et al. 2007) and a retrospective survey of archived museum specimens in Thailand (McLeod et al. 2008) failing to identify the fungus. A recent region-wide survey concluded that *Bd* is present at low prevalence, based on samples collected in 15 Asian countries (Swei et al. 2011). In Asia, ranavirus has been documented in mass die-offs of introduced North American bullfrogs and farmed populations of Chinese giant salamanders (*Andrias davidianus*) and have also been shown to be widespread among wild populations of Dybowski's frog (*Rana dybowskii*) in Heilongjiang, China (Geng et al. 2011, Une et al. 2009, Xu et al. 2010).

As of date no studies have evaluated the role that the extensive Southeast Asian trade in amphibians might play in the dissemination of amphibian pathogens. Herein we describe the first survey of amphibians in the pet or food trade in Indochina (Lao PDR, Cambodia and Vietnam) and Singapore to determine the relative risk these activities present for the spread of *Bd*, ranavirus and other amphibian pathogens in the region.

Materials and Methods

Study Sites

Amphibian sampling in Lao PDR and Cambodia focused on animals intended for sale in rural food markets between June 2010 and February 2011. In Lao PDR sampling took place in five provinces (Vientiane, Khammouane, Luang Nam Tha, Xieng Khouang, and Houapanh) and in Cambodia samples were collected in four provinces (Takeo, Kandal, Battambang, and Kampong Cham). Sellers reported that these animals had been captured locally in

rice paddies, although in Lao PDR some animals had been sourced at more distant markets (usually within fifty, but sometimes up to several hundred kilometers away) for resale at a higher price. Live animals were held collectively in sacks, bamboo cages or buckets prior to preparation for sale, with some in Lao PDR being tethered via twine or bamboo stakes threaded through hind legs or foot webs.

In Vietnam, samples were collected from 13 farms and two food markets during January 2011. Sample locations included Soc Son District, Hanoi Province, in Northern Vietnam, and Cu Chi District, District Nine, District Six, and Binh Chanh District of Ho Chi Minh City in Southern Vietnam. Samples were all collected from farms dedicated to growing stock that had been bred on site, although three farms supplemented their holdings with animals purchased from breeder farms elsewhere. Frogs were held in groups consisting of fewer than 10 to approximately 2,000 individuals in artificial tanks constructed from a variety of materials including cement, ceramic tile, or plastic tarpaulin. Others were housed in floating enclosures constructed within naturally occurring waterways and man-made ponds using knitted polyethylene fabric ("shade cloth") stretched over bamboo frames. Frogs were held in tanks with variable water depths (from <1 cm up to approximately 2 m where tanks were within natural ponds). Animals were provided shade from direct sunlight and occasionally offered substrate of rice straw or polystyrene sheets.

Both free-living and captive animals were sampled in Singapore. Free-living frogs included both native and introduced species, and were captured adjacent to nature reserves, in urban town parks, on state land, or on agricultural farmland. Captive animals included those sold for pets in local pet stores, aquariums, and farmed animals that were intended for human consumption. One of the farms-segregated animals that had been bred locally from those imported from Malaysia and Taiwan.

No compensation was offered to trappers, farmers, or traders in return for samples.

Sample Collection

Frogs in most market or farmed settings were held in "collective units" (CU) consisting of groups of individuals in close contact (e.g., within a bucket, a pen, a bag, etc.). These CUs may contain one or multiple species and represent anywhere from one to approximately 2,000 individuals. Skin swab samples were collected from individual frogs to determine the presence of *Bd* within a CU. A subsample of animals was selected from each CU for sample collection. The number of animals sampled per CU varied and was calculated to ensure the detection of at least one positive sample with 95% confidence using the tables given in Cannon and Roe (1982), assuming at least 10% of animals were positive for *Bd*. Each CU was given a unique identifier to insure that positive samples could be traced to an individual CU. For captive animals in Singapore the same protocols for sampling CUs were used, with only one or two individuals sampled from smaller CUs (i.e., buckets with <12 individuals) and no more than ten individuals sampled from any single seller. All wild frogs captured in Singapore were individually sampled, as the CU concept did not apply in a wild setting.

Skin swabs were collected for *Bd* detection using sterile, 0.635 mm diameter polyester swabs with an aluminum shaft (Fisherbrand). Each frog was swabbed 4–5 times each on the underside of the hind feet, thighs, abdomen, and forefeet using moderate pressure to insure collection of skin tissue without causing injury to the animal following standard procedures (Brem et al. 2007). One swab was used to sample up to five individuals of the same species within a single CU before being placed in a dry, 2.0 ml cryovial. Each tube was labeled with a unique sample identifier. Samples were stored in airtight plastic containers with desiccant at room temperature, or were refrigerated in areas where extreme high temperatures were expected. Handlers changed latex gloves between CUs and before handling different species within a CU to reduce potential contamination. Calipers were used to record linear snout to vent measurements from at least one individual in each sample, and these individual animals were photographed in dorsal, ventral, palmar and plantar orientation to aid in species identification when not possible in the field. A record was made of the date and location of sample collection, the species and number of individuals in the sample, as well as details of number of individuals and species in the CU.

Sampling for ranavirus was limited to dead frogs. Liver samples were collected and stored in 70% ethanol. Skin or visceral lesions were stored in duplicate in 10%-buffered formalin and 70% ethanol for histological examination. Tissue samples were held at room temperature until export. Sample collection from dead frogs was limited to cases where specimens had no economic value or where samples could be obtained opportunistically when animals were being butchered for sale.

Sample Analysis

Tissue samples and swabs were shipped to the Wildlife Conservation Society's Pathology and Molecular Diagnostics Laboratories, Bronx Zoo, New York for analysis. For *Bd* analysis, DNA from the swabs was extracted using PrepMan (Applied Biosystems, Foster City, CA) and extracts were diluted 1:10 in RNAse/DNAse-free water. The samples were then analyzed by real-time quantitative polymerase chain reaction (qPCR) amplification of the Internal Transcribed Spacer (ITS-1) and 5.8S rDNA region using established methods (Boyle et al. 2004). Taqman PCR assays were conducted using a Bio-Rad Mini-Opticon Real-Time PCR detection system. Twenty-microlitre reactions containing 10 µl of 2× Taqman Master Mix (Applied Biosystems), 900 nM of each primer (ITS-1 Chytr3 and 5.8S Chytr), 250 nM of Chytr MGB probe (Applied Biosystems) and 5 µl of diluted DNA were added to a 48-well plate. PCR amplification was run under the following thermal profile: 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Positive control material consisting of purified genomic *Bd* DNA (provided by Dr. Allan Pessier, San Diego Institute for Conservation Research, CA) was diluted to a range of concentrations to generate a standard curve for quantifying zoospore equivalents.

Samples found to be positive for *Bd* using qPCR were retested using conventional PCR using primers for the ITS/5.8S region as described by Annis et al. (2004). Positive bands obtained from these samples were purified using ExoSAP-IT (Affymetrix; Santa Clara, CA, USA) then submitted for DNA sequencing in both the forward and reverse direction (Genewiz, Inc. South Plainfield, NJ). Sequences were analyzed and trimmed using Geneious software (Geneious Pro 5.6.3, Biomatters LTD. Auckland NZ) and run through the GenBank database using BLASTn to determine the identity of the sequences (GenBank, National Center for Biotechnology Information).

For ranavirus analysis, DNA was purified from ethanol-fixed liver tissue using a Qiagen QIAamp DNA mini kit (Qiagen) and analyzed by qPCR for a conserved region of the major capsid protein (MCP) gene according to established methods (Pallister et al. 2007). Twenty-microlitre reactions containing 10 µl of 2× Taqman Master Mix, 900 nM of each primer (CON F and CON R), 250 nM of CON MGB probe, and 5 µl of DNA sample were prepared in a 48-well plate. PCR amplification was run using the same thermal profile as for *Bd* analysis. Positive control material consisting of purified genomic ranavirus DNA (provided by Dr. Jesse Brunner, SUNY College of Environmental Science and Forestry, NY) was diluted to a range of concentrations to generate a standard curve.

All samples from Lao PDR and Cambodia were initially run in singlicate with appropriate positive (known ranavirus or *Bd* standards), negative (no DNA template), and inhibition controls (unknown samples spiked with a known quantity of ranavirus or *Bd* DNA). Samples collected in Vietnam and Singapore were pooled in groups of four, and individual samples in positive pools were retested to determine individual infection status. Suspected positive samples were then retested in duplicate reactions, with positive and negative controls included. A weak positive for *Bd* was defined as a Ct value between 0.01 and 1 zoospore equivalents per swab in the PCR reaction. A weak positive for ranavirus was represented by Ct value greater than 37, and samples exceeding 45 were considered negative.

Pathogen prevalence was expressed as the proportion of animals tested that were found to be positive, and was expressed using confidence limits calculated using the adjusted Wald method (Agresti and Coull 1998).

All necropsy tissues fixed in 10% neutral-buffered formalin were processed routinely, embedded in paraffin, cut at 5 m and stained with hematoxylin, and eosin. Special staining for pathogens (Gram's, Ziehl–Neelsen acid fast, and Fites acid fast) was performed, as indicated, according to standard protocols. A certified Veterinary Pathologist reviewed all cases.

Results

A total of 1,225 skin swab samples representing 2,389 individual animals were collected across the four countries (Table 1). In Indochina (Vietnam, Lao PDR and Cambodia) samples were collected from CUs containing 8,933 animals (4,111 in Vietnam, 3,770 in Lao PDR and 1,052 in Cambodia). Samples were collected at 51 locations, including 15 farms, 19 food markets, 7 pet stores and 10 sites where free-ranging animals were targeted (Figure 1). All swabs collected in Lao PDR and Vietnam were negative for *Bd*. In Cambodia one sample, from a *H. rugolosus* intended for food consumption, tested positive for *Bd*. In Singapore, 13 samples were found to be weak positives for *Bd* and included frogs sampled in the wild and in pet stores. Positive wild frogs were found at two sites and included one common tree frog (*Polypedates leucomystax*) at one site, and a Malayan giant frog (*Limnonectes blythii*) at the

Table 1. Summary of Samples Collected and Test Results by Location

Country	Site name	Type	Number of animals sampled for *Bd*	Number of animals positive for *Bd*	Number of animals sampled for Ranavirus	Number of animals positive for Ranavirus
Cambodia	Boeung Sdao	Food market	19	0	0	0
	Chey Chum Nea	Food market	46	0	0	0
	Chhour Teal	Food market	71	1	0	0
	Ow Phea Sang	Food market	130	0	0	0
	Ow Phea Sang 2	Food market	81	0	70	0
Lao PDR	Kasi Market	Food market	93	0	0	0
	Khan Mark Market	Food market	56	0	0	0
	Luang Nam Tha Market	Food market	159	0	0	0
	Muang Sing Market	Food market	34	0	0	0
	Phahom	Food market	49	0	0	0
	Sam Neua Market	Food market	53	0	0	0
	Vang Vieng Market	Food market	215	0	0	0
	Viengthong Market	Food market	65	0	0	0
	Xieng Khouang Market	Food market	200	0	0	0
	Gnommalath and Nakhai Markets	Food market	202	0	0	0
Vietnam	Farm 1, Yên Tang, Bac Phu, Soc Son, Hanoi	Farm	83	0	1	0
	Farm 2, Yên Tang, Bac Phu, Soc Son, Hanoi	Farm	31	0	0	0
	Farm 3, Yên Tang, Bac Phu, Soc Son, Hanoi	Farm	37	0	0	0
	Farm 4, Yên Tang, Bac Phu, Soc Son, Hanoi	Farm	32	0	0	0
	Farm 5, Xom Moi, Bac Vong, Soc Son, Hanoi	Farm	20	0	1	0
	Farm 6, Bac Vong, Bac Phu, Soc Son, Hanoi	Farm	26	0	0	0
	Farm 7, Bac Vong, Bac Phu, Soc Son, Hanoi	Farm	26	0	0	0
	Farm 8, Ap An Thach, Trung An, Cu Chi, HCMC	Farm	94	0	0	0
	Farm 9, Ap An Thach, Phuoc Hiep, Cu Chi, HCMC	Farm	27	0	2	0
	Farm 10, Phuoc Hiep, Cu Chi, HCMC	Farm	26	0	0	0
	Farm 11, Phuoc Hiep, Cu Chi, HCMC	Farm	26	0	0	0
	Farm 12, Cay Cham, Phuoc Hiep, Cu Chi, HCMC	Farm	28	0	0	0
	Farm 13, Long Thanh My, District 9, HCMC	Farm	26	0	0	0
	Phu Lam Market, District 6, HCMC	Food market	9	0	0	0
	Vinh Loc Market, Binh Chanh, HCMC	Food market	6	0	0	0

Table 1. continued

Country	Site name	Type	Number of animals sampled for *Bd*	Number of animals positive for *Bd*	Number of animals sampled for Ranavirus	Number of animals positive for Ranavirus
Singapore	Farm 1	Farm	48	0	0	0
	Farm 2	Farm	20	0	0	0
	Chinatown Wet Market	Food market	9	0	0	0
	Seng Siong Market	Food market	2	0	0	0
	Pet Store 1	Pet store	20	4	0	0
	Pet Store 2	Pet store	20	1	0	0
	Pet Store 3	Pet store	10	2	0	0
	Pet Store 4	Pet store	10	0	0	0
	Pet Store 5	Pet store	20	3	0	0
	Pet Store 6	Pet store	10	1	0	0
	Pet Store 7	Pet store	10	0	0	0
	Bukit Batok Town Park	Wild	21	0	0	0
	Dairy Farm Road, adjacent to Nature Reserve	Wild	8	0	0	0
	Hort Park	Wild	21	0	0	0
	Kent Ridge Park adjacent to Nature Reserve	Wild	5	0	0	0
	Lim Chu Kang Orchid Farm adjacent to Nature Reserve	Wild	23	0	0	0
	Lorong Halus	Wild	19	0	0	0
	Lower Pierce Road Trail adjacent to Nature Reserve	Wild	23	0	0	0
	Upper Peirce Road Pond adjacent to Nature Reserve	Wild	20	1	0	0
	West Coast Park	Wild	45	0	0	0
	Venus Drive adjacent to Nature Reserve	Wild	55	1	0	0
		Total	2,389	14	74	0

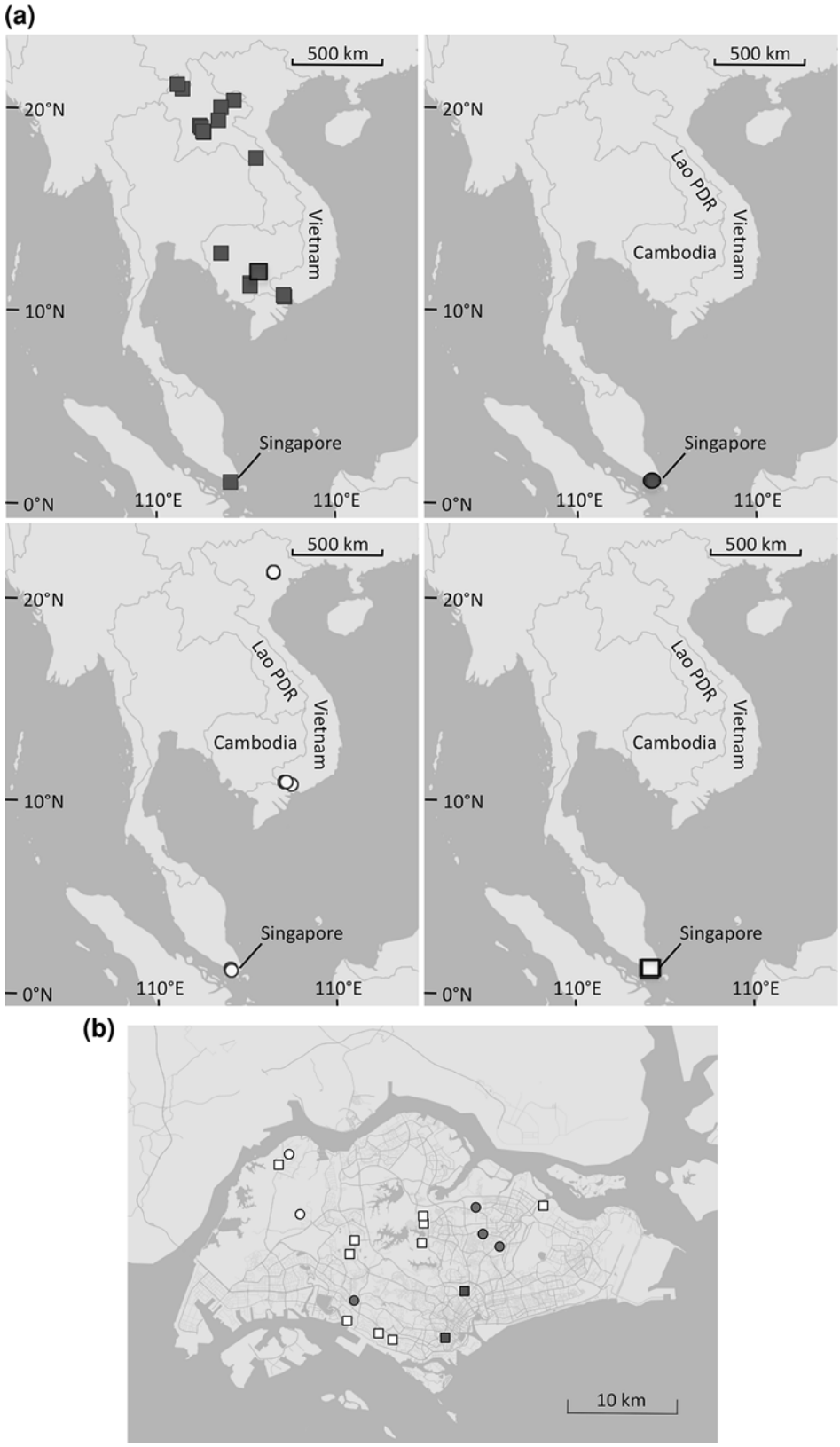

Figure 1. **a** Southeast Asian sampling sites at food markets (*shaded squares*), pet shops (*shaded circles*), farms (*white circles*), and in the wild (*white squares*). **b** Sampling sites in Singapore, including food markets (*shaded squares*), pet shops (*shaded circles*), farms (*white circles*) and in the wild (*white squares*).

Table 2. Summary of Samples Collected and Test Results in each Country by Species

Country	Family	Species name	Common name	Number of animals sampled for *Bd*	Number of animals positive for *Bd*	Number of animals sampled for Ranavirus	Number of animals positive for Ranavirus
Cambodia	Bufonidae	*Duttaphrynus melanostictus*	Black-spined toad	6	0	0	0
Cambodia	Ranidae	*Fejervarya limnocharis*	Paddy frog	86	0	0	0
Cambodia	Ranidae	*Hoplobatrachus rugulosus*	East Asian bullfrog	255	1	70	0
Lao PDR	Ranidae	*Fejervarya limnocharis*	Common pond frog	659	0	0	0
Lao PDR	Ranidae	*Hoplobatrachus rugulosus*	East Asian bullfrog	104	0	0	0
Lao PDR	Ranidae	*Hylarana cubitalis*	None	3	0	0	0
Lao PDR	Ranidae	*Hylarana nigrovittata*	None	22	0	0	0
Lao PDR	Ranidae	*Limnonectes kuhlii*	Large-headed frog	223	0	0	0
Lao PDR	Ranidae	*Limonectes sp.*	None	9	0	0	0
Lao PDR	Ranidae	*Odorrana chloronota*	None	28	0	0	0
Lao PDR	Ranidae	*Odorrana sp.*	None	43	0	0	0
Lao PDR	Ranidae	*Pelophylax lateralis*	Kokarit frog	17	0	0	0
Lao PDR	Ranidae	*Polypedates leucomystax*	White-lipped tree frog	18	0	0	0
Vietnam	Ranidae	*Hoplobatrachus rugolosus*	East Asian bullfrog	497	0	4	0
Singapore	Bufonidae	*Duttaphrynus melanostictus*	Black-spined toad	72	0	0	0
Singapore	Megophryidae	*Leptobrachium nigrops*	Black eyed litter frog	3	0	0	0
Singapore	Microhylidae	*Kaloula pulchra*	Banded bullfrog	40	0	0	0
Singapore	Microhylidae	*Microhyla butleri*	Painted chorus frog	17	0	0	0
Singapore	Microhylidae	*Microhyla heymonsi*	Dark-sided chorus frog	4	0	0	0
Singapore	Pipidae	*Hymenochirus boettgeri*	African dwarf frog	10	0	0	0
Singapore	Pipidae	*Xenopus laevis*	African clawed frog (albino)	10	0	0	0
Singapore	Ranidae	*Fejervarya cancrivora*	Crab-eating frog	43	3	0	0
Singapore	Ranidae	*Fejervarya limnocharis*	Field frog	21	0	0	0
Singapore	Ranidae	*Limnonectes blythii*	Malayan giant frog	25	1	0	0
Singapore	Ranidae	*Limnonectes malesianus*	Malesian frog	1	0	0	0
Singapore	Ranidae	*Polypedates leucomystax*	Common tree frog	48	1	0	0
Singapore	Ranidae	*Pulchrana laterimaculate*	Masked rough-sided frog	1	0	0	0
Singapore	Ranidae	*Rana catesbeiana*	American bullfrog	124	8	0	0
				2,389	14	74	0

other (both within the family Ranidae). Eleven positive animals were found in four of the seven pet stores sampled, and included eight North American bullfrogs and three crab-eating frogs (*Fejervarya cancrivora*).

Of the 14 weak positive samples identified, three were found to have positive bands when they were retested for *Bd* by conventional PCR using the methods of Annis et al. (2004). Sequences obtained from these bands included 295 bp from a *F. cancrivora*, in Singapore (GenBank Accession No. JX993750), 283 bp from a *R. catesbeiana* in Singapore (GenBank Accession No. JX993751), and 292 bp from a pooled sample representing five juvenile *H. rugolosus* from Cambodia (GenBank Accession No. JX993752). DNA sequences were analyzed using BLASTn (National Center for Biotechnology Information, GenBank) and all three were found to match with >99% sequence identity to known *Bd* haplotypes of a South African strain CW34 from *Xenopus laevis*. We found that the sequence from *F. cancrivora* (JX993750) was 99.7% identical to CW34 clone F haplotype (GenBank Accession No. JQ582927). While that from the *R. catesbeiana* (JX993751) was 99.6% identical to CW34 clone D haplotype (GenBank Accession No.

JQ582906), and the *H. rugolosus* (JX993752) was 99.3% identical to CW34 clones A, B, S, O, and N haplotypes (GenBank Accession Nos. JQ582924, JQ582926, JQ582920, JQ582928, JQ582929). Using a distance matrix analysis, we found that the three sequences were between 94.6 and 96.7% identical to each other (Table 2).

Samples for ranavirus analysis were collected in Cambodia and Vietnam. All 70 liver samples collected in Cambodia and all necropsy tissues collected from four frogs in Vietnam tested negative by qPCR and histopathology.

Dermatological lesions were observed on all 497 of the frogs (100%) sampled from the 13 frog farms in Vietnam. Clinical signs ranged from mild to severe, with the vast majority of frogs showing moderate to severe lesions (Figure 2a). Lesions included erythema of the ventral aspect of the abdomen, inguinal region and the medial aspect of the limbs; swelling of the distal limbs; erosive to ulcerative lesions of variable size and depth, concentrated on the limbs, the lips and the digits; small (<1 mm diameter), round clear vesicles visible in the inguinal region and the hindlimbs; and missing or deformed digits.

Owing to the severity of dermatologic lesions, necropsy was performed at the request of farmers at one farm on two animals immediately following euthanasia (via pithing). The body condition of one animal was normal, with mild to moderate erythema visible on all ventral surfaces and multiple vesicles and full thickness ulcerative lesions on all limbs. The liver appeared pale, with focal areas of congestion and hemorrhage visible on the surface and within the parenchyma. The body condition of the second animal was very poor, with multiple, irregular, full thickness ulcerative lesions on all four limbs, and both palmar and plantar surfaces. The tips of all digits were ulcerated, and digits appeared misshapen and shortened. Intra-abdominal fat was pale yellow in color, the gastrointestinal tract-appeared inflamed, and the surface of the liver was rough.

Tissues collected from the two necropsied animals (including heart, lung, liver, spleen, kidneys, stomach, intestines and skin, including digital lesions) and limited tissues from two further cases at a second farm in Vietnam (including liver from both animals, as well as pancreas, tongue, gonad, gall bladder, esophagus, and eye from one animal) were exported for histopathologic examination and ancillary diagnostics at the Bronx Zoo, New York. Three of the four animals showed evidence of severe granulomatous and granulocytic hepatitis, a finding consistent with bacterial sepsis. Additional organs from one of these animals were examined and showed similar inflammation in the spleen and kidneys. In the two animals for which samples of the digital lesions were submitted, there was evidence of mild erosive to severe ulcerative inflammation of the skin (Figure 2b). In one animal there was extensive spread into the local subcutaneous tissues. Special stains for bacterial organisms (Gram's and Ziehl–Neelsen acid fast) were performed on the liver in the three animals with evidence of sepsis and the digit of the animal with mild pododermatitis. One of the three animals had Gram-negative bacilli within the liver lesions (Figure 2c). A second had acid-fast organisms within the liver consistent with *Mycobacterium*. There were no bacteria evident in the digit sample from the animal with severe pododermatitis and sepsis lesions. In the animals that were negative for acid-fast organisms on Ziehl–Neelsen staining, an additional acid-fast stain for atypical mycobacteria (Fite-faraco acid fast) was performed and also gave negative results. The formalin-fixed paraffin embedded livers of three animals were submitted for mycobacterial PCR in an attempt to further characterize the mycobacterial species seen in the one animal, and to identify *Mycobacterium* in the two remaining animals. The animal with mild digital lesions was not submitted for further testing due to the limited tissue available in that block. PCR was negative in all of the animals submitted for analysis; however, this assay is not as sensitive as culture for detection of mycobacterial species. The negative results, particularly in the case where acid-fast staining demonstrated organisms, may indicate that the organisms were not present in sufficient numbers for amplification.

Histologic findings in these four cases of clinical dermatitis in amphibians from two farms in Vietnam indicate that in three of the four animals morbidity and mortality was the result of bacterial sepsis, likely due to the clinically described ulcerative skin lesions.

Discussion

The findings of this study support the conclusions of Swei et al. (2011) who reported that *Bd* infections are rare or absent across much of East Asia. In contrast to that study, our sampling approach focused almost exclusively on amphibians in trade settings, where animals were being reared or sold for food or as pets, as this has been proposed as a key mechanism in the spatial dissemination of *Bd* (Cunningham et al. 2005; Picco and Collins 2008; Une et al. 2008). In Indochina *H. rugulosus* was the most frequently encountered species in farms and markets, with only one

animal testing positive for *Bd* among 856 individuals sampled. This low prevalence mirrors findings in Hong Kong, where samples collected from 129 individuals of this species imported from Thailand in 2005 and 2006 also tested negative (Rowley et al. 2007). In our study, *Bd* infections were most prevalent in *R. catesbeiana* (2.7%, n = 124, 95% adjusted Wald confidence limits of 1.8–4.0%). This species is able to tolerate *Bd* infections and has been implicated in its spread through trade (Weldon et al. 2004; Fisher and Garner 2007), with infections in as many as 62% (n = 493) of *R. catesbeiana* imported into the United States from Asian sources (Schloegel et al. 2009). This disparity in prevalence may relate to differences in handling conditions for frogs intended for food versus those being sold as pets. However, the detection of *Bd* in four of the seven pet stores surveyed is a cause for concern, as it suggests that amphibian imports, particularly of *R. catesbeiana*, may act as a portal for introducing *Bd* into Southeast Asia.

The detection of *Bd* among amphibians in trade and in the wild in Singapore is an important finding and represents the first report of *Bd* in the territory (Global Bd-Mapping Project 2011). Moreover, it appears highly likely that trade is responsible for its dissemination, as the only cases of *Bd* found in native habitats were those known to be sites for Buddhist practitioners of animal releases. However, it is important to emphasize that all infected individuals, with the exception of the Cambodia sample which was slightly higher, were determined to be weakly positive by qPCR (a zoospore equivalents of 0.01 to 1.00 per swab). It is very unlikely that such low intensity infections are associated with disease, although they contribute to pathogen persistence and as a source of infection into naïve species. Vredenburg et al. (2010) only observed population declines in yellow-legged frogs (*R. muscosa* and *R. sierrae*) above a population average threshold of 10,000 zoospore equivalents per swab. The detection of *Bd* in 2/240 wild amphibians in Singapore is similar to reports from neighboring areas in Peninsular Malaysia, where previous studies found occurrences of 2/111 (Swei et al. 2011) and 10/127 (Savage et al. 2011). Furthermore, considering the detection of high infection loads in two animals in Malaysia by Savage et al. (2011), qPCR should be combined with histological and population surveys wherever possible to determine whether clinical chytridiomycosis is present in some of these species.

To determine whether these cases represent infections of novel exotic or endemic Asian strains or ones associated

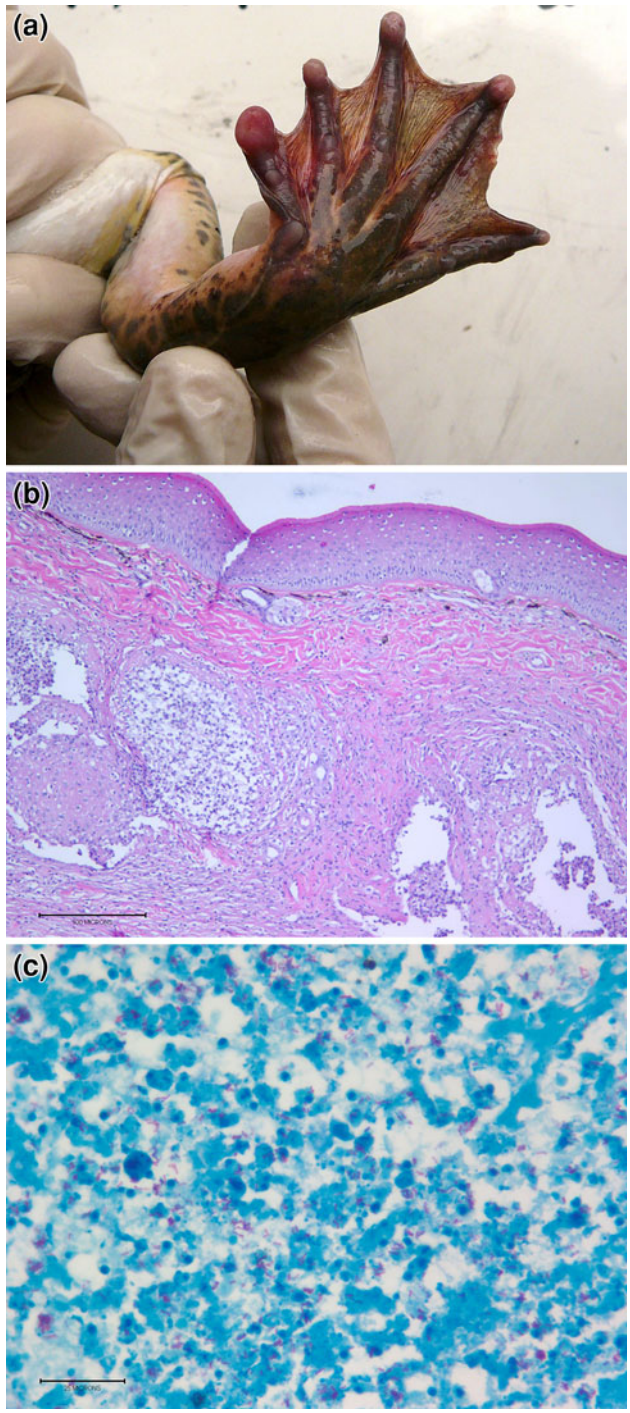

Figure 2. Lesions of farmed *Hoplobatrachus rugolosus* in Vietnam, illustrating (**a**) gross pathology, including ulcerative and vesicular cutaneous lesions, and inflammation on the legs. **b** Histological images of epidermis from the plantar surface of the digit of an affected frog, including multiple areas of necrosis, granulocytic, and histiocytic inflammation throughout the dermis (HE, 10), and **c** acid fast stain of the liver from an affected frog, including a myriad intra and extracellular acid fast organisms throughout the areas of necrosis (Ziehl–Neelsen (ZN), 63).

with the bullfrog trade we analyzed DNA sequences spanning part of the ITS gene. Analysis of the sequences obtained from frogs in Cambodia and Singapore confirms that the weak positives detected through qPCR corresponded to the presence of *Bd* in the samples, and were not the result of an artifact of non-specific amplification. Furthermore, all three sequences were closely related to the *Bd* strain CW34 isolated from *Xenopus laevis* in South Africa, a strain that falls within the phylogenetic lineage *Bd*-GPL-2 (Global panzootic lineage) which is a globally dispersed clade (Schloegel et al. 2012).

The ubiquity of skin lesions observed among farmed *H. rugulosus* in Vietnam is a cause for concern, both in terms of animal welfare and potential production losses, but also for wider environmental impacts. The similar findings in the cases examined suggest a common environmental (water quality) or husbandry issue that either led to skin irritation and secondary bacterial infection, or resulted in immune suppression causing susceptibility to opportunistic infection. Furthermore, these infections may be of public health significance, as some Gram-negative organisms and *Mycobacterium* can have zoonotic implications. Although it was not possible to definitively identify the mycobacteria that were associated with these cases, nor determine their role as a primary or secondary pathogen, there is clearly a risk that frog farms could act as a source of infection. At all farms surveyed, untreated wastewater was disposed into natural watercourses, representing a potential avenue for infecting neighboring farms, wild amphibians, and other aquatic species (e.g., chelonians and fish). In some cases, frogs were reared in ponds along with Chinese softshell turtle (*Pelodiscus sinensis*) and fish including tilapia (*Tilapia* sp.), koi (*Cyprinus carpio*) and grass carp (*C. idella*), thus increasing the potential for inter-species contamination and amplification of pathogens. There is strong evidence to support interspecies transmission of ranaviruses between amphibians, reptiles, and fish (Jancovich et al. 2010), and although we failed to detect ranavirus, our sample sizes were not sufficient to conclude absence of the pathogen. Ranaviruses closely related to amphibian viruses have been associated with mortality in chelonians (Johnson et al. 2008). With all but one of the 25 species of freshwater chelonian in Vietnam classified as threatened with extinction by the IUCN (2011), there is a strong imperative for future studies to examine the potential of frog and chelonian farms as a source of pathogens that might impact remaining wild turtle and amphibian populations.

Recognizing the importance of international trade in the dissemination of amphibian pathogens has prompted the World Organization for Animal Health (OIE) to list *Bd* and ranavirus as notifiable under the Aquatic Animal Health Code. This listing requires all 174 member countries that trade in amphibians to conduct surveillance for these pathogens, report confirmed cases to the OIE and implement OIE recommendations to limit their spread (Schloegel et al. 2010). Measures to reduce spread of *Bd* via live frogs in the pet and food trade include quarantine and treatment of infected individuals with pharmaceuticals (e.g., itraconazole), or potential use of temperature regimens to reduce zoospore load. A number of pre-export processing options are available to eliminate *Bd* burdens in deceased amphibians being shipped for food, including skinning and preservation methods such as canning or drying (Schloegel et al. 2010). Unfortunately, at present there are no treatment options for controlling ranavirus infections, so importing countries may be advised to obtain animals from disease-free sources.

Conclusion

This study showed that at the time of sampling, *Bd* was rare or absent in several sectors of the amphibian trade in Southeast Asia, but detection in animals sold for pets in Singapore represents a potential route of introduction to the region. Measures to treat animals being imported for this purpose warrant serious consideration. No evidence of ranavirus infection was detected, but sample sizes were not sufficient to draw conclusions on the occurrence of this pathogen. Ubiquitous skin lesions among frogs being reared in commercial facilities in Vietnam and putatively most commonly resulting from bacterial infections represent a cause for concern, particularly considering the low level of biosecurity observed at these farms and the potential for contaminating water systems and neighboring farms. Findings of mild *Bd* infections of low prevalence among wild amphibians in Singapore are a cause for concern, particularly in view of similar findings in neighboring countries. There is an urgent need to conduct wider surveys of wild amphibians in Southeast Asia to determine the extent and severity of *Bd* and other infectious diseases among a range of species, and whether and how these change over time. Studies should focus on differentiating *Bd* strains that may be endemic to the region from exotic strains that may be introduced through routes including international trade.

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