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Assessment of *Batrachochytrium dendrobatidis* and Ranavirus Among Wild Amphibians from Four Philippine Islands

SIERRA N. SMITH*
Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, 2401 Chautauqua Ave., Norman, Oklahoma 73072, USA; Department of Biology, University of Oklahoma, 730 Van Vleet Oval, Room 314, Norman, Oklahoma 73019, USA
JESSA L. WATTERS
Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, 2401 Chautauqua Ave., Norman, Oklahoma 73072, USA
ELYSE D. ELLSWORTH
Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, 2401 Chautauqua Ave., Norman, Oklahoma 73072, USA; Department of Biology, University of Oklahoma, 730 Van Vleet Oval, Room 314, Norman, Oklahoma 73019, USA
DREW R. DAVIS
School of Earth, Environmental, and Marine Sciences, University of Texas Rio Grande Valley, 100 Marine Lab Dr., South Padre Island, Texas 78597, USA
CAMERON D. SILER
Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, 2401 Chautauqua Ave., Norman, Oklahoma 73072, USA; Department of Biology, University of Oklahoma, 730 Van Vleet Oval, Room 314, Norman, Oklahoma 73019, USA

**Corresponding author, e-mail: sierrasmith@ou.edu*

Amphibian populations continue to face rapid declines worldwide due to a number of factors, including emerging infectious diseases such as chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), and ranavirosis, caused by ranaviruses (RV; Daszak et al. 1999; Berger et al. 2016; Rollins-Smith 2016). The presence of RV in Asia has been documented by several studies, with an infection prevalence of 5.7% in adults and 42.5% in larval *Rana dybowskii* from China (Xu et al. 2010; Duffus et al. 2015) and a mass mortality of *Rana catesbeiana* tadpoles in Japan (Une et al. 2009). Interestingly, studies conducted on the prevalence of *Bd* among wild amphibian populations across several mainland east Asian countries—China, Japan, and South Korea—have reported low numbers of individuals testing positive for the *Bd* pathogen (Goka et al. 2009; Swei et al. 2011; Bai et al. 2012; Bataille et al. 2013). One hypothesis put forward recently to explain low prevalence of *Bd* in east Asia points to the region as the source of the *Bd* strain responsible for the worldwide panzootic, conferring amphibians in parts of the Old-World tropics an evolutionary advantage (O'Hanlon et al. 2018).

The most comprehensive study on amphibian chytridiomycosis in southeast Asia to date was conducted by Swei et al. (2011), who screened over 3,000 individuals and found only 2.35% were positive for *Bd*. Those individuals observed to be *Bd*-positive were sampled from Indonesia (0.25% infection rate), Laos (0.73%), Malaysia (0.90%), and the Philippines (8.01%). Although Philippine samples showed a higher infection rate in the study, the infected individuals came from a single, highly disturbed mountain on Luzon Island (Swei et al. 2011). Given the unique biogeographic history of the Philippine archipelago, and its importance to global amphibian diversity as a megadiverse nation and biodiversity hotspot (Myers et al. 2000), additional studies are needed across a broader region of the country to better evaluate the prevalence of infectious amphibian diseases.

TABLE 1. Comparison of *Bd* and RV screening results by site and then by species, including numbers of *Bd*+ and RV+ individuals over sample size (N), and mean infection load. Anuran families shown for reference and invasive species are indicated (*).

Site	Family	Species	Number *Bd*+/N	Number RV+/N	Mean infection load	Infection load Std. Dev.
Negros Occidental;	Bufonidae	*Rhinella marina**	0/12	0/12	N/A	N/A
9.6132°N, 122.4786°E	Dicroglossidae	*Limnonectes visayanus*	0/3	0/3	N/A	N/A
(Site 1)		*Occidozyga laevis*	0/6	0/6	N/A	N/A
Negros Oriental;	Bufonidae	*Rhinella marina**	0/1	0/1	N/A	N/A
9.2875°N, 123.2090°E	Dicroglossidae	*Limnonectes visayanus*	1/24	0/24	147	N/A
(Site 2)		*Occidozyga laevis*	0/7	0/7	N/A	N/A
	Ranidae	*Sanguirana everetti*	0/7	0/7	N/A	N/A
Gattaran;	Bufonidae	*Rhinella marina**	0/7	0/7	N/A	N/A
17.9701°N, 121.6544°E	Ceratobatrachidae	*Platymantis* cf. *mimulus*	0/5	0/5	N/A	N/A
(Site 3)		*Platymantis* sp.	0/4	0/4	N/A	N/A
	Dicroglossidae	*Limnonectes macrocephalus*	0/20	0/20	N/A	N/A
		Occidozyga laevis	0/1	0/1	N/A	N/A
	Microhylidae	*Kaloula picta*	0/1	0/1	N/A	N/A
		Kaloula pulchra	0/6	0/6	N/A	N/A
	Ranidae	*Pulchrana similis*	0/5	0/5	N/A	N/A
	Rhacophoridae	*Polypedates leucomystax*	0/8	0/8	N/A	N/A
Quezon;	Bufonidae	*Rhinella marina**	0/1	0/1	N/A	N/A
16.4623°N, 121.2198°E	Ceratobatrachidae	*Platymantis cornutus*	0/1	0/1	N/A	N/A
(Site 4)		*Platymantis corrugatus*	0/1	0/1	N/A	N/A
		Platymantis pygmaeus	0/4	0/4	N/A	N/A
		Platymantis sp. 1	0/21	0/21	N/A	N/A
		Platymantis sp. 2	0/1	0/1	N/A	N/A
		Platymantis sp. 3	2/20	0/20	100	28
	Dicroglossidae	*Limnonectes macrocephalus*	1/21	0/21	221	N/A
		Limnonectes woodworthi	0/1	0/1	N/A	N/A
		Occidozyga laevis	0/27	0/27	N/A	N/A
	Microhylidae	*Kaloula kalingensis*	0/7	0/7	N/A	N/A
		Kaloula rigida	0/1	0/1	N/A	N/A
	Ranidae	*Pulchrana similis*	0/2	0/2	N/A	N/A
		Sanguirana aurantipunctata	0/7	0/7	N/A	N/A
	Rhacophoridae	*Polypedates leucomystax*	0/8	0/8	N/A	N/A
Calayan;	Bufonidae	*Rhinella marina**	0/7	0/7	N/A	N/A
19.2748°N, 121.4470°E	Dicroglossidae	*Hoplobatrachus rugulosus*	0/6	0/6	N/A	N/A
(Site 5)	Microhylidae	*Kaloula picta*	0/28	0/28	N/A	N/A
Camiguin Norte;	Bufonidae	*Rhinella marina**	0/1	0/1	N/A	N/A
18.9178°N, 121.8925°E	Ceratobatrachidae	*Platymantis* sp.	0/14	0/14	N/A	N/A
(Site 6)	Dicroglossidae	*Limnonectes woodworthi*	0/7	0/7	N/A	N/A
	Rhacophoridae	*Polypedates leucomystax*	0/1	0/1	N/A	N/A
Albay;	Bufonidae	*Rhinella marina**	0/1	no data	N/A	N/A
13.3056°N,123.6890°E	Ceratobatrachidae	*Platymantis corrugatus*	1/1	no data	39	N/A
(Site 7)		*Platymantis dorsalis*	0/34	no data	N/A	N/A
	Dicroglossidae	*Limnonectes macrocephalus*	0/2	no data	N/A	N/A
		Limnonectes woodworthi	0/23	no data	N/A	N/A
	Ranidae	*Occidozyga laevis*	2/36	no data	42	10
		Pulchrana similis	0/3	no data	N/A	N/A
		Sanguirana luzonensis	0/5	no data	N/A	N/A
	Rhacophoridae	*Polypedates leucomystax*	0/3	no data	N/A	N/A

Table 1. Continued.

Site	Family	Species	Number *Bd*+/N	Number RV+/N	Mean infection load	Infection load Std. Dev.
Aurora;	Bufonidae	*Rhinella marina**	0/4	no data	N/A	N/A
15.7294°N,121.4038°E	Ceratobatrachidae	*Platymantis corrugatus*	0/1	no data	N/A	N/A
(Site 8)		*Platymantis luzonensis*	0/3	no data	N/A	N/A
		Platymantis polillensis	0/10	no data	N/A	N/A
		Platymantis sp. 1	0/11	no data	N/A	N/A
		Platymantis sp. 2	0/16	no data	N/A	N/A
	Dicroglossidae	*Hoplobatrachus rugulosus**	0/2	no data	N/A	N/A
		Limnonectes macrocephalus	1/23	no data	240	N/A
		Limnonectes woodworthi	0/10	no data	N/A	N/A
	Microhylidae	*Kaloula kalingensis*	0/4	no data	N/A	N/A
	Ranidae	*Occidozyga laevis*	0/9	no data	N/A	N/A
		Pulchrana similis	3/21	no data	57	23
		Sanguirana cf. *luzonensis*	0/1	no data	N/A	N/A
		Sanguirana luzonensis	0/16	no data	N/A	N/A
		Sanguirana tipanan	0/1	no data	N/A	N/A
	Rhacophoridae	*Polypedates leucomystax*	0/2	no data	N/A	N/A

Here, we present novel data on the presence and distribution of both *Bd* and RV pathogens among wild amphibian populations on the islands of Calayan, Camiguin Norte, Luzon, and Negros in the central and northern Philippines.

Fieldwork was conducted in February–March 2016 (Luzon), June 2016 (Negros), May–June 2017 (Luzon), and May–June 2018 (Calayan and Camiguin Norte; Fig. 1). Together, these expeditions surveyed populations at eight distinct sites spanning two provinces on Negros Island (Fig. 1, Sites 1, 2; Table 1), four provinces on Luzon Island (Fig. 1, Sites 3, 4, 7, and 8; Table 1), and two isolated deep-ocean islands of the Babuyan Island Group (Calayan Island: Fig. 1, Site 5; Camiguin Norte Island: Fig. 1, Site 6; Table 1). Each site was sampled for five to seven days. Amphibians were captured by hand and then kept in individual collection bags until they were swabbed and euthanized within 24 h of capture (Watters et al. 2018). Only adult frogs were collected; no tadpoles were sampled. We did not encounter or sample any individuals that were dead or showed clinical symptoms of *Bd* or RV infection. We swabbed amphibian skin on the ventral, lateral, and dorsal portions of the individual, on the hind limbs (five swipes per regions), and on the webbing between the hind limb toes (Watters et al. 2018). Animals were euthanized by submersion in a chlorobutanol solution prior to the collection of liver tissue for RV screening (St-Amour and Lesbarrères 2007; Gray et al. 2012). Individuals collected at Sites 7 and 8 were sampled for *Bd* only, no RV samples were collected from these two sites (Table 1). Throughout the swabbing and tissue collection, sterile techniques were employed to avoid cross-contamination (Gray et al. 2017). Each swab was stored dry in a 1.5-mL microcentrifuge tube, and tissues were stored in 95% ethanol in a 2-mL cryovial.

Swabs and liver tissues were extracted at the Genomic Core Facility located within the Sam Noble Oklahoma Museum of Natural History, using the PrepMan Ultra (Life Technologies) reagent following the protocol of Cheng et al. (2011) for *Bd* and the High Salt Extraction method for RV (Esselstyn et al. 2008). Tissue and swab extracts were stored at -20°C until used for pathogen screening. Quantitative PCR (qPCR) techniques were utilized to determine the presence/absence of *Bd* or RV and to estimate the number of gene copies (infection load) per sample (Kerby et al. 2013; Davis and Kerby 2016; Watters et al. 2018). Prior to qPCR analysis, DNA extracts were diluted 1:10 for *Bd* swab samples and 1:2 for RV tissue samples with a 0.25× TE buffer solution to reduce the effects of potential inhibitors, following standardized protocols (Hyatt et al. 2007; Watters et al. 2016, 2018; Marhanka et al. 2017). Primers used for the *Bd* assay target the internal transcribed spacer (ITS-1) ribosomal RNA gene as described by Boyle et al. (2004). Primers used for the RV assay target the Major Capsid Protein (MCP) gene as described by Forson and Storfer (2006). All qPCR assays of samples were run in triplicate (three reactions per sample) for 50 cycles, with standard dilutions of known pathogen gene copy number (DNA quantities) based on customized, double-stranded DNA fragments (gBlocks; Integrated DNA Technologies) from ITS-1 (*Bd*) and MCP (RV; Watters et al. 2018). Inhibition controls were not run to account for false negatives, but dilution has resulted in positive samples in previous studies that have used the identical protocol (Watters et al. 2018). The 750-base-pair gBlocks contain known copy numbers of the PCR fragment target for each pathogen, which allowed us to quantify the gene copy number amplified in each sample. Four standards were created for each pathogen gBlock by serially diluting the gBlock stock of known DNA quantities into concentrations ranging from $1e^1$–$1e^4$. As with study samples, each gBlock standard was run in triplicate on each qPCR assay plate, with the exception of the $1e^4$ standard which was run in duplicate next to a single negative control sample (sterile, molecular grade water). Pathogen loads for samples collected from Sites 1–6 were quantified using a QuantStudio 3 machine using QuantStudio 3 Design and Analysis software. Samples from Sites 7 and 8 were run on a StepOnePlus Real-Time PCR system and quantified using StepOne software v2.3, following the protocols of Kerby et al. (2013) and Davis and Kerby (2016), with 3 µL of DNA added to a 7 µL cocktail solution (TaqMan Fast Advanced Master Mix, ABI; forward primer; reverse primer; TaqMan probe, ABI; and molecular grade water). For all samples from all sites, samples were considered positive for a pathogen if:

(1) at least two of the three (triplicate) wells were amplified, and (2) the resulting mean gene copy number was above 1.0 (Davis and Kerby 2016; Watters et al. 2016, 2018; Marhanka et al. 2017). To determine final infection load per sample, *Bd* and RV gene copy numbers were multiplied by the dilution factor (10 for *Bd*; two for RV) and then were multiplied again by a value in order to reach the total original extraction volume for each sample (Watters et al. 2016, 2018; Marhanka et al. 2017).

In total, 546 individuals were screened for *Bd* only, and 304 individuals were screened for both pathogens, representing 30 species, 10 genera, and six families of anurans. Despite sampling several distinct amphibian communities in the country, we found only 11 individuals (2%) infected with *Bd*, and no samples tested positive for RV (Table 1). These results suggest that *Bd* and RV may not be widespread among wild amphibian populations in the Philippines. However, we note that due to limited sample sizes per species, we would only have been able to detect *Bd* or RV infections in a species if the pathogens occurred at fairly high prevalence (Gray et al. 2017). Of 30 species sampled, *Bd*-positive individuals were found from six species (*Limnonectes macrocephalus*, *L. visayanus*, *Occidozyga laevis*, *Platymantis corrugatus*, *Platymantis* sp. 3, *Pulchrana similis*), all with relatively low infection loads (Table 1). One *Bd*-positive individual (*L. visayanus*) came from Negros Oriental Province, Negros Island (Fig. 1; Site 2; Table 1), three *Bd*-positive individuals (*L. macrocephalus* and *Platymantis* sp. 3) from Quezon Province, Luzon Island (Fig. 1, Site 4; Table 1), three *Bd*-positive individuals (*O. laevis* and *P. corrugatus*) from Albay Province, Luzon Island (Fig. 1, Site 7; Table 1), and four *Bd*-positive individuals (*L. microcephalus* and *P. similis*) from Aurora Province, Luzon Island (Fig. 1, Site 8; Table 1).

The low number of *Bd*-positive individuals observed in our study is consistent with previous pathogen research conducted in the Philippines (Swei et al. 2011; Diesmos et al. 2012), including no observations of *Bd*-infected invasive species. However, our results present several new findings concerning infectious disease among Philippine amphibian populations. First, Diesmos et al. (2012) detected *Bd* in a total of seven anuran species; of those, we confirmed *Bd* presence in only three (*Limnonectes macrocephalus*, *Occidozyga laevis*, *Pulchrana similis*; Table 1). Second, we found *Bd*-infected individuals of three additional species not detected previously by Diesmos et al. (2012): *Limnonectes visayanus*, *Platymantis corrugatus*, and *Platymantis* sp. 3 (Table 1). Third, we detected fairly low levels of *Bd* prevalence at our sites, which is in contrast to the higher *Bd* infection prevalence recorded previously from Mt. Palay-palay and the Cotabato Cordillera (Swei et al. 2011; Diesmos et al. 2012). Although this may be the result of small sample sizes, it may also be related to the relatively undisturbed and remote nature of the sites we sampled (Beebee and Griffiths 2005; Greer and Collins 2008).

With respect to ranaviruses, there are two factors that may have contributed to the absence of positive individuals detected in our study. First, it is possible that the life stage we tested may have reduced our ability to detected RV because the viruses tend to be most prevalent in tadpoles (Duffus et al. 2015) whereas our study focused on terrestrial frogs. Second, there may have been false negatives in our study due to primer specificity. The primers used in our study were initially designed for *Ambystoma tigrinum* virus isolated from tiger salamander populations in Arizona, USA (Forson and Storfer 2006). Although these primers have subsequently been shown to detect a range of amphibian

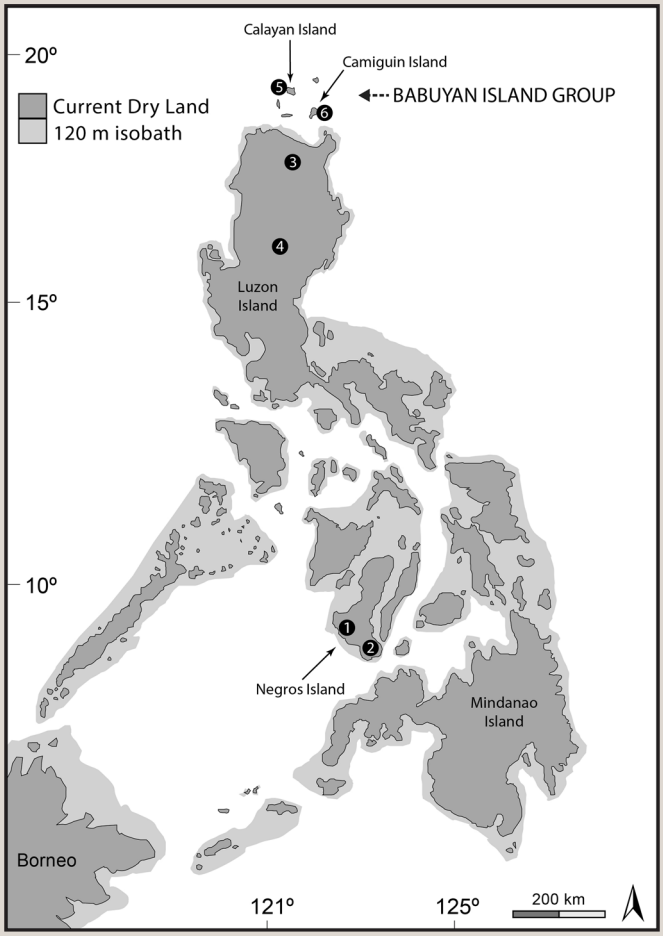

FIG. 1. Map of the Philippines showing the pathogen survey sites in the central and northern regions of the country. Site numbers correspond with text descriptions and Table 1.

ranaviruses from North America (Hall et al. 2016), their utility for ranaviruses in Asian amphibian populations is untested. To explore whether assay biases may have contributed to our negative results, we compared the specificity of the RV assay of Leung et al. (2017), which has been applied to several amphibian species in Europe (Price et al. 2017; Miaud et al. 2019), to the Forson and Storfer (2006) assay, using a broad sample of MCP sequences available on GenBank. First, we downloaded 35 sequences for MCP and aligned using default parameters for MUSCLE (Edgar 2004) in Geneious v9.05 (Biomatters Ltd.; GenBank Accession Nos.: AB500273, AF080218, AY033630, AY548303, AY548304, AY585203, FJ358612, FM213466, GU391285, HM133594, HQ684750, KC440841, KC440842, KC465189, KC756956, KC756963, KC756964, KC816423, KM516715, KM516716, KM516717, KM516718, KM516719, KM516720, KM516721, KR809884, KT265736, KU507315, KU507317, KY207443, KY207444, MF359927, MF678512, MF678523, MG637360). The final aligned dataset included ranaviruses from 11 species of amphibians (five frogs, six salamanders), four species of fish, and six species of reptiles, with representation of New- and Old-World lineages. Second, we aligned the forward and reverse primer and probe sequences for both RV assays (Forson and Storfer 2006; Leung et al. 2017) to the final alignment and examined the universality of the fit of both assays across the broad taxonomic diversity of hosts sampled. The comparison revealed both assays to have a suite of nucleotide differences between the primer and/

or probe sequences and vertebrate barcodes on GenBank, which is not unexpected given the broad taxonomic diversity of hosts examined. Interestingly, the Forson and Storfer (2006) assay we used appears to have fewer unique nucleotide differences across vertebrate groups sampled, including fewer differences among amphibian and reptile hosts, in comparison with the Leung et al. (2017) assay. We recognize that the results of this investigation do not indicate that one assay performs better than the other—simply that comparisons of pairwise sequence differences do not support a geographic specificity of either assay. Given the highly conserved nature of the region of the MCP targeted by the qPCR assay we used (Forson and Storfer 2006), it is unlikely that the negative results we obtained in our study were due to primer specificity.

The results of this study add to an early body of literature indicating that *Bd* and RV may not be negatively impacting amphibian populations in southeast Asia. Combined with the two previous disease studies in the archipelago (Swei et al. 2011; Diesmos et al. 2012), it appears that Philippine amphibians may not be highly susceptible to *Bd* infection. However, increased and continued monitoring throughout a broader region of this biodiversity hotspot is critical to conservation efforts to ensure a disease outbreaks do not go unnoticed.

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