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Low Prevalence or Apparent Absence of *Batrachochytrium dendrobatidis* Infection in Amphibians from Sites in Vietnam and Cambodia

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Batrachochytrium dendrobatidis (*Bd*), the causative agent for the amphibian disease chytridiomycosis, is widespread, but patchily distributed throughout Asia. Within Asia, *Bd* has so far been detected from amphibians in Cambodia, China, India, Indonesia, Japan, Kyrgyzstan, Laos, Malaysia, the Philippines, South Korea, Sri Lanka, and Vietnam (Bai et al. 2010; Goka et al. 2009; Kaiser and Grafe 2012; Kusrini et al. 2008; Mendoza II. et al. 2011; Nair et al. 2011; Savage et al. 2011; Swei et al. 2011; Vörös et al. 2012; Wei et al. 2010; Yang et al. 2009). The pattern of *Bd* prevalence in Asia appears drastically different to that in Australia, Africa, the Americas, and Europe, with isolated cases and low infection prevalence (or apparent absence) at most sites (Swei et al. 2011). To date, there have been no reports of *Bd*-associated morbidity or mortality and no evidence of enigmatic amphibian population declines in Southeast Asia (Rowley et al. 2010).

Bd was first reported to occur in Vietnam in 2011, with seven samples taken in 2008 from Bidoup-Nui Ba National Park, Lam Dong Province, testing positive for *Bd* (Swei et al. 2011). To date, these are the only positive records published for Vietnam. Here we carried out an additional survey for *Bd* at Bidoup-Nui Ba National Park, and performed surveys at localities in central and southern Vietnam, and in adjacent eastern Cambodia (Fig. 1; Table 1).

Amphibians were sampled for *Bd* as part of broader amphibian surveys in evergreen forest areas between May 2009 and July 2010. During nocturnal surveys, conducted along rocky streams and adjacent evergreen forest, adult amphibians were captured by hand and placed in individual plastic bags. Immediately after capture, or the following morning (<8 h after collection), the ventral surface of each frog was swabbed using

TABLE 1. Locations and taxonomic breadth of amphibians sampled for *Batrachochytrium dendrobatidis* (*Bd*) in Cambodia and Vietnam.

Country	Site	Location	Elevation (m)	No. Families	No. Genera	Approx. No. Species
Cambodia	Seima	12.32°N, 107.10°E	485–585	7	11	16
Vietnam	Bidoup-Nui Ba	12.18°N, 108.68°E	1470–1625	5	10	15
	Ngoc Linh	15.22°N, 107.73°E	1085–2300	5	15	27
		15.06°N, 107.86°E	935–2105	5	12	15
	Nui Ong	11.02°N, 107.72°E	140–1020	5	14	21

FIG. 1. Survey sites in Vietnam and adjacent eastern Cambodia. *Batrachochytrium dendrobatidis* was only detected at Ngoc Linh Nature Reserve (closed circle) in 2009.

TABLE 2. Number of individual amphibians in each family and genus sampled for *Batrachochytrium dendrobatidis* (*Bd*) in Cambodia and Vietnam.

Family	Genus	No. individuals sampled
Bufonidae	*Duttaphrynus*/*Ingerophrynus*	7
Dicroglossidae	*Fejervarya*	1
	Limnonectes	69
	Occidozyga	6
	Quasipaa	4
Icthyophiidae	*Icthyophis*	1
Megophryidae	*Leptobrachium*	71
	Leptolalax	40
	Ophryophryne	56
	Xenophrys	12
Microhylidae	*Kalophrynus*	3
	Microhyla	16
Ranidae	*Amolops*	3
	Hylarana	39
	Odorrana	48
Rhacophoridae	*Gracixalus*	4
	Kurixalus/*Raorchestes*	73
	Polypedates	1
	Rhacophorus	41
	Theloderma	5
Total		500

a sterile cotton swab (Medical Wire & Equipment, Potley, UK). Due to taxonomic uncertainty and the presence of undiagnosed diversity within the amphibians of the area, only the generic identity of the individuals swabbed is given (Table 2).

Samples were stored in the field at ambient temperatures (below that known to reduce the amount of *Bd* detectable; Van Sluys et al. 2008) for 5–14 days, and then at approximately –20°C for 3 weeks to 4 months prior to analysis. Storage conditions are unlikely to have affected our results, as storing swabs for 18 months at 4°C, 23°C, and –20°C does not reduce the amount of *Bd* detected (Hyatt et al. 2007). Due to the cost of PCR and our previous negative results (Swei et al. 2011), our 2009 samples from three sites were analyzed in batches of up to 8 samples via PCR (full volume or half volume pooling; Table 3). Samples from 2010 were analyzed individually via qPCR (Table 3).

All samples were analyzed by Pisces Molecular (Boulder, Colorado, USA). Samples pooled by full volume were transferred into a 50-ml tube of ethanol and vortexed to dislodge any zoospores from the swabs before pooling. Samples pooled by half volume had 1.1 ml of 70% ethanol added to the sample tubes and 500µl was transferred into a 15 ml screw-capped centrifuge tube for each pool. Individually analyzed samples had 1.0 ml of 70% ethanol added before mixing by vortexing, and then the entire volume was transferred into a clean microfuge tube. Total DNA was extracted from all samples using Qiagen DNeasy tissue kits. Samples were evaluated for the presence of *Bd* using diagnostic PCR (Annis et al. 2004), modified for greater specificity and sensitivity at Pisces Molecular. Samples were assayed in triplicate and each PCR run included three controls: a positive control (*Bd* DNA), negative control (sample from an uninfected amphibian), and No DNA control (remains uncapped during addition of sample DNA to detect contaminating DNA in the PCR reagents or carryover of positive DNA during reaction set-up). PCR results were scored as very strong positive (equivalent to the positive control), strong positive, positive, weak positive and negative (no signal/below the limit of detection). Each qPCR run included positive controls of *Bd* DNA in serial ten-fold dilutions from 3×10^6 to 3×100 molecules per reaction (used to generate the standard curve). Each qPCR run also included a No DNA control (as above). The detection sensitivity of these assays is three target sequence molecules (~0.02 zoospore equivalents).

TABLE 3. *Batrachochytrium dendrobatidis* (*Bd*) infecting amphibians in Cambodia and Vietnam. Prevalence and lower and upper 95% binomial confidence limits (CL; singlicate) or bias-corrected maximum likelihood estimates (MLE) for *Bd* infection from samples analyzed using diagnostic PCR.

Country	Site	Date	Analysis	N	Prevalence	95% CL/MLE Lower	95% CL/MLE Upper
Cambodia	Seima	July/August 2009	PCR; Pooled (full volume)	100	0.00	0.00	3.62
Vietnam	Bidoup Nui-Ba	July 2010	qPCR singlicate	124	0.00	0.00	3.39
	Ngoc Linh	July 2009	PCR; Pooled (full volume)	133	3.29	1.08	7.86
		March/April 2010	qPCR; singlicate	43	0.00	0.00	19.51
	Nui Ong	May 2009	PCR; Pooled (half volume)	100	0.00	0.00	3.62

For individually analyzed samples, we calculated the lower and upper 95% binomial confidence limits of infection prevalence. For pooled samples, we calculated infection prevalence and lower and upper confidence intervals using a bias-corrected maximum likelihood estimation for pooled samples of unequal sample size (Hepworth 2005), using PooledInfRate 4.0 (Biggerstaff 2009).

A total of 500 skin swab samples from at least 52 species of amphibian was analyzed for *Bd*. Only samples from the July 2009 survey of Ngoc Linh tested positive for *Bd* using diagnostic PCR (Table 3). Four of the 18 pooled samples collected from the site in 2009 tested strong positive, giving an overall estimate of 3.29% infection prevalence and maximum likelihood estimates of confidence intervals of 1.08–7.86%. Follow-up surveys at Ngoc Linh during 2010 failed to detect *Bd* at the site, with a maximum likelihood estimate of upper confidence interval of 19.51%. Because samples were not pooled by species, the identity of infected species could not be determined. However, positive pools included sampled from the families Megophryidae (*Leptobrachium, Leptolalax, Ophryophryne, Xenophrys*), Microhylidae (*Microhyla*), Ranidae (*Odorrana*) and Rhacophoridae (*Gracixalus, Kurixalus, Rhacophorus*). Samples from the three survey sites at which *Bd* was not detected had upper limits of infection prevalence ranging from 3.39–7.86%. We did not detect *Bd* on samples from Bidoup-Nui Ba, a site at which we previously detected *Bd* at low prevalence and at low intensity of infection in 2008 (6.36% "low" infection prevalence in May 2008 [7/110]), but failed to detect *Bd* again in March 2008 (0/155) (Swei et al. 2011).

Our results are consistent with a previous, large-scale survey of Asia, which found a surprisingly low *Bd* prevalence throughout Asia (2.35%; Swei et al. 2011). Surveys at many sites in the region have failed to detect *Bd*, despite often relatively large sample sizes including Ratanakiri Province, Cambodia (0/178); Lao Cai Province, Vietnam(0/82); Savannahkhet, Laos (0/191), Hong Kong (0/274), and Thailand (0/123) (Rowley et al. 2007; McLeod et al. 2008; Swei et al. 2011). Recent surveys of captive native frogs in the trade markets in Cambodia, Laos, Singapore, and Vietnam also reported low infection prevalence, with <1% (14/2389) of amphibians testing weakly positive for *Bd* (Gilbert et al. 2013).

Although most surveys for *Bd* in Asia have reported low prevalence or apparent absence, *Bd* has been reported at high infection prevalence at some sites (Yang et al. 2009; Swei et al. 2011), including four sites in Cambodia, where *Bd* was reportedly detected on 41% (59/144) of swab samples from southwestern Cambodia (Mendoza et al. 2011), and on 36% (86/238) of swab samples at three lowland sites in Cambodia (Gaertner et al. 2011). These survey results are in contrast to our survey at Seima and our previous surveys in eastern Cambodia, which did not detect the pathogen (Swei et al. 2011). Differences in the habitat and the amphibian species sampled makes comparisons between surveys difficult. However, Mendoza et al. (2011) and Gaertner et al. (2011) surveyed a very different suite of amphibians (most of which were lowland, pond-breeding amphibians).

The global distribution (Olson et al. 2013) and emergence of *Bd* remains far from clarified, and recent evidence suggests that *Bd* may have evolved from or at least have a long history in Asia (Goka et al. 2009; Bai et al. 2012). Additional disease surveys in Asia, molecular analysis of *Bd* from throughout Asia, and infection trials to assess disease susceptibility in Asian amphibians are required in order to assess the threat of *Bd* to amphibians in Asia. We also recommend assessing the identity and distribution of *Bd* haplotypes present throughout Asia. Given that qPCR appears unable to detect all strains of *Bd* present in Asia (Goka et al. 2009), it may also be necessary to identify the *Bd* haplotypes present at a site in order to assess the accuracy of *Bd* infection prevalence estimates obtained using qPCR.

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