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Amphibian Surveys Reveal No Instances of *Batrachochytrium dendrobatidis* and Suggest Low Prevalence of Chytrid Fungus in Thailand

Chytridiomycosis, the amphibian infectious disease caused by the fungal organism *Batrachochytrium dendrobatidis* (*Bd*), occurs globally with high prevalence and has been implicated in the population declines of amphibians in Africa (Hopkins and Channing 2003), Australia (Berger et al. 1998), Central America (Berger et al. 1998), Europe (Bosch et al. 2001), North America (Vredenburg et al. 2010), and South America (de Queiroz Carnaval et al. 2006). However, studies on the prevalence of *Bd* in East Asian amphibians present a different story—surveys conducted in parts of China, Japan, and the Korean peninsula have found low prevalence of *Bd* and have observed few negative effects on individuals that have tested positive for *Bd* (e.g., Goka et al. 2009; Swei et al. 2011; Bai et al. 2012; Bataille et al. 2013). Additionally, when sequencing the *Bd* strains found in the region, high diversity of *Bd* haplotypes were detected across sampling localities, suggesting that *Bd* has been evolving in the region for an extended period (Bai et al. 2012; Bataille et al. 2013). Recently, whole-genome sequencing of 234 isolates of *Bd* revealed a hyperdiverse East Asian *Bd* lineage with the highest SNP diversity found in wild South Korean frogs (O'Hanlon et al. 2018). Based on subsequent demographic and diversification analyses, it is hypothesized that *Bd* originated in the Korean Peninsula and spread globally throughout the 20th century (O'Hanlon et al. 2018).

Nevertheless, there remain several regions of the globe that have not been well tested for *Bd* presence; this lack of data hinders our understanding of the origin, direction, and timing of the pathogen's global spread. Additionally, without *Bd* prevalence data, it is difficult to develop strategies for conservation and disease management of sensitive species or habitats. One of

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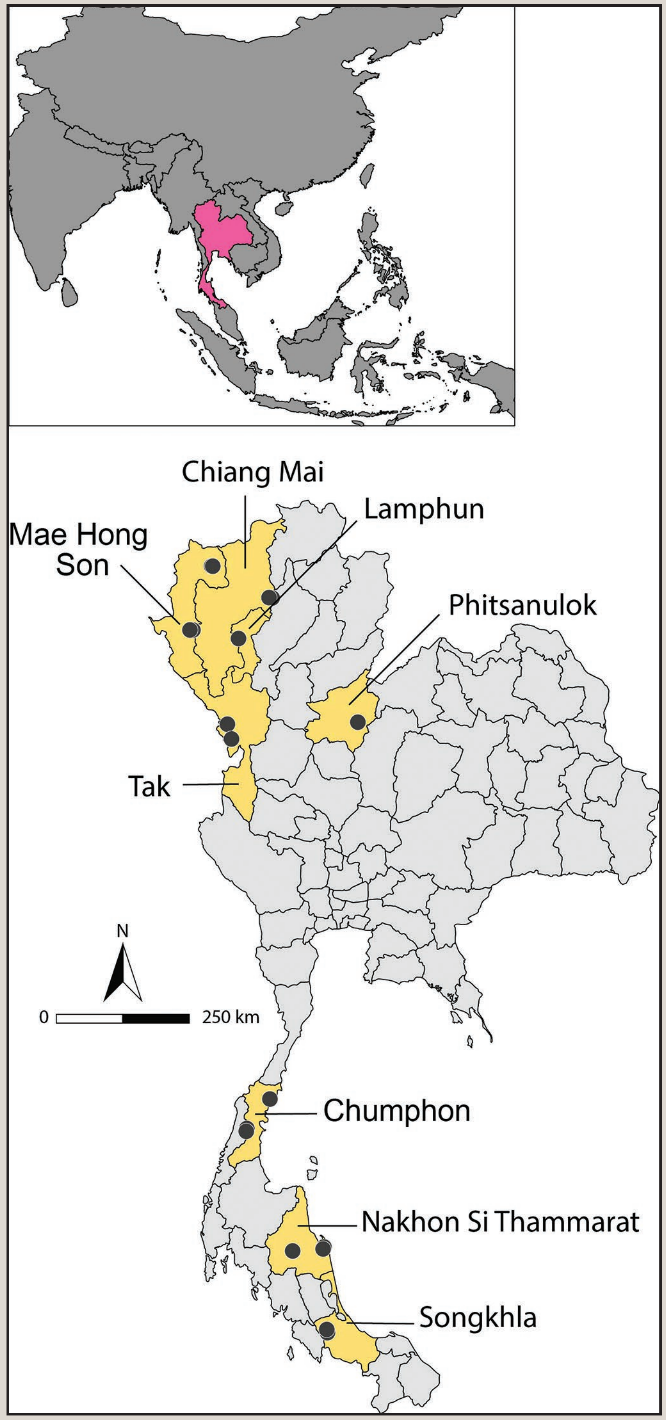

FIG. 1. Map of Thailand showing locations where amphibians were sampled for *Batrachochytrium dendrobatidis* (black circles show specific localities, orange regions show the sampled provinces). The inset map shows Southeast Asia with Thailand highlighted in pink.

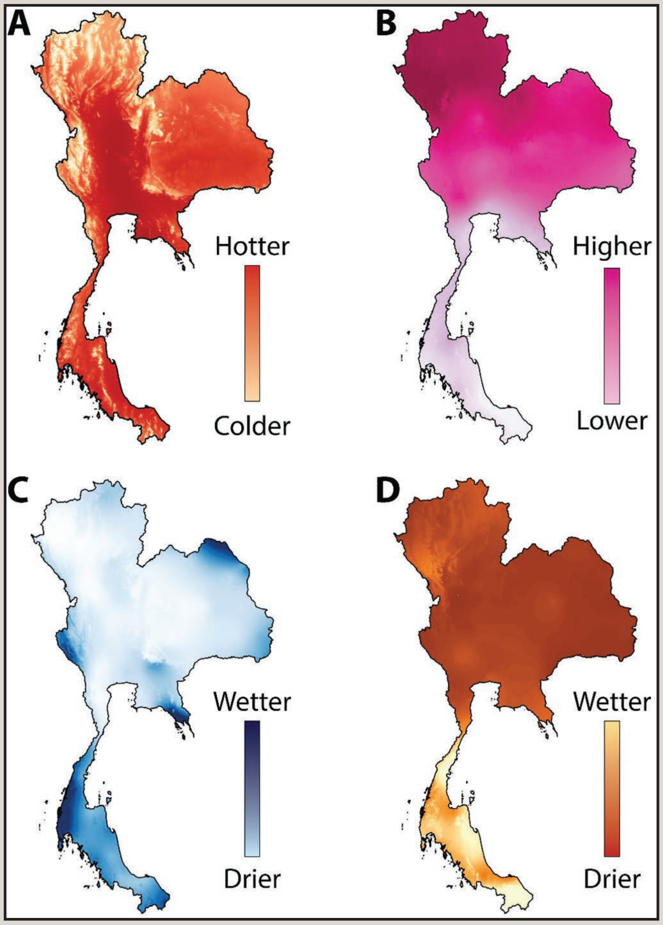

FIG. 2. Map of Thailand showing gradients in: A) annual mean temperature; B) annual temperature differential (difference between the maximum temperature and the minimum temperature; C) annual mean precipitation; and D) the mean precipitation of the warmest month. Northern Thailand has an overall higher temperature differential and lower precipitation than southern Thailand. Data from WorldClim (2017).

these understudied regions is Southeast Asia. Despite comprising four major biodiversity hotspots (Myers et al. 2000), there is a paucity of studies on *Bd* prevalence across the region (but see Diesmos et al. 2012 [Philippines]; Rowley et al. 2013 [Cambodia and Vietnam]; and LeBlanc et al. 2014 [Peninsular Malaysia], which suggested extremely low *Bd* prevalence; and Chong et al. 2018 [Singapore], which suggested moderate *Bd* prevalence). The most thorough survey of Southeast Asia to date, conducted by Swei et al. (2011), found that only 1.4% of more than 2,800 individuals tested positive for *Bd*, with positive samples found in Indonesia (0.25% infection rate), Laos (0.73%), Malaysia (0.90%), and the Philippines (8.01%).

Despite the high numbers of samples collected throughout Southeast Asia by Swei et al. (2011), sampling was opportunistic and large areas of the region were missed, including Thailand. The general paucity of countrywide data regarding *Bd* prevalence in Thailand is surprising given its unique and complex biogeography. Within Southeast Asia, Thailand represents a major biogeographic transition zone between Indochinese and South Asian species in the north and Sunda Shelf species in the south (Woodruff 2003; Parnell 2013), making it an important region for biodiversity. Currently, there are 145 species of amphibians recognized in the country, second only to Vietnam (241 species) in mainland Southeast Asia (AmphibiaWeb 2018). Therefore, understanding the prevalence of *Bd* in Thailand is critical for higher-level analyses of the pathogen's prevalence and distribution across Southeast Asia.

There have been three studies to date on the occurrence of *Bd* in Thailand: 1) McLeod et al. (2008) examined histological samples from 123 fluid-preserved historical museum specimens housed at the Natural History Museum of Chulalongkorn University in Bangkok, representing 28 species from localities across the country, and found no instances of *Bd* infection; 2) Vörös et al. (2012) tested skin swabs of six individuals of the toad *Ingerophrynus parvus* at the Prince of Songkla University Protected Area in southern Thailand and found one individual that was positive for *Bd*, suggesting an overall high prevalence of disease in that region, albeit with limited sampling; 3) Techangamsuwan et al. (2017) tested skin swabs of 259 wild-caught amphibians and 233 captive amphibians at zoos and parks across Thailand, including a zoo near Prince of Songkla University, and found *Bd* presence only in captive non-native species. Here, we present the first study investigating the prevalence of chytrid fungus on wild amphibians in non-protected areas in northern and southern Thailand using the most comprehensive spatial sampling effort to date.

We conducted amphibian surveys in five provinces in northern Thailand in March 2017 and in three provinces in southern Thailand in June 2017 (Fig. 1). In general, sampling was opportunistic and occurred as part of biodiversity surveys targeting northern and southern Thailand amphibian species for vouchered phylogenetic analyses. All surveys were carried out after dark between 1900–2130 h, and temperature and humidity measurements were taken at the start of each sampling period with a Kestrel 2000 pocket weather meter (Kestrel Instruments, Boothwyn, Pennsylvania, USA). For samples collected in northern Thailand, the temperature and relative humidity for each sampling locality ranged from 21.3–32.2°C and 27.4–70.1%, respectively, and in southern Thailand measured 20.3–30.0°C and 68.7–100%, respectively. Amphibians were captured by hand, placed into individual plastic bags to avoid potential cross-contamination of individuals, and euthanized the same night of capture with an injection of MS-222 (Conroy et al. 2009). After euthanization, each individual was rinsed briefly with tap water to remove any loose debris and mud on the body that may have interfered with our ability to detect *Bd* (i.e., PCR inhibitors). We acknowledge that this may have decreased our ability to detect *Bd* in an individual with low loads of infection (Brem et al. 2007). Individuals were swabbed with a sterile, rayon-tipped swab (Medical Wire; Corsham, Wiltshire, England) ten times each on the dorsum, venter, medial surface of the left thigh, left foot webbing, throat, and mouth (juveniles and adults) or ten times on the oral disc (tadpoles) (modified from Hyatt et al. 2007). A control swab was dipped and swirled in the tap water used for rinsing and tested for chytrid to ensure that there was no contamination of individuals from the water source—all control swabs tested negative for chytrid. Each swab was then placed into individual 1.5-ml Eppendorf tubes and allowed to dry unsealed for an hour, before being stored after the completion of field surveys in a -20°C freezer until extracted. We sexed each specimen via gonadal inspection and vouchered individuals at the Zoological Museum of Kasetsart University, Bangkok, Thailand (ZMKU). Extractions of *Bd* DNA were done at the Sam Noble Museum Genomics Core Facility (University of Oklahoma, Norman, Oklahoma, USA) with PrepMan Ultra

TABLE 1. The number of amphibian individuals for each age class (tadpole, juvenile, adult) and sex (male, female) sampled for *Batrachochytrium dendrobatidis* (*Bd*) at each locality in Thailand (coordinates; elevation). The localities are listed in the chronological order that they were sampled. The right column shows the calculated 95% confidence interval for the maximum potential *Bd* prevalence in each species for each locality, given the sample size, and for each locality, given the total number of individuals sampled.

Locality	Species	Tadpole	Juvenile	Adult male	Adult female	Max. chytrid prevalence in population (%)
19 March 2017: Tak Province: Mae Sot District: Mae Kasa Subdistrict; (16.86129°N, 98.63038°E; 246 m)	*Duttaphrynus melanostictus*	0	0	0	2	77.6
	Fejervarya limnocharis	0	4	0	1	45.1
	Kaloula pulchra	0	1	0	0	95.0
	Leptobrachium smithi	0	2	0	0	77.6
	Microhyla butleri	0	2	0	0	77.6
	Microhyla fissipes	0	3	1	6	25.9
	Microhyla heymonsi	0	3	0	1	52.7
	Microhyla pulchra	0	0	2	0	77.6
	Polypedates megacephalus	0	2	0	1	63.2
	Sylvirana nigrovittata	0	1	2	1	52.7
	TOTAL	0	18	5	12	8.2
20 March 2017: Tak Province: Mae Sot District: Mahawan Subdistrict; (16.57016°N, 98.69524°E; 656 m)	*Fejervarya chiangmaiensis*	0	1	0	0	95.0
	Leptobrachium smithi	0	1	0	0	95.0
	Limnonectes doriae	0	0	1	0	95.0
	Limnonectes limborgi	0	0	1	0	95.0
	Limnonectes taylori	0	0	0	1	95.0
	Microhyla butleri	0	1	0	0	95.0
	Microhyla heymonsi	0	1	0	0	95.0
	TOTAL	0	4	2	1	34.8
20 March 2017: Tak Province: Mae Sot District: Mahawan Subdistrict; (16.57339°N, 98.69710°E; 668 m)	*Chiromantis hansenae*	0	0	1	0	95.0
	Fejervarya limnocharis	0	1	0	0	95.0
	Ingerana tenasserimensis	0	2	0	0	77.6
	Leptobrachium smithi	0	1	0	0	95.0
	Micryletta inornata	0	1	0	0	95.0
	Polypedates megacephalus	0	1	0	0	95.0
	TOTAL	0	6	1	0	34.8
21 March 2017: Mae Hong Son Province: Mae La Noi District: Huai Hom Subdistrict; (18.34089°N, 98.07134°E; 975 m)	*Ingerana tenasserimensis*	0	6	0	0	39.3
	Microhyla fissipes	0	1	2	0	63.2
	Microhyla heymonsi	0	7	1	0	31.2
	TOTAL	0	14	3	0	16.2
22 March 2017: Mae Hong Son Province: Mae La Noi District: Tha Pha Pum Subdistrict; (18.34223°N, 98.02317°E; 991 m)	*Amolops marmoratus*	0	3	2	0	45.1
	Ansonia inthanon	0	2	0	0	77.6
	Graxcixalus seesom	0	1	0	0	95.0
	Ingerana tenasserimensis	0	9	0	0	28.3
	Leptobrachium smithi	0	1	0	0	95.0
	Leptolalax minimus	1	0	1	0	77.6
	Limnonectes limborgi	0	6	2	0	31.2
	Limnonectes taylori	0	0	1	0	95.0
	Megophrys major	3	0	0	0	63.2
	Microhyla butleri	0	2	0	0	77.6
	Microhyla heymonsi	0	6	0	1	34.8
	Raorchestes parvulus	0	8	0	0	31.2
	TOTAL	4	38	6	1	5.9
23 March 2017: Mae Hong Son Province: Pai District: Mae Na Toeng Subdistrict; (19.37854°N, 98.37448°E; 882 m)	*Amolops marmoratus*	1	2	2	3	31.2
	Duttaphrynus melanostictus	0	0	1	0	95.0
	Fejervarya limnocharis	0	3	2	1	39.3
	Leptolalax minimus	8	0	0	0	31.2
	Limnonectes taylori	0	1	2	1	52.7
	Microhyla fissipes	0	2	0	1	63.2
	Microhyla heymonsi	0	1	0	0	95.0
	Microhyla pulchra	0	1	0	0	95.0
	Odorrana livida	0	0	3	2	45.1
	Polypedates megacephalus	0	0	4	0	52.7
	Sylvirana nigrovittata	0	1	1	2	52.7
	TOTAL	9	11	15	10	6.4

Table 1. Continued.

Locality	Species	Tadpole	Juvenile	Adult male	Adult female	Max. chytrid prevalence in population (%)
24 March 2017: Mae Hong Son Province: Pai District: Mae Na Toeng Subdistrict; (19.37669°N, 98.39301°E; 728 m)	*Chiromantis hansenae*	0	0	1	0	95.0
	Duttaphrynus melanostictus	0	0	3	0	63.2
	Fejervarya limnocharis	0	8	1	0	28.3
	Microhyla fissipes	0	3	0	0	63.2
	Microhyla pulchra	0	0	0	1	95.0
	Polypedates megacephalus	0	1	0	0	95.0
	Sylvirana nigrovittata	0	1	8	1	25.9
	TOTAL	0	13	13	2	10.2
25 March 2017: Lamphun: Wang Laung Waterfall (18.20337°N, 98.81094°E; 522 m)	*Duttaphrynus melanostictus*	0	0	3	0	63.2
	Fejervarya limnocharis	0	1	0	2	63.2
	Limnonectes gyldenstolpei	0	1	0	0	95.0
	Microhyla butleri	0	4	0	0	52.7
	Microhyla fissipes	0	11	1	1	20.6
	Microhyla heymonsi	0	5	0	1	39.3
	Microhyla pulchra	0	0	1	2	63.2
	Micryletta inornata	0	11	3	0	19.3
	Occidozyga martensii	0	1	0	1	77.6
	Polypedates megacephalus	0	3	2	2	34.8
	Sylvirana nigrovittata	6	4	12	2	11.7
	TOTAL	6	41	22	11	3.7
26–27 March 2017: Chiang Mai Province: Mae On District: Huai Kaeo Subdistrict; (18.86129°N, 99.35742°E; 1213 m)	*Fejervarya limnocharis*	0	0	1	0	95.0
	Huia melasma	0	1	2	5	31.2
	Leptobrachium sp.	3	0	0	0	63.2
	Leptolalax minimus	2	0	3	4	34.8
	Limnonectes taylori	0	1	1	2	52.7
	Megophrys major	3	0	0	0	63.2
	Megophrys sp.	1	0	0	0	95.0
	Odorrana chloronata	0	4	12	5	13.3
	Raorchestes parvulus	0	3	0	1	52.7
	Sylvirana nigrovittata	0	0	6	1	34.8
	TOTAL	9	9	25	18	7.1
26 March 2017: Chiang Mai Province: Mae On District: Huai Kaeo Subdistrict; (18.86751°N, 99.30668°E; 706 m)	*Microhyla fissipes*	0	0	1	0	95.0
	Polypedates megacephalus	0	0	1	0	95.0
	Sylvirana nigrovittata	0	0	1	1	77.6
	TOTAL	0	0	3	1	52.7
28 March 2017: Phitsanulok Province: Wang Thong District: Kaeng Sopha Subdistrict; (16.84531°N, 100.74796°E; 223 m)	*Fejervarya limnocharis*	0	3	4	3	25.9
	Hylarana erythraea	0	4	0	0	52.7
	Kaloula pulchra	0	1	1	1	62.3
	Microhyla fissipes	0	20	0	1	13.3
	Microhyla pulchra	0	0	0	1	95.0
	Occidozyga lima	0	0	0	1	95.0
	Polypedates megacephalus	0	3	4	0	34.8
	Sylvirana mortenseni	0	1	2	1	52.7
	TOTAL	0	32	11	8	5.7
4 June 2017: Chumphon Province: Sawi District: Thung Raya Subdistrict; (10.23400°N, 98.94609°E; 34 m)	*Kaloula pulchra*	0	0	1	0	95.0
	Limnonectes hascheanus	0	0	4	2	39.3
	Microhyla butleri	0	6	0	0	39.3
	Micryletta inornata	0	1	0	0	95.0
	Sylvirana nigrovittata	0	1	3	0	52.7
	TOTAL	0	8	8	2	15.3
4 June 2017: Chumphon Province: Sawi District: Thung Raya Subdistrict; (10.25289°N, 98.94605°E; 39 m)	*Fejervarya limnocharis*	0	0	1	0	95.0
	Kaloula pulchra	0	0	1	0	95.0
	Limnonectes doriae	0	0	1	0	95.0
	Limnonectes hascheanus	0	0	1	1	77.6
	Microhyla butleri	0	2	0	0	77.6
	Microhyla fissipes	0	3	0	0	62.3
	Micryletta inornata	0	1	0	0	95.0
	TOTAL	0	6	4	1	23.8

Table 1. Continued.

Locality	Species	Tadpole	Juvenile	Adult male	Adult female	Max. chytrid prevalence in population (%)
5 June 2017: Chumphon Province: Sawi District: Khao Thalu Subdistrict; (10.20659°N, 98.93649°E; 70 m)	*Duttaphrynus melanostictus*	0	0	2	0	77.6
	Fejervarya limnocharis	0	0	3	1	52.7
	Kaloula pulchra	0	0	2	1	62.3
	Limnonectes hascheanus	0	0	2	1	62.3
	Microhyla butleri	0	1	0	0	95.0
	Microhyla fissipes	0	1	1	1	62.3
	Microhyla heymonsi	0	1	0	0	95.0
	Micryletta inornata	0	0	0	2	77.6
	Occidozyga martensii	0	0	0	1	95.0
	Polypedates leucomystax	0	1	2	0	62.3
	TOTAL	0	4	12	7	12.2
6–7 June 2017: Nakhon Si Thammarat Province: Thung Song District: Na Luang Sen Subdistrict; (8.26059°N, 99.68733°E; 170 m)	*Chalcorana eschatia*	0	0	2	1	62.3
	Icthyophis supachaii	0	1	0	0	95.0
	Limnonectes hascheanus	0	0	3	3	39.3
	Microhyla berdmorei	0	0	1	0	95.0
	Pulchrana glandulosa	0	0	1	0	95.0
	TOTAL	0	1	7	4	22.1
6–7 June 2017: Nakhon Si Thammarat Province: Thung Song District: Na Luang Sen Subdistrict; (8.26533°N, 99.68964°E; 212 m)	*Chalcorana eschatia*	0	0	1	1	77.6
	Fejervarya limnocharis	0	0	1	0	95.0
	Microhyla berdmorei	0	0	1	0	95.0
	Odorrana chloronota	0	0	1	1	77.6
	Odorrana hosii	0	0	4	1	45.1
	Sylvirana nigrovittata	0	0	2	1	62.3
	TOTAL	0	0	10	4	19.3
8 June 2017: Songkhla Province: Hat Yai District: Thung Tam Sao Subdistrict; (6.94010°N, 100.25426°E; 72 m)	*Chalcorana eschatia*	0	0	1	0	95.0
	Fejervarya limnocharis	0	2	2	1	45.1
	Humerana miopus	1	0	1	0	77.6
	Leptobrachium hendricksoni	6	2	3	0	23.8
	Microhyla berdmorei	0	2	0	0	77.6
	Occidozyga martensii	0	0	2	1	62.3
	Odorrana hosii	0	0	3	1	52.7
	Polypedates discantus	14	0	6	0	13.9
	Polypedates leucomystax	9	0	0	0	28.3
	Pulchrana glandulosa	0	0	0	1	95.0
	TOTAL	30	6	18	4	5.0
9 June 2017: Songkhla Province: Hat Yai District: Chalung Subdistrict; (6.99054°N, 100.24322°E; 133 m)	*Chalcorana eschatia*	0	0	1	1	77.6
	Icthyophis supachaii	0	1	0	0	95.0
	Pulchrana glandulosa	0	1	0	0	95.0
	TOTAL	0	2	1	1	52.7
10 June 2017: Nakhon Si Thammarat Province: Pak Phanang District: Pak Phanang Subdistrict; (8.33083°N, 100.19784°E, 0 m)	*Fejervarya moodiei*	9	4	5	7	11.3
	TOTAL	9	4	5	7	11.3
10 June 2017: Nakhon Si Thammarat Province: Pak Phanang District: Pak Phanang Subdistrict; (8.29819°N, 100.18362°E; 6 m)	*Duttaphrynus melanostictus*	0	0	0	1	95.0
	Fejervarya limnocharis	0	5	0	2	34.8
	Hylarana erythraea	0	0	0	1	95.0
	Polypedates leucomystax	0	1	0	0	95.0
	TOTAL	0	6	0	4	25.9
11 June 2017: Chumphon Province: Pathio District: Chum Kho Subdistrict; (10.73106°N, 99.32079°E; 10 m)	*Fejervarya limnocharis*	0	0	0	1	95.0
	Hoplobatrachus rugulosus	0	1	0	0	95.0
	Kaloula pulchra	0	0	2	0	77.6
	Microhyla heymonsi	0	0	2	4	39.3
	Micryletta inornata	0	0	1	0	95.0
	Polypedates leucomystax	0	0	3	0	62.3
	TOTAL	0	1	8	5	19.2
	THAILAND TOTALS	67	224	179	103	0.5

Table 2. The total number of individuals tested for *Batrachochytrium dendrobatidis* (*Bd*) for each sampled amphibian species in Thailand. The two right columns show the calculated 95% confidence interval for the maximum potential *Bd* prevalence per species across the country. NA indicates no data were available in the IUCN database; — denotes species not sampled by Techangamsuwan et al. (2017). The listed IUCN status is as of December 2018 (IUCN 2018).

Family	Species	IUCN Conservation Status	Number sampled	Maximum chytrid prevalence: This study (%)	Maximum chytrid prevalence: This study + Techangamasuwan et al. 2017 (%)
Bufonidae	*Ansonia inthanon*	Least Concern	2	77.6	—
Bufonidae	*Duttaphrynus melanostictus*	Least Concern	12	22.1	7.8
Dicroglossidae	*Fejervarya moodiei*	Data Deficient	25	11.3	—
Dicroglossidae	*Fejervarya chiangmaiensis*	NA	1	95.0	—
Dicroglossidae	*Fejervarya limnocharis*	Least Concern	54	5.4	4.4
Dicroglossidae	*Hoplobatrachus rugulosus*	Least Concern	1	95.0	20.6
Dicroglossidae	*Ingerana tenasserimensis*	Leas Concern	17	16.2	—
Dicroglossidae	*Limnonectes doriae*	Least Concern	2	77.6	63.2
Dicroglossidae	*Limnonectes gyldenstolpei*	Least Concern	1	95.0	—
Dicroglossidae	*Limnonectes hascheanus*	Least Concern	17	16.2	—
Dicroglossidae	*Limnonectes limborgi*	Least Concern	9	28.3	—
Dicroglossidae	*Limnonectes taylori*	Least Concern	10	25.9	—
Dicroglossidae	*Occidozyga lima*	Least Concern	1	95.0	45.1
Dicroglossidae	*Occidozyga martensii*	Least Concern	6	39.3	17.1
Icthyophiidae	*Icthyophis supachaii*	Data Deficient	2	77.6	—
Megophryidae	*Leptobrachium hendricksoni*	Least Concern	11	23.8	19.3
Megophryidae	*Leptobrachium smithi*	Least Concern	5	45.1	25.9
Megophryidae	*Leptobrachium* sp.	NA	3	63.2	—
Megophryidae	*Leptolalax minimus*	Least Concern	19	14.6	—
Megophryidae	*Megophrys major*	Least Concern	6	39.3	34.8
Megophryidae	*Megophrys* sp.	NA	1	95.0	—
Microhylidae	*Kaloula pulchra*	Least Concern	11	23.8	11.7
Microhylidae	*Microhyla berdmorei*	Least Concern	4	52.7	39.3
Microhylidae	*Microhyla butleri*	Least Concern	18	15.3	—
Microhylidae	*Microhyla fissipes*	Least Concern	60	4.9	—
Microhylidae	*Microhyla heymonsi*	Least Concern	34	8.4	6.4
Microhylidae	*Microhyla pulchra*	Least Concern	8	31.2	25.9
Microhylidae	*Micryletta inornata*	Least Concern	20	13.9	10.1
Ranidae	*Amolops marmoratus*	Least Concern	13	20.6	8.9
Ranidae	*Chalcorana eschatia*	NA	8	31.2	—
Ranidae	*Huia melasma*	Data Deficient	8	31.2	—
Ranidae	*Humerana miopus*	Least Concern	2	77.6	—
Ranidae	*Hylarana erythraea*	Least Concern	5	45.1	23.8
Ranidae	*Odorrana chloronota*	Least Concern	23	12.2	—
Ranidae	*Odorrana hosii*	Least Concern	9	28.3	22.1
Ranidae	*Odorrana livida*	Data Deficient	5	45.1	39.3
Ranidae	*Pulchrana glandulosa*	Least Concern	3	63.2	—
Ranidae	*Sylvirana mortenseni*	Near Threatened	4	52.7	15.3
Ranidae	*Sylvirana nigrovittata*	Least Concern	58	5.0	3.7
Rhacophoridae	*Chiromantis hansenae*	Least Concern	2	77.6	—
Rhacophoridae	*Gracixalus seesom*	NA	1	95.0	—
Rhacophoridae	*Polypedates discantus*	NA	20	13.9	—
Rhacophoridae	*Polypedates leucomystax*	Least Concern	16	17.1	6.3
Rhacophoridae	*Polypedates megacephalus*	Least Concern	24	11.7	—
Rhacophoridae	*Raorchestes parvulus*	Least Concern	12	22.1	—

lysis buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA) following standard protocols (Cheng et al. 2011). DNA extractions were diluted 1:10 using 0.25x TE buffer and the internal transcribed spacer (ITS-1) nuclear DNA fragment was amplified via quantitative PCR (qPCR) using the primers ITS1-3 and 5.8S (Boyle et al. 2004). The reactions were run in triplicate on a QuantStudio 3 Real-Time PCR (qPCR) machine (ThermoFisher Scientific, Waltham, Massachusetts, USA) along with gBlock standards (Integrated DNA Technologies, Skokie, Illinois, USA) in order to quantify the chytrid load for each sample. In general, samples are considered positive for *Bd* if two of three replicate reactions are positive, with a mean gene copy per sample above 1.0; if only a single reaction tests positive, the sample is rerun and if reruns detect one or more positive replicate samples, the sample is considered positive for *Bd*.

Sampling occurred at 21 distinct localities in eight provinces across northern and southern Thailand (Fig. 1). We collected a total of 573 swabs representing 44 frog species and one caecilian species across all age classes (tadpoles, juveniles, adults; Table 1). All sampling localities were human-impacted local and regional recreation spots, or agricultural areas.

All 573 samples tested negative for *Bd*, in line with the observations of several previous studies in Thailand (McLeod et al. 2008; Techangamsuwan et al. 2017), suggesting that the pathogen is not widespread among wild amphibian populations in the country. However, despite our large sample size, our species-level sampling within each locality was low, which, in some cases, may mean that our negatives are misleading. In particular, if *Bd* has a low infection rate in Thailand, detection of the disease is more difficult and requires a larger sample size—at least 59 individuals are required to have a 95% chance of finding one *Bd*-positive individual when the infection rate is 5% (Hanley and Lippman-Hand 1983; Skerratt et al. 2008; Gray et al. 2017). Given our sample size and zero positive observations, we cannot determine the rate of *Bd* infections in wild amphibian populations. However, we can use our sample size to calculate the maximum possible *Bd*-positive infection rate within a 95% confidence interval, assuming an infinite population size, that would yield negative results, using the equation:

$$\text{maximum 95\% confidence prevalence (\%)} = (1 - 0.05^{1/n}) \times 100$$

With n being the sample size of each species at each locality (Hanley and Lippman-Hand 1983). This metric can be useful in giving context to our negative results and secondarily, illustrates the importance of sample size when investigating *Bd* prevalence (species with fewer individuals sampled have a higher potential *Bd*-positive infection rate). Furthermore, this metric illustrates that despite our large total sample size and the apparent lack of *Bd*-positive individuals, *Bd* may still be present in Thailand.

Using our sample size, we find a maximum possible *Bd* infection rate for each species at each site ranging between 11.7–95.0% (Table 1). Alternatively, if we consider each species at each locality equally likely to contract the infection, we find a maximum possible *Bd* infection rate for each site ranging between 3.7–52.7%. Finally, if we consider each species equally likely to contract the infection across the country, we find a maximum possible *Bd* infection rate of 0.5% for wild amphibians in Thailand. Furthermore, if we combine our sampling of wild-caught amphibians (N = 573) with the sample of wild-caught amphibians (N = 259) from Techangamsuwan et al. (2017), we find a maximum possible *Bd* infection rate of just 0.3% across Thailand, and we can infer that less than 1% of all amphibians in Thailand are infected with *Bd*. Additionally, we calculated the maximum possible *Bd* infection rate for each individual species across all sampling localities, which ranged from 4.9–95.0% (average = 41.3 ± 30.2%; Table 2), with rarer species having a higher potential *Bd* infection rate given that our statistical analyses are based on our sample size. Combining our species sampling with the number of wild individuals swabbed by Techangamsuwan et al. (2017) results in additional decreases in the maximum potential *Bd* infection rate per species (Table 2).

Although site- and species-specific statistics suggest that *Bd* could be widespread in Thailand, especially in rare species, our total lack of positive results adds to the growing body of literature that suggests that *Bd* either is absent, or present at extremely low rates, throughout large regions of the country. Therefore, while swabs have been shown to be less effective at detecting *Bd* in individuals when the intensity (load) of the infection is low (Shin et al. 2014), we feel confident in the results and implications given our study's robust sampling, especially when combined with the results of previous surveys (Techangamsuwan et al. 2017). Nevertheless, *Bd* surveys targeting our sites and additional sites across the country with greater intraspecific sampling are needed to corroborate these findings. Additionally, increasing the number of *Bd* surveys in Thailand is all the more imperative given that our results contradict those of Vörös et al. (2012), who detected *Bd* in the toad *I. parvus* in southern Thailand in one of six individuals sampled. Their results suggest that *Bd* may be present in localized areas across the country; however, given the small sample size included in the study, more data is needed to confirm and document disease distribution.

Our failure to detect the *Bd* pathogen in amphibians across sites in northern Thailand is not surprising given the high seasonality in temperature and precipitation in the region (Fig. 2). Laboratory experiments have shown that the fungus does best at temperatures between 25–28°C and dies at temperatures above 30°C (Piotrowski et al. 2004). Central and northern Thailand experience high temperatures at least one-third of the year, particularly at the end of the dry season (March–April; Singhrattna et al. 2005). During our amphibian surveys in northern Thailand in March, daytime temperatures were consistently above 30°C, the lethal cut-off for *Bd*. Not only should future survey efforts expand the geographic and taxonomic sampling in the region, but also, they should investigate seasonality in *Bd* prevalence to ascertain whether *Bd* is present in northern Thailand during the cooler rainy season.

In southern Thailand, where there is higher precipitation and lower seasonality in both temperature and precipitation (Fig. 2) and the predicted habitat suitability for *Bd* is higher (Rödder et al. 2009), we had expected to find higher rates of *Bd* infection. Our taxonomic sampling did not include *I. parvus*, the only wild-caught species to exhibit *Bd* prevalence in previous studies, although our geographic sampling included several sites in Songkhla Province, close to where the *Bd*-positive individual had been found. However, these sites yielded no *Bd*-positive individuals. Whereas statistical analyses suggest that the site-specific *Bd*-infection rate in southern Thailand potentially could be between 5.0–52.7% (Table 1), our absence of positive samples suggests that *Bd* is present only at low infection rates in the region and may be confined to localized water bodies.

The results of this research, combined with previous studies in Thailand, present a hopeful picture for the country's amphibians in terms of conservation threats from chytrid fungus. Combined with additional data for Southeast Asia (Swei et al. 2011; Diesmos et al. 2012; Rowley et al. 2013; LeBlanc et al. 2014; Chong et al. 2018) it appears that amphibians in the region are not highly susceptible to *Bd* infection. However, continuing to survey for the pathogen throughout the region across seasons is warranted in order to determine the overall risk of *Bd* to wild amphibians and to identify species and sites that may be at risk. Furthermore, future studies should employ additional techniques, such as environmental DNA, to detect *Bd* in the environment (Kirshtein et al. 2007) and to investigate non-amphibian species that may act as reservoir hosts for the pathogen (McMahon et al. 2013) across Southeast Asia.

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