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A SURVEY FOR CHYTRID FUNGUS IN THAI AMPHIBIANS

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ABSTRACT. **–** The chytrid fungus, *Batrachochytrium dendrobatidis*, is implicated as a major cause of global die-offs of amphibians. Curiously, it has not been reported to date from Asia. The presence of this fungus in many other parts of the world suggests that it is already present, but undetected, in Asia or that it will be introduced in the future through human or animal movements. We undertook a histological screening of museum specimens to test for the presence of *B. dendrobatidis* in Thailand in the last 60 years. Histological results for 123 adult specimens representing 28 species were all negative. We propose several follow-up studies to continue testing for the presence of this pathogen.

KEY WORDS. **–** Amphibian population declines; Asia; *Batrachochytrium dendrobatidis*; histochemical; Thailand.

INTRODUCTION

A great deal of recent herpetological research has focused on identifying the causes of amphibian population declines. One potential cause implicated in mass amphibian die-offs and population declines is the fungus *Batrachochytrium dendrobatidis* (Berger et al., 1998; Collins et al., 2003; Daszak et al., 2003). This fungus has been detected in populations of frogs on six continents, yet there is no documentation of its existence in Asia, one of the world's biodiversity hot spots (Speare & Berger, 2000). The presence of *B. dendrobatidis* in the Americas, Africa, Australia, New Zealand and Europe suggests that its occurrence in Asia is plausible, and that its apparent absence most likely represents a sampling bias. Recent modeling work by Ron (2005) predicts that the fundamental niche (i.e. the theoretical range of ecological conditions within which a species can persist without immigration) of this fungus occurs in SE Asia, suggesting that this region is particularly suitable for the establishment of this pathogen (Fig. 1).

Chytridiomycete fungi are common decomposers found in aquatic and soil systems. Some species are parasites of invertebrates, other fungi, plants and algae. Apart from *B. dendrobatidis*, no other species of Chytrid are known to be vertebrate pathogens (Berger et al., 2005). Zoospores of *B. dendrobatidis* are motile and easily dispersed in an aquatic system (Berger et al., 1999). Chytridiomycosis in anurans develops only in keratinized cells, and causes hyperkeratosis and death; however, the mechanism for this is still unknown (Mazzoni et al., 2003). For thorough treatments of the Batrachiochytrium, see Longcore et al. (1999); Berger et al. (1999); and Berger et al. (1999).

The lethality of this fungus in anurans is variable. Apparently, it is not lethal in larvae, and infected tadpoles can act as vectors of this pathogen. In larvae, the fungus digests only mouthparts (beaks and labial denticles), because larval skin does not contain keratin. After the larvae metamorphose, infection can spread to the epidermis (containing keratin) and may result in death within 2–3 weeks of metamorphosis

(Berger et al., 1998). In some cases, infection can occur without causing death, and it may be possible for anurans to develop resistance to this pathogen over time (Retallick et al., 2004).

Several modes of global transmission of *Batrachochytrium dendrobatidis* have been proposed (Daszak et al., 1999). Of immediate relevance to Thailand is the introduction of non-native anurans such as the pipid, *Xenopus laevis*, and the ranid, *Rana catesbeiana*. Based on a survey of museum specimens, Weldon et al. (2004) proposed that *B. dendrobatidis* originated in Africa and was spread as a result of the increase in the global transport of *X. laevis*, a species commonly used in research and sold in the pet trade. Given that *X. laevis* is a known carrier of *B. dendrobatidis*, it is likely that the trafficking of *X. laevis* through the Thailand pet trade (pers. obs.) has introduced *B. dendrobatidis* into Asia. Introduced extensively around the globe as a human food source, *Rana catesbeiana* may act as a silent carrier of the *B. dendrobatidis* pathogen, because larvae and adults can be infected, but appear asymptomatic (Mazzoni et al., 2003; Daszak et al., 2004). *Rana catesbeiana* was first introduced into Thailand in 1980 for farming purposes because of its higher growth rate when compared to local edible frogs (Pariyanonth & Daorerk, 1994). This introduction, coupled with the cultural practices of releasing live animals purchased at markets or temples in order to make merit, poses a serious conservation threat to many native species, especially with respect to the spread of this pathogen.

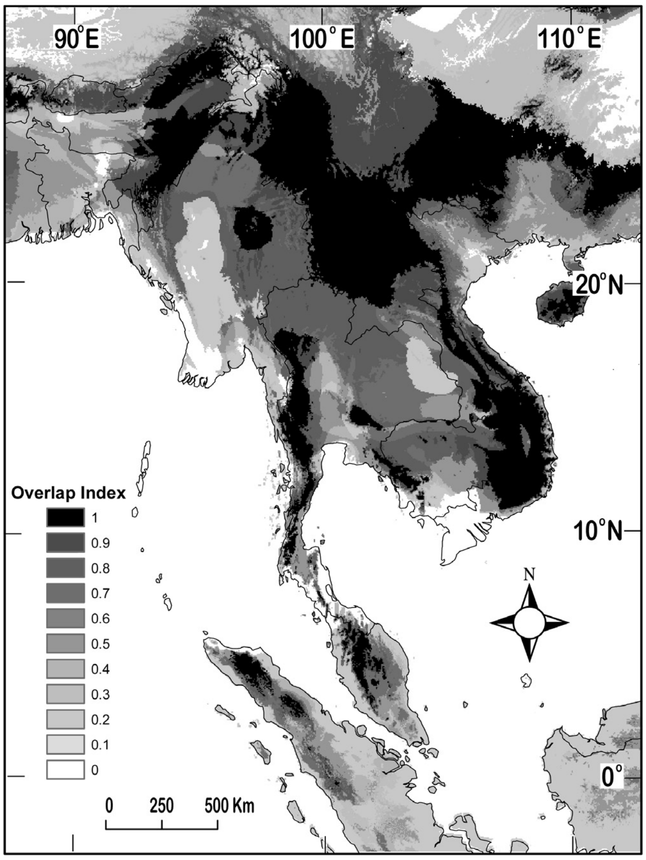

Fig. 1. Predicted distribution of the fundamental niche of *Batrachochytridium dendrobatidis* in Southeast Asia (redrawn from Ron, 2005). Dark areas represent those regions where niche presence was predicted by more models (i.e., Overlap Index 1 means that 10 of 10 models predicted niche presence; 0 means that none of the 10 models predicted niche presence). Models are based on known New World occurrences of *B. dendrobatidis*.

This investigation began in 2003 when one of us (JS) observed six dead and dying frogs in Khao Yai National Park. Four frogs had blue/white opaque eyes, one was unable to move its lower body, and two were dead. None of these frogs showed any other signs of trauma (no obvious lesions or wounds), nor did they appear to be starved. Although it was not possible to study these individuals it prompted us to organize a survey for *Batrachochytrium dendrobatidis* in both historical and recent amphibian specimens from Thailand.

MATERIALS AND METHODS

Between 2004 and 2005, we surveyed preserved amphibian specimens housed at the Natural History Museum of Chulalongkorn University. Two batches of samples were prepared for histological examination. The first sample was focused on specimens collected from 1998–2004 at various sites within Thailand, the second on historical specimens from 1957–1960 (Fig. 2). A total of 123 amphibian specimens representing 28 species (Table 1) was sampled during this preliminary study. Of these, 59 specimens were collected in the past 7 years, and 64 were taken more than 45 years ago. In all cases, skin samples (approx. 1 cm^2) were taken from the venter just anterior to the groin, approximately along the midline of the body. In frogs with more than one-third webbing between toes, a second sample was taken from between Toes 4 and 5 on the animal's right hind foot; the webbing was simply cut from between the digits in a V-pattern to produce a tissue sample that was slightly smaller than that removed from the venter.

Whole specimens were originally preserved in a variety of ways, but most were originally fixed in 10% formalin, and then stored in 70% EtOH. Others were preserved directly in 95% EtOH without formalin fixation. To allow for histochemical treatment of specimens, all samples were soaked in water overnight in a refrigerator and then transferred to 10% buffered formalin.

Sampling efforts were focused on those taxa most closely associated with riparian habitats (primarily ranids). Additional specimens were included to represent rhacophorids and bufonids, the larvae of which occur in aquatic environments, in contrast to the adults, which tend to be less closely tied to water. Our purpose was to sample a breadth of taxa from as many locations in Thailand as possible; thus, taxonomic and locality sampling was non-random and reflects the availability of materials.

We used standard histochemical staining techniques (Humason, 1972) to screen tissues for *B. dendrobatidis* because of the availability, accuracy, and cost effectiveness of this diagnostic tool. Skin samples were fixed in 10% buffered formaldehyde for 24 hours, washed with 70%

ethanol, dehydrated and cleared with a series of ethanol and xylene solutions. Samples were then embedded in paraffin, sectioned at 5 µm thickness with a rotary microtome (HistoSTAT, Reichert, USA), and stained with hematoxylin and eosin. All histological specimens were examined for abnormalities using a Nikon E600 light microscope and photographed by a Nikon DXM 1200 digital camera. Alan Pessier, pathologist at the Center for Reproduction of Endangered Species (CRES) of the San Diego Zoological Society examined photographs and confirmed our initial diagnoses.

RESULTS

Batracochytridium dendrobatidis was not observed in any of the tissues examined. These negative results were confirmed by Dr. Pessier (Table 1).

DISCUSSION

This work represents the first effort to test the hypothesis that *Batrachochytrium dendrobatidis* is present, but yet undetected, in Thai frogs. All specimens sampled in our study returned negative results for the presence of *B. dendrobatidis* in Thailand. However, our efforts were by no means exhaustive, and it is important that the search for this amphibian pathogen in Asia continues. We hope this work will serve as a springboard for a more thorough search for *B. dendrobatidis* in Thailand and other parts of Asia.

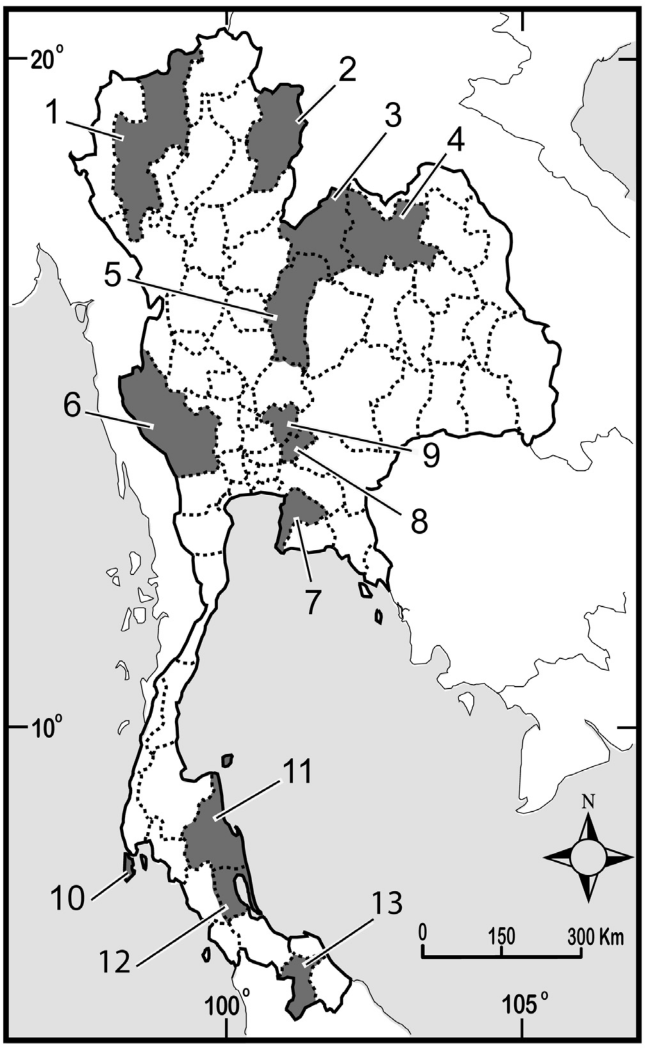

Fig. 2. Distribution of museum specimens sampled from Thailand for the presence of *Batrachochytridum dendrobatidis*. Numbers correspond to provincial names in Table 1.

The development of frog farms in Thailand, particularly those where non-native species (i.e. *Rana catesbeiana*) are maintained, poses some potentially serious ecological threats. There is the immediate problem of the introduction of non-native anurans into the Thai herpetofaunal community. Moreover, because *R. catesbeiana* may act as a reservoir for *B. dendrobatidis* (Mazzoni et al., 2003), the threat of introducing this pathogen into native populations of Thai amphibians is even more imminent. Standard procedures for monitoring farmed populations of anurans for the chytrid fungus would help to ensure that this pathogen is not present. If *B. dendrobatidis* is found, measures should be taken to quarantine infected frogs and additional tests performed to determine whether the fungus has spread to local frog populations.

To detect the presence of *B. dendrobatidis* in Thailand and elsewhere in Asia will require a collaborative effort on the part of field biologists making first-hand observations and collections for study, pathologists capable of screening specimens, and funding agencies that will support these efforts to assess and protect the health of the region's amphibians. On the basis of our work and other contemporary studies, we recommend the following. First, additional attention should be paid to historical museum specimens to test for the presence of this fungus. Second, field projects designed to test the predictive niche models of Ron (2005) should be undertaken. When conducting field studies, data collection should include geo-referenced localities and elevation records. Third, standard-practice methodologies for regularly screening captive-bred populations of native and non-native amphibians should be developed and implemented. Finally, amphibians available in the pet trade should be screened regularly using non-invasive methods to ensure that this pathogen is not being inadvertently introduced to native stocks.

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Table 1. Specimens examined in Thailand for infection by *B. dendrobatidis* in this study. In each case the test was negative. Original collection date and locality information are presented whenever available. NA = information not available. WKC = un-numbered research collection of Wichase Khonsue (Chulalongkorn University, Bankgok, Thailand). Numbers in parentheses associated with locality data refer to provincial numbers in Fig. 2.

Family Species	**CUBMZA No.**	**Collection Date**	**Locality**	**No. of specimens**
Bufonidae				
Bufo asper	1579–1580	16 Jun.1960	(13) Betong, Yala	2
Bufo macrotis	756	27 Sep.1958	(1) Mong Haut, Chiang Mai	1
Bufo melanostictus	WKC	12–15 Jul.2003	(3) Phu Luang, Loei	1
	2005.07.30.59	25 Nov.2004	(11) Khao Luang, Nakhon Si Thammarat	1
Ranidae				
Amolops sp.	WKC	9 Feb.2002	(6) Kanchanaburi	1
Fejervarya cancivora	35061,65,67	14–16 Mar.1958	NA	3
	711–12	9 Feb.1960	NA	2
	34968	NA	NA	1
	1005	16 Apr.1960	NA	1
	2000.32	NA	NA	1
Fejervarya limnocharis	WKC	17–18 Oct.2003	(2) Nan	1
	WKC	22 Mar.2003	(3) Wangoapung, Loei	5
	WKC	31 May–1 Jun.2003	(8) Nakhon Nayok	5
	WKC	16–17 May 2004	(5) Nam Now, Petchaboon	1
Hoplobatrachus chinensis	WKC	17–18 Oct.2003	(2) Nan	1
Ingerana tassanae	WKC	9 Feb.2002	(6) Kanchanaburi	1
Leptobrachium sp.	WKC	12–15 Jul.2003	(3) Phu Luang, Loei	1
Limnonectes blythi	WKC	9 Feb.2002	(6) Kanchanaburi	1
	1154–57, 59–60	NA	NA	6
	2005.07.30.57 2005.07.30.60	5 Nov.2004	(11) Khao Luang, Nakhon Si Thammarat	2
Limnonectes kuhlii	1183, 85–88; 1190–92,95; 1200–02	14–16 Mar.1958	(3) Phu Kra Dung, Loei	12
	WKC	9 Feb.2002	(6) Kanchanaburi	2
Limnonectes laticeps	1142–46	NA	NA	5
Limnonectes macrognathus	2005.07.30.130	15 Nov.2004	(11) Khao Luang, Nakhon Si Thammarat	1
Limnonectes pileata	WKC	12–15 Jul.2003	(3) Phu Luang, Loei	1
Megophrys aceras	2005.07.30.10	25 Nov.2004	(11) Khao Luang, Nakhon Si Thammarat	1
Occidozyga martensi	1203	21 Dec.1957	(1) Doi Suthep, Chiang Mai	1
	1287; 1328; 1418	23 Apr.1960	(7) Sri Racha, Chonburi	3
	1380	Mar.1958	(4) Udon Thani	1
	1381	18 Mar.1958	(3) Phu Kru Dung, Loei	1
	1337	14–16 Mar.1958	(3) Phu Kading, Loei	1
	WKC	16–17 May 2004	(5) Nam Now, Petchaboon	1
	WKC	12–15 Jul.2003	(3) Phu Luang, Loei	1
Occidozyga lima	WKC	17–18 Oct.2003	(2) Nan	1

Table 1. Cont'd.

Family Species	**CUBMZA No.**	**Collection Date**	**Locality**	**No. of specimens**
Rana erythraea	1853	NA	NA	1
	1111	NA	"South Thailand"	1
	1112	6 Jun.1960	(10) Bhuket Besa, Phuket	1
	1109; 1118	1960	(13) Bethong, Yala	2
	1115	NA	(11) Ronpibul; Nakhon Si Thammarat	1
	1110	26 Jan.1958	(13) Bethong, Yala	1
	1113	Dec.1957	(1) Doi Suthep, Chiang Mai	1
	WKC	17–18 Oct.2003	(2) Nan	1
	2005.07.30.5	16 Nov.2004	(11) Khao Luang, Nakhon Si Thammarat	1
	2005.07.30.8	8 Nov.2004	(11) Khao Luang, Nakhon Si Thammarat	1
	WKC	16–17 May 2004	(5) Nam Now, Petchaboon	1
Rana hosii	WKC	9 Aug.2003	(8) Khao Yai, Nakhon Nayok	1
Rana nigrovittata	WKC	14 Aug.2004	(8) Khao Yai, Nakhon Nayok	5
	WKC	9 Aug.2003	(8) Khao Yai, Nakhon Nayok	3
	2005.07.30.3	17 Nov.2004	(11) Khao Luang, Nakhon Si Thammarat	1
	2005.07.30.145	24 Nov.2004	(11) Khao Luang, Nakhon Si Thammarat	1
Rhacophoridae				
Polypedates leucomystax	WKC	16–17 May 2004	(5) Nam Now, Petchaboon	1
	1720	Mar.1958	(4) Udon Thani	1
	1718	21–26 Mar.1958	(4) Udon Thani	1
	1716–17	16–23 Apr.1960	(7) Sri Racha, Chonburi	2
	1712, 14, 21	21 Dec.1957	(1) Chiang Mai	3
	1719	8–9 Dec.1957	(7) Ang Hin, Chonburi	1
Chirixalus sp.	WKC	11 Jan.2003	(1) Kam Phang Pet, Chiang Mai	5
Icthyophidae				
Ichthyophis kohtaoensis	WKC	14 Jan.1998	(12) Pattalung	2
Microhylidae				
Microhyla berdmorei	WKC	12–15 Jul.2003	(3) Phu Luang, Loei	1
	2005.07.30.129	1 Dec.2004	(11) Khao Luang, Nakhon Si Thammarat	1
Microhyla ornata	WKC	16–17 May 2004	(5) Nam Now, Petchaboon	1
	WKC	17–18 Oct.2003	(2) Nan	1
Microhyla pulchra	803–4; 815–16	NA	(1) Kaeng Pang Tao, Chiang Mai	4
	805	5–8 Dec.1957	(1) Chiang Mai	1
	808	16 Dec.1957	(1) Doi Suthep, Chiang Mai	1
	806	NA	"Village on Mae Kong River"	1
Salamandridae				
Tylototriton verrucosus	WKC	2004	(3) Pu Luang, Loei	1
	WKC	2004	(3) Puttin Long Cha, Loei	2

incorporate these into this manuscript. Special thanks to Alan Pessier of CRES for generously lending his time and advice, and to Dr. Kumthorn Thirakhupt, Anchalee Aowphol and the other members of Chulalongkorn University's "Thai Turtle lab" who helped secure data and make this work possible.

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