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Survey of Turkey's endemic amphibians for chytrid fungus *Batrachochytrium dendrobatidis*

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ABSTRACT: We report a new survey for *Batrachochytrium dendrobatidis* (*Bd*) in Turkey. We swabbed 228 individuals of 7 amphibian species (from 5 families) living in 2 locations (26-August National Park and the Turkish Lakes District) in the southwestern Anatolian region. The infection intensity of all the samples was determined using quantitative PCR. All 4 amphibian breeding sites and 4 amphibian species in 26-August National Park were infected by *Bd*, with the prevalence at each site ranging from 8 to 29%. Only 1 species was sampled from Beysehir Lake near the conservation area Beysehir Natural Park, but these samples were notable for their high detection rates (prevalence of 32.11%). This study reports the first records of *Bd* infecting wild *Pelophylax ridibundus, Hyla orientalis, Pseudepidalea variabilis*, and endemic Beysehir frogs *Pelophylax caralitanus*.

KEY WORDS: *Bd* · Chytridiomycosis · qPCR · Endemic frogs

INTRODUCTION

Chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is an emerging infectious amphibian disease that has been associated with population declines in multiple regions worldwide (Berger et al. 1998, Longcore et al. 1999). Both natural and anthropogenic causes can affect declines via emergence of *Bd* in amphibian populations (Bosch et al. 2001, Bradley et al. 2002, Daszak et al. 2003, Stuart et al. 2004, Bosch & Martinez-Solano 2006). *Bd* has been hypothesized to spread through amphibian dispersal, downstream via water and sand, and possibly by bird, human, insect, or other animal movement (Kilpatrick et al. 2010). Europe's amphibians have been declining for decades, and habitat fragmentation and/or destruction are the major causes (Stuart et al. 2004). To date, the rapid and widespread distribution of *Bd* infection across hundreds of amphibian species is alarming.

Bd invades the surface layer of the animal's skin and infects the superficial, keratin-containing layers. In frog tadpoles, the mouthparts are keratinized and are thus susceptible to *Bd* infection, leading to mouth-part depigmentation and defects (Berger et al. 1998, Rachowicz & Vredenburg 2004). Additionally, an amphibian with chytridiomycosis can present a wide variety of clinical signs. Some of the most common signs are abnormal postures and unnatural behaviors. Many of these signs are considered 'nonspecific,' and many different amphibian diseases cause symptoms that overlap with those of chytridiomycosis. Infected frogs begin to die roughly 21 d post-infection, and although larval stages are susceptible to infection, deaths are generally restricted to the adult life stage.

A number of researchers have reported that quantitative PCR (qPCR) is generally a more sensitive technique than histological examination for detecting *Bd* infections in amphibians (Kriger et al. 2006,

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Hyatt et al. 2007). For example, Baiet al. (2010) did not find typical histological signs of *Bd* in qPCR-positive samples, possibly because the zoospore loads in the samples were too low to be detected by a histological examination. Other advantages of the qPCR assay are that many samples can be processed within a single day with small amounts of skin swabs, and that samples can be obtained non-destructively, e.g. when endangered species are being examined.

Of the 26 amphibian species found in Turkey, many are listed in the IUCN Red List (www.iucnredlist.org). Many of the causes of amphibian declines are still poorly understood in Turkey. Although rapid declines are often obvious and are easily detected, gradual declines are more difficult to identify without long-term monitoring (Erismis 2011). According to Ron (2005), *Bd* was predicted to spread in Anatolia, but the geographic distribution of *Bd* and its effect on Turkish amphibians is poorly understood (Farrer et al. 2011; www.bd-maps.net). Recently, its presence in Levant green frogs *Pelophylax bedriagae* was shown by Göçmen et al. (2013). However, both endemic and non-endemic frog species need to be screened for *Bd* infection. The aim of our study was to screen for *Bd* in 2 different geographical regions of western Turkey (Table 1).

MATERIALS AND METHODS

The study was carried out in 2 different areas. The first location, 26-August National Park, is a protected area (40.742 ha) at 950 to 1900 m altitude in the central West Anatolian Region of Turkey, with 2 natural lakes (Akoren Lake, AKL, and Golcuk Lake, GCL), and 2 ponds (Tınaztepe pond, TNP, and Erkmen pond, EKP). Seven amphibian species (*Pelophylax caralitanus*, *Pelobates syriacus*, *Pseudepidalea variabilis*, *Hyla orientalis, Triturus karelinii, Pelophylax ridibundus,* and *Rana macrocnemis*) are known to inhabit the area (see Table 2; Kaya & Erismis 2001, Erismis 2011). Field work was conducted between 15 March and 18 April 2013, principally in the vicinity of 26-August National Park; weather conditions were also recorded during the field work at this site.

The second location was in the Lakes District of southwestern Anatolia, where we surveyed 3 lakes, viz. Beysehir Lake (BYL), Egirdir Lake (EGL), and Isikli Lake (ISL) (Table 1), which range in altitude between 918 and 1135 m above sea level. The specimens from this location were collected between 21 and 26 June 2012. Air temperature, water pH, and humidity were recorded during the field work.

In the *Bd* sampling process, adults were swabbed 30 times along keratinized tissues where *Bd* zoospores are highly concentrated (Marantelli et al. 2004). We followed the standardized sampling protocol detailed by Hyatt et al. (2007), Marantelli et al. (2004), and Kriger & Hero (2007). Swabs (Medical Wire Company MW113) were stored in 95% ethanol. The infection intensity of all samples was determined by using qPCR (Boyle et al. 2004, Hyatt et al. 2007), with modifications of the methods described by Boyle et al. (2004): we extracted nucleic acids using 50 µl PrepMan Ultra (Applied Biosystems), and the tip of the swab was used instead of a toe. To ensure the integrity of our results, a negative control (dH_2O) was run in triplicate on every 96-well PCR plate (Kriger et al. 2006). Infection intensity was measured as the

Table 1. Study sites in Turkey where amphibians were examined for *Batrachochytrium dendrobatidis* infection. Depth: maximum depth of the lake; Temp.: mean air temperature during the study period; pH: water pH; Area: water surface area. The dominant vegetation in surrounding habitats at all sites were *Ceratophyllum demersum, Phragmites australis*, and *Schoenoplectus lacustris* on the muddy banks

Site	Code	Coordinates N	Coordinates E	Elevation (m a.s.l.)	Depth (m)	Temp. (°C)	pH	Area (km^2)	Anthropogenic influences
26-August National Park, Afyonkarahisar Province									
Akoren Lake	AKL	38°47′37″	30°22′49″	1064	2.5	22	6.9	16.5	Domestic waste, urban sewage water
Golcuk Lake	GCL	38°41′37″	30°30′27″	1375	3.5	9	7.2	7.5	Agricultural chemicals
Tınaztepe Pond	TNP	38°43′04″	30°23′14″	1147	5	17	7.3	10.5	Domestic waste, municipal sewage water
Erkmen Pond	EKP	38°45′13″	30°29′20″	1128	8	21	7.6	9.6	Agricultural chemicals, domestic waste
Turkish Lake District, Afyonkarahisar and Isparta Provinces									
Isikli Lake	ISL	37°40′08″	31°42′51″	930	5	30.1	7.1	49	Agricultural chemicals
Egirdir Lake	EGL	37°53′01″	30°52′12″	970	14	25.7	7.7	482	Agricultural chemicals, municipal waste
Beysehir Lake	BYL	37°40′08″	31°42′51″	1135	10	24.9	8.2	656	Agricultural chemicals, industrial discharges, municipal waste

number of genome equivalents (GE) per swab, calculated by multiplying the genome equivalent values generated during the qPCR by the dilution factor of the template DNA. Swabs were categorized as *Bd*-positive at ≥1 GE and as *Bd*-negative at <1 GE (Savage et al. 2011). All analyses were performed in triplicate.

The percentages of infected individuals and GEs were compared among and within sites using chi-squared contingency tests and 1-way ANOVAs, respectively. In order to determine the accuracy of these percentages, their respective 95% confidence intervals (CIs) were calculated.

RESULTS

During the work at 26-August National Park, the temperatures ranged from 9 to 22°C (mean = 17.25°C), and humidity was between 38 and 56%. Adults of 7 species (*Pelophylax caralitanus*, *Pelobates syriacus*, *Pseudepidalea variabilis*, *Hyla orientalis*, *Triturus karelinii*, *Pelophylax ridibundus*, and *Rana macrocnemis*) were analyzed for *Bd* infection at this location. We observed that 19 of 104 adult specimens were infected by *Bd*. We also found that 4 amphibian breeding sites in this area were infected by *Bd*, with prevalence ranging from 8 to 29% (Table 2, data on negative sites not shown). The prevalence of *Bd* infection among EKP, TNP, AKL, and GCL did not differ significantly ($\chi^2 = 5.08$, df = 3, p = 0.16).

P. ridibundus and *P. variabilis* were notable for their higher *Bd* infection rates at AKL, with prevalences of ~42.9% and 23.1%, respectively. In addition, the populations of both *P. variabilis* and *H. orientalis* exhibited great developmental plasticity, and they did not differ significantly in their *Bd* infection prevalence ($\chi^2 = 0.41$, df = 3, p = 0.09); however, we detected *Bd* in only a single specimen of oriental tree frog *H. orientalis*. Furthermore, we did not detect any *Bd* on Syrian spadefoots *P. syriacus*. The low estimated zoospore load in mean genome equivalents (GE) ± SE across all sites with positives was 0.35 ± 0.04 for adult amphibian species in 26-August National Park. The average GEs among the EKP, TNP, AKL, and GCL amphibians infected by *Bd* were analyzed through multiple comparisons based on a Tukey-HSD post hoc test, which indicated a significant difference among them ($F = 0.26$, df = 3.15 p = 0.08). The average GE of amphibian species infected by *Bd* at AKL was slightly higher than at the other 3 localities (Table 2).

Table 2. Amphibian species examined for *Batrachochytrium dendrobatidis* (*Bd*) infection in Turkey: number of animals sampled (n), number of animals in which infection was detected (+ve), prevalence (Prev.), and mean *Bd* genome equivalents (GE, a proxy for infection intensity). See Table 1 for study site details

Species	Parameter	26-August National Park				Turkish Lake District			All sites
		AKL	GCL	TNP	EKP	ISL	EGL	BYL	
Beysehir frog	n (+ve)	–	9 (1)	–	–	39 (8)	57 (8)	28 (9)	133 (26)
Pelophylax caralitanus	Prev. (%)	–	11.1	–	–	20.5	14.0	32.1	19.5
	GE	–	20.4	–	–	350.5	365.4	246	306.2
Variable toad	n (+ve)	13 (3)	7 (1)	7 (1)	9 (2)	–	–	–	36 (7)
Pseudepidalea variabilis	Prev. (%)	23.1	14.2	14.2	22.2				19.4
	GE	30.6	10.8	30.3	30.2	–	–	–	27.6
Oriental tree frog	n (+ve)	6 (1)	3 (0)	–	1 (0)	–	–	–	10 (1)
Hyla orientalis	Prev. (%)	16.7	0	–	0	–	–	–	10
	GE	20.8	–	–	–	–	–	–	20.8
Marsh frog	n (+ve)	14 (6)	–	11 (2)	15 (2)	–	–	–	40 (10)
Pelophylax ridibundus	Prev. (%)	42.9	–	18.2	13.3	–	–	–	25.0
	GE	50.7	–	40.8	40.4	–	–	–	46.7
Long-legged wood frog	n (+ve)	–	–	3 (0)	–	–	–	–	3 (0)
Rana macrocnemis									
Syrian spadefoot	n (+ve)	1 (0)	–	–	–	–	–	–	1 (0)
Pelobates syriacus									
Southern crested newt	n (+ve)	–	6 (0)	–	–	–	–	–	6 (0)
Triturus karelinii									
All species	n (+ve)	34 (10)	25 (2)	21 (3)	25 (4)	39 (8)	57 (8)	28 (9)	229 (44)
	Prev. (%)	29.4	8.0	14.3	16.0	20.5	14.0	32.1	19.2
	GE	40.2	20.1	40	3.8	350.5	365.4	246	196.4

In the second sampling location, the temperatures ranged from 23.3 to 33.6°C (mean = 27.42°C), and humidity was between 44 and 68% (mean 55.83%). The samples from BYL, near the conservation area Beysehir Natural Park, were notable for their higher *Bd* infection rates with a prevalence of ~32.11% (CI 20–44%). *Bd* infection prevalence was 20.5% (CI 10.7–30.5%) at ISL. Furthermore, *Bd* was detected in Beysehir frogs from EGL with a minimal prevalence of 14% (CI 6–23%). Infection percentages of *Bd* did not differ significantly among the lakes at this location (χ^2 = 3.81, df = 2, p = 0.14; Table 2).

The highest total GE was found at EGL, followed by ISL. However, GE of *Bd* did not differ significantly among lakes (1-way ANOVA, F = 0.46; df = 7, p = 0.65).

DISCUSSION

Anatolia is at the intersection point of Asia, Europe, and Africa, and this feature makes it rich in biological diversity, due to its varied habitats suitable for foraging, finding shelter, and migrating. The geographical features of Turkey contribute not only to a rich biodiversity but also to epidemics of some life-threatening and pathogenic illnesses. A long-term monitoring study by Erismis (2011) revealed a gradual decline in populations of *Pelophylax ridibundus* from 26-August National Park. Many of the amphibian populations in Turkey are classed as Near Threatened. Local declines of several species have been principally attributed by the IUCN to 2 threats: habitat destruction and pollution, whereas pathogens have not been considered as threats for decades. We have shown that *Bd* constitutes a third potential threat to Turkish amphibians.

To our knowledge, there is only 1 previous report on *Bd* among amphibians in Turkey, which indicated infection in Levant green frogs *Pelophylax bedriagae*, although this species is widely dispersed in Europe and has also been reported from Asia and Africa (Göçmen et al. 2013, Olson et al. 2013). With Turkey's location at the nexus of 3 continents, the presence of *Bd* is not surprising. Furthermore, Mediterranean and the Central Anatolian environmental conditions are considered favorable for the persistence of *Bd*, based on ecophysiological studies of this pathogen (Piotrowski et al. 2004, Kriger & Hero 2007). BYL and EGL have distinct climate conditions because of their location between the Mediterranean and the Central Anatolian transition zone. The climate of these 2 lakes is cool, with annual average temperatures ranging between –0.3 and +26.5°C and an annual total precipitation of 700 to 1300 mm. ISL has a mild climate, a combination of Aegean, continental, and Mediterranean climates. Winters at ISL are rainy and mild, and the average annual average temperature is 14.2°C (range = 2.3–28.5°C; Yılmaz 2009).

According to Ron (2005), *Bd* is predicted to spread to areas that support its ecological niche, which in most models includes Anatolian forests and steppes, and Europe south of about 55° N. Our study suggests that the Anatolian climate is indeed favorable for the spread of chytridiomycosis.

We detected *Bd* in 19 out of 105 individuals from 4 sites at the first study location. Four of 7 amphibian species were infected, and marsh frogs *Pelophylax ridibundus* had the highest prevalence (42.85% at AKL). We also detected the first record of *Bd* infection in endemic Beysehir frogs *Pelophylax caralitanus*. The low estimated zoospore load in mean GE ± SE across all sites with positives was 0.35 ± 0.04 for adult amphibian species in the central West Anatolian Region of Turkey.

Although the samples from BYL were notable for their higher *Bd* infection rates, infection percentages and GEs of *Bd* did not differ significantly among the lakes studied.

This study is the first report of *Bd* in wild *P. ridibundus, Hyla orientalis, Pseudepidalea variabilis*, and *P. caralitanus* (Table 2). Although our sampling sizes and study areas were limited, our results provide some more evidence for *Bd* infection among Turkey's amphibian populations. Our findings of *Bd*-positive individuals within a limited study area underscores the need for a larger survey in Turkey to determine the level of threat. If chytridriomycosis becomes a serious problem for amphibians in such montane refuges, the survival of these species will be seriously jeopardized.

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