Potential Distribution of the Amphibian Pathogen, *Batrachochytrium dendrobatidis* in the Eastern Black Sea Region of Turkey

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**A B S T R A C T**

Although *Batrachochytrium dendrobatidis*, pathogen for amphibians, has been reported in Anatolia, its geographical distribution, as well as its impact on the amphibians in Turkey, remained obscure. In this study, 62 adult individuals belonging to ten different species (*Pelodytes caucasicus, Rana dalmatina, Rana macrocnemis, Bufo bufo, Bufo verrucosissimus, B. variabilis, Hyla savignyi, Pelophylax ridibundus, Ommatotriton ophryticus, and Mertensiella caucasicus*) were collected from five wetland habitats in Eastern Black Sea Region of Turkey. The prevalence and the intensity of *B. dendrobatidis* infections in all the individuals were investigated by using quantitative Real-time PCR technique and the presence of *B. dendrobatidis* infection was reported for the first time in 13 of the 62 individuals collected from 10 amphibian species from Eastern Black Sea Region of Turkey. The intensity of *B. dendrobatidis* infection ranged from 403.520 to 534.280 genomic equivalents (GE) was detected. The highest GE between amphibian species were determined in *P. caucasicus* (534.280 GE) in Uzungöl (Çaykara-Trabzon) and *B. bufo* (504.00 GE) in Lake Karagöl (Şavşat- Artvin).

Keywords: Chytridiomycosis, *Batrachochytrium dendrobatidis*, Anatolia, Amphibia

**Introduction**

Infectious diseases are one of the factors implicated in the declines and extinctions of amphibians in worldwide. *Batrachochytrium dendrobatidis* (*Bd*) is a fungus that colonizes amphibian skin and the associated disease, chytridiomycosis, can disturb cutaneous respiration and osmoregulation and result in the death of the host (Carver et al. 2010). Differences in ecological factors such as host population density, habitat, and age structure may influence the rate at which chytrid spreads through the environment (Daszak et al. 1999). 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). Though 26 Turkish amphibian species are listed in the International Union for Conservation of Nature (IUCN) Red List,
investigation of the decrease in frog population has become mandatory (Başkale et al. 2013). Only two works were carried out on the distribution of Bd in Turkey. Previously, Göçmen et al. (2013) reported that one of two P. bedriagae specimens from Göynük Canyon (Antalya) was found as positive for Bd. Erişmiş et al. (2014) reported Bd infecting wild P. ridibundus, H. orientalis, B. variabilis as well as endemic Beysehir frogs P. caralitanus in West Anatolian Region and the District Lakes of South Western Turkey.

Thirty-six amphibian species were recorded in Turkey. Due to Turkey’s geographical position, different species spread in different regions and they are exposed to a great number of threats (Şekercioğlu et al. 2011). This includes a number of restricted and rare amphibian species such as Rana tavaensis (Franzen et al. 2008), R. holtzi (Yildiz and Göçmen 2012), R. macrocnemis (Veith et al. 2003), P. caralitanus (Bülbül et al. 2011). If such species were susceptible to the fungal infection, the local and isolated populations might easily become extinct. Hence, the location of pathogens and susceptible species are needed to be determined in Turkey. Management strategies for the containment of Bd spreading include the detection of wild and captive populations infected with chytrid disease, the identification of infected geographical areas, and the control of infected animal’s movement from one location to another.

Therefore, the main objective of the present study was to determine Bd infected amphibian species through quantitative polymerase chain reactions (qPCR) (Kriger et al. 2006; Hyatt et al. 2007) in the Eastern Black Sea Region of Turkey.

**Materials and Methods**

The study was carried out in 6 different areas [Uzungöl (Çaykara-Trabzon, UZL), Karagöl (Şavşat-Artvin, KRL), Sahara Natural Park (Şavşat-Artvin, SNP), Ardeşen (Rize, ARD), Lake Şavsat (Şavşat-Artvin, SVT)] at 39 to1876 m elevation (E) in the eastern Black Sea region (EBS) of Turkey (Figure 1).

The specimens were collected during the summer season of 2014 (June through August). Air temperature, water pH, and humidity (H) were recorded during the fieldwork (Table 1). For each captured animal, surveyors recorded its GPS coordinates. To prevent the transfer of infected materials among sites, we rinsed all equipment with 5% bleach before entering each location. All of the frogs were handled with latex gloves and gloves were discarded after examination of each animal (Bai et al. 2010). The distribution of zoosporangia of Bd was studied in collected 10 species [P. caucasicus (Pc), R. dalmatina (RD), R. macrocnemis (Rm), B. bufo (Bb), B. verrucosissimus (Bv), B. variabilis (Bvs), H. savignyi (Hs) P. ridibundus (Pr), O. ophryticus (Oo), M. caucasicus (Mc)].

To determine whether the animal was infected with Bd or not by PCR analysis, tissue samples were collected using swab method with a sterile cotton tip swab to take the keratinized tissues where Bd zoospores were highly concentrated (Marantelli et al. 2004). During Bd sampling process, each individual
was swabbed 30 times. We followed the standardized sampling protocol detailed by Hyatt et al. (2007). Samples were stored in 95% ethanol and were kept on ambient temperature (≥10°C) in the field and transported back to the laboratory and stored in a −80°C freezer (Hyatt et al. 2007). The intensity of infection in all samples was determined by using qPCR (Boyle et al. 2004; Hyatt et al. 2007), with the modifications of methods described by Boyle et al. (2004). It was 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 (dH2O) was run in triplicate on every 96-well PCR plate (Kriger et al. 2006). We constructed a standard curve to determine the zoospore load. A standard curve was constructed from the control reactions containing 100, 10, 1 and 0.1 Bd zoospores and the concentration determined for the test samples expressed as the number of zoospore equivalents. The intensity of infection was measured as the number of genome equivalents (GE) per swab, calculated by multiplying the GE 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 (Kriger et al. 2006; Hyatt et al. 2007; Anna et al. 2011; Erismis et al. 2014). All analyses were performed in triplicate. The percentages of infected individuals and GE were not compared among within sites or the species due to the low statistical power of small sample sizes for each species at a site. In the localities with positive Bd, we used the zonal statistic routine to extract from the digital maps the environmental variables values from each point (ArcView 3.2, Spatial Analyst). These values were also used to run Principal Component Analysis (PCA; implemented in XLSTAT v.3.0) to visualize the degree of clustering in environmental space among EBS region of Turkey where Bd was found.

Results

We swabbed 62 individuals from the 8 genera, including 10 species that occur in EBS region of Turkey. The prevalence and the intensity of Bd infections in all the individuals were investigated by using quantitative real-time-PCR technique and the presence of Bd infection was reported for the first time in 13 of the 62 individuals collected from 10 amphibian species from EBS Region of Turkey (Figure 2, Table 1).

*Bd* was not detected only in ARD region. We determined the presence of *Bd* infection in 13 out of 62 (20.9%) samples comprising six species: *Pelobates caucasicus* (Caucasian type-specific), *Bufo bufo*, *B. verrucosissimus*, *Bufotes variabilis*, *Pelophylax. ridibundus*, *Ommatotriton ophryticus* (Table 1).

![Figure 2](image_url). Map of *Bd* prevalence on EBS of Turkey. EBS from all states sampled tested positive and negative for *Bd*. Positive (black) and negative (white) proportions by the state were indicated by pie charts.
**Table 1.** Prevalence (Prev) and Average Genomic Equivalents (GE) of *Bd* in Eastern Black Sea Region of Turkey

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
<th>Coordinates</th>
<th>Air °C</th>
<th>H %</th>
<th>E (a.s.l)</th>
<th>N (+ve) (%)</th>
<th>GEs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pc</td>
<td>UZL</td>
<td>40°37′23″N - 40°16′50″E</td>
<td>26.0</td>
<td>56.0</td>
<td>1164</td>
<td>12(2)</td>
<td>16.0</td>
</tr>
<tr>
<td>Rd</td>
<td>UZL</td>
<td>40°36′24″N - 40°18′48″E</td>
<td>19.5</td>
<td>78.0</td>
<td>1271</td>
<td>1(0)</td>
<td>-</td>
</tr>
<tr>
<td>Rm.</td>
<td>UZL</td>
<td>40°35′18″N - 40°21′19″E</td>
<td>18.5</td>
<td>82.0</td>
<td>1701</td>
<td>2(0)</td>
<td>-</td>
</tr>
<tr>
<td>Bb</td>
<td>KRL</td>
<td>40°37′07″N - 40°18′45″E</td>
<td>19.5</td>
<td>78.0</td>
<td>1272</td>
<td>4(0)</td>
<td>-</td>
</tr>
<tr>
<td>Bv</td>
<td>UZL</td>
<td>40°37′11″N - 40°17′36″E</td>
<td>19.5</td>
<td>78.0</td>
<td>1278</td>
<td>10(1)</td>
<td>10.0</td>
</tr>
<tr>
<td>Mc</td>
<td>UZL</td>
<td>40°35′27″N - 40°20′45″E</td>
<td>27.5</td>
<td>72.3</td>
<td>1702</td>
<td>3(0)</td>
<td>-</td>
</tr>
<tr>
<td>Bvs</td>
<td>SNP</td>
<td>41°14′24″N - 41°13′12″E</td>
<td>23.0</td>
<td>77.0</td>
<td>1876</td>
<td>2(1)</td>
<td>0.50</td>
</tr>
<tr>
<td>Hs</td>
<td>ARD</td>
<td>41°11′13″N - 40°59′19″E</td>
<td>28.0</td>
<td>68.0</td>
<td>39.0</td>
<td>2(0)</td>
<td>-</td>
</tr>
<tr>
<td>Pr</td>
<td>KRL</td>
<td>41°14′24″N - 41°13′12″E</td>
<td>23.0</td>
<td>77.0</td>
<td>1876</td>
<td>15(5)</td>
<td>33.3</td>
</tr>
<tr>
<td>Oo</td>
<td>SVT</td>
<td>41°17′47″N - 42°28′37″E</td>
<td>30.5</td>
<td>66.0</td>
<td>1409</td>
<td>3(2)</td>
<td>66.6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>62(13)</td>
<td></td>
<td></td>
<td>20.9</td>
<td>473.66±18.44</td>
</tr>
</tbody>
</table>

Sampling localities and examined frog species abbreviations used: UZL: Uzungöl/Trabzon, KRL: Karagöl/Artvin, SNP: Sahara Natural Park/Artvin, ARD: Ardeşen/Artvin, SVT: Savsat Lake/Artvin; Pc: Pelodytes caucasicus, Rd: Rana dalmatina, Rm: Rana macrocnemis, Bb: B. bufo, Bv: Bufo verrucosissimus, Mc: Mertensiella caucasicus; Bv: B. variabilis, Hs: Hyla savigyi, Pr: Pelophylax ridibundus, Oo: O. ophryticus respectively, and H: Humidity, E: Elevation. GE genomic equivalent (including positive samples and negative samples, GE represents the burden of infection with *Bd*).

*Bd* was detected at 4 of 5 sites in EBS Region of Turkey were not being specifying *Bd* may be due to the small number of samples (Figure 1). *B. bufo* were sampled at two locations (UZL and KRL), only one of these locations tested positive for *Bd*. Although *P. ridibundus* were sampled at two regions (SNP and KRL) but 6 tested positive for *Bd*. The population of both *P. ridibundus* and *B. bufo* (at KRL) with a prevalence of 25%. However, we detected *Bd* in only one specimen of *B. verrucosissimus* (*n*=10). Furthermore, we did not detect any *Bd* on *R. dalmatina, R. macrocnemis, B. bufo, and M. caucasicus* (at UZL, *H. savigyi* (at ARD). In addition, Northern banded newts (*O. ophryticus*) were notable for their highest *Bd* infection rate at SVT with the prevalence of 66.6 % than other frog species (Table 1). Therefore, the prevalence of *Bd* infection on the populations of 10 frogs species among EBS regions (UZL, KRL, SNP, SVT) did differ significantly (*χ²* = 8.43, *df* = 3, *P* < 0.03).

We also detected the rate of *Bd* infection as the mean number of GE per sample in 3 replicates. The mean number of GE for individual positive samples ranged from 405±9.0 (for *O. ophryticus* at SVT region) to 527±16.0 (for *P. caucasicus* at UZL region). The highest intensity of zoospores was found at *P. caucasicus* (527±16.00) at UZL region followed by *B. bufo* (504.00) at KRL region (Table 1). However, the average GEs among the four regions (UZL, KRL, SNP, SVT) individuals of frogs infected by *Bd* were analyzed through multiple comparisons based on a Tukey-HSD post-hoc test, which indicated a not significant difference among them (*F* = 3.27, *df* = 3, *P* = 0.08). Increasing suitability for both prevalence and GE of *Bd* was widely distributed on EBS regions of Turkey but was detected lowest in the ARD region (Tablo 1).

Final map resulted with areas highly suitable for the presence of *Bd* (Niche Overlap Index (NOI) > 0.70) dispersed irregularly overall EBS Region of Turkey (Figure 3). There are localities with highly suitable for the fungus in north regions of phytogeographic provinces of Trabzon and Artvin (Northeast of Turkey). NOI was varied among regions and was represented by different percentages of covered surface. Areas with 0.70 > OI < 1 (highest suitability for chytrid development) covered only 32.72 % of the total surface while areas with 0.50 > OI < 0.70 covered 56.86 %. Areas with 0.00 > OI < 0.50 only 10.42 of the total surface of EBS region of Turkey (Figure 3).

With eigenvalues >1, Principal Component I was positively correlated with (1) Elevation, (2) mean annual temperature, (2) precipitation of wettest explaining 89.6 % of the variance of the system. Principal Component II explained 29.0% and was
highly positively correlated only with seasonal temperature. The environmental variables in the localities where *Bd* was found show that suitable locations for the fungus are possible across a wide range of habitats (Table 1). In the localities with known presence of the fungus, the annual mean temperature ranged from 5°C (*UZL* and *SVT*) to 23.5°C (*SNP* and *SVT*), moreover in areas where the OI = 0.89. In addition, our analysis shows that the presence of the fungus in *EBS* region of Turkey is related to precipitations between 68 mm (*UZL* and *SVT*) and 235 mm (*UZL* and *SVT*).

**Figure 3.** Niche Overlap Index (NOI) map of the potential distribution of *Bd* in *EBS* region of Turkey

**Discussion**

Distribution models showed that *Bd* has likely already spread to most climatically suitable regions (Fisher et al. 2009). Turkey’s diverse regions have different climates because of irregular topography. Black Sea region has an oceanic climate (Köppen climate classification: *Cfb*), wet and humid (summer 23°C, winter 7°C) (Sensoy et al. 2008). Our previous study suggests that the Anatolian climate is indeed favorable for the spread of chytridiomycosis (Erismis et al. 2014). Intercalarly, *Bd* has been found in five mainlands including North and South America, Europe, Oceana, Africa, and Asia. However, there have only been two published studies up to date describing the presence of *Bd* in Anatolia at the crossroads of three continents. Therefore, it is not surprising that the *Bd* present in Anatolia.

In this study, a total of 62 specimens from 8 genera, including 10 species was sampled. We detected the presence of *Bd* infection in 13 of 62 (20.09%) samples comprising four species: *P. caucasicus* (*Caucasian type-specific*), *B. bufo*, *B. verrucosissimus*, *P. ridibundus*, *O. ophryticus* (Table 1). *Bd* was detected at 4 of 5 sites. We detected *Bd* in only one from ten swamp samples of *B. verrucosissimus*. We did not detect any *Bd* on *R. dalmatina*, *R. macrocnemis*, *B. bufo* (at *UZL*), *B. variabilis*, *H. savignyi* and *M. caucasicus*. In addition, Northern banded newts (*O. ophryticus*) were notable for their higher *Bd* infection rate at *SVT* prevalence of 75% than other frogs species. *P. ridibundus* were sampled at two locations (*SNP* and *KRL*) but 6 tested positive for *Bd*. We found a moderate *Bd* rate (20.09 %, *N* = 62) and low zoospore loads (473.66±18.44). The number of zoospores increases during infection. Low numbers may represent an earlier stage of infection, but the shedding of the skin may also contribute to low counts. We observed a widespread prevalence of *Bd* zoospore in apparently healthy adult amphibians in the study areas. Recent work suggests that *Bd* may produce tiny, non-pathogenic resting spores that attach to the amphibian skin surface but without causing disease (Di Rosa et al. 2007). The competing hypothesis contends that chytrid is endemic to many regions and that climate or other factors have altered the host-pathogen relationship, resulting in the recent outbreaks of chytridiomycosis (Morehouse et al. 2003; Weldon et al. 2004). As is also known adults may be protected by acquired immunity (Richmond et al. 2009) and thus may clear or prevent infections more efficiently than juveniles that are naive to *Bd*, infects some amphibian species with little negative effects on the host and do not die therefore may serve
as reservoirs of the disease (Mazzoni et al. 2003). Many amphibian species such as *Xenopus spp*, *R. catesbeiana*, and *B. marinus* carry this disease, also terrestrial species of anurans have been observed with *Bd*, suggesting frog to frog transmission is possible (Kriger et al. 2007).

Differences in morbidity and mortality in experimentally infected amphibians indicate that *Bd* virulence can vary between strains of the same and different lineages. Increased *Bd* growth rate, zoospore production, and sporangial size in pure culture, have been linked with increased host mortality and immunosuppressive activity (Fisher et al. 2009). Our studies suggested that amphibians can evolve resistance to *Bd* and may have the ability to coexist with the disease. The Eastern Black Sea region has unique reptile fauna. These regions are the corridors of the species coming from the Caucasus and the south. The high Anatolian diagonal mountains are a barrier to colonization (Ansell et al. 2011). This study showed that Anatolian diagonal mountains are not a barrier for the colonization of *Bd* in Anatolia. If amphibians can evolve resistance to *Bd* and may have the ability to coexist with the disease, testing for the presence of *Bd* should be mandatory other regions of Turkey.

In conclusion, the uncertain distribution and potential impact of *Bd* presence in Turkey require additional investigation before accurate evaluations can be made. Standardized field surveillance methods and laboratory diagnostic techniques are needed to more carefully investigate. The presence, distribution, virulence to native species and clade membership of *Bd* in Turkey must be verified before its potential impact on Anatolian amphibians can be accurately predicted.

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