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Distribution, prevalence, and amphibian hosts of *Batrachochytrium dendrobatidis* in the Balkans

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Abstract. The Mediterranean region, consisting of, amongst other areas, three main southern European peninsulas (the Iberian, Apennine, and Balkan) is known as one of the world's biodiversity hotspots. The Iberian Peninsula was the first place in Europe where amphibians were confirmed to be infected by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). Whereas the Iberian and Apennine peninsulas have been subject to intensive chytridiomycosis research, this study represents the first large-scale *Bd* survey in the Balkans. Over a two-year period, 454 skin swab samples of amphibians were collected in Montenegro, Albania, and the Republic of Macedonia of which 65 samples (14.3%) were *Bd*-positive. *Bd* was detected in 5 out of 11 sampled species (*Bombina variegata*, *Hyla arborea*, *Lissotriton vulgaris*, *Pelophylax* sp., and *Triturus macedonicus*) at 13 out of 38 localities. Infection rates did not differ between countries but varied greatly between species with a maximum in *Pelophylax* sp. (> 30%). Within positive *Pelophylax* samples, infection loads were constant across areas and age groups. Considering the Balkans' biodiversity and the potential threat to local endemics and genetic richness, future monitoring is vital for assessing *Bd* presence, prevalence and infection trends in the region.

Key words. Amphibian infectious disease, Balkan Peninsula, chytridiomycosis.

Introduction

With its extraordinary species richness and high level of endemism, the Mediterranean region is known as one of the world's biodiversity hotspots (cf. Cox et al. 2006). Old radiation centres and glacial refuges of various organisms, including amphibians, in the Mediterranean (Taberlet et al. 1998, Husemann et al. 2014) have contributed to the current species and genetic diversity in Europe. Compared to the Iberian and Apennine peninsulas, the Balkans encompass a lower overall amphibian species diversity (Cox et al. 2006). However, with its 29 species of amphibians (excluding the batrachofauna of Crete and the eastern Aegean Islands), the region is nevertheless considered a centre of species diversity and endemism of the European herpetofauna (e.g., Džukić & Kalezić 2004 with respect to the recent taxonomy sensu Speybroeck et al. 2010).

The greatest species diversity within the Balkans was recorded for the so-called "Adriatic triangle" in Montenegro (Džukić & Kalezić 2004). This area's remarkable geological, soil, and climatic diversity has shaped the highest diversity index recorded for all of Europe (number of species/area unit = 0.84; Šćepanović et al. 2010). The Balkans region has played an important role as a crossroad for species entering Europe from eastern radiation centres (e.g., the *Triturus karelinii* complex, *Hyla orientalis* or *Bufotes viridis* complex; Wielstra et al. 2010, Stöck et al. 2012, Özdemir et al. 2014). Several amphibian species presently express high phenotypic and especially unique genetic diversity or are endemic to the Balkans region (*Proteus anguinus*, *Triturus* spp., *Ichthyosaura alpestris*, *Bombina* spp., *Hyla arborea*, *Pelophylax shqipericus*, *Pelophylax epeiroticus*, *Rana graeca*; Džukić & Kalezić 2004, Sotiropoulos et al. 2007, Fijarczyk et al. 2011, Dufresnes et al. 2013, Wielstra et al. 2013).

Amphibians are presently facing a global decline that is in many cases caused by invasive species and diseases (Berger et al. 1998, Stuart et al. 2004). Knowledge of presence of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), and its impact on amphibians in the main Mediterranean peninsulas varies greatly, while following a west-to-east gradient of "scientific interest". The Iberian region became the centre of European chytridiomycosis research due to the declining populations of three species in the genus *Alytes* (Bosch et al. 2001, Bosch & Rincón 2008, Bosch et al. 2013, Rosa et al. 2013). The Apennine Peninsula has been the subject of several local *Bd* mapping projects (e.g., Simoncelli et al. 2005, Canessa et al. 2013, Tessa et al. 2013) that also included northern Italy (Stagni et al. 2004) and the island of Sardinia (Bielby et al. 2009). In the Balkans region, on the other hand, only few samples have been analysed and published to date, of which none (Garner et al. 2005, Vörös & Jelić 2011) or only small numbers proved *Bd*-positive (Vörös et al. 2013). Asia Minor, meanwhile, has had a proven presence of *Bd* in just a single individual (Göçmen et al. 2013). Given the low surveying effort, it is possible that even mass mortalities with an intensity equivalent to that of *Alytes obstetricans* in Spain (Bosch et al. 2001) or of *Discoglossus sardus* in Sardinia (Bielby et al. 2009) have gone unrecorded in the eastern Mediterranean region. The aim of our study was to conduct the first extensive survey of *Bd* occurrence in the Balkan Peninsula.

Material and methods

The present *Bd* survey focused on Montenegro, Albania, and the Republic of Macedonia (Fig. 1). In 2013, samples were collected haphazardly at one locality as a pilot study to provide preliminary information on the presence of *Bd* in the area. During spring of the following year, more intense *Bd* sampling was conducted in Montenegro. At each of 13 localities there, we endeavoured to sample a minimum of 30 individuals per species in order to ensure an acceptable probability of *Bd* detection (DiGiacomo & Koepsell 1986). Overall, sampling was conducted in two consecutive years (2013 and 2014) at 14 localities in Montenegro (one locality in 2013, 13 in 2014), 16 localities in Albania, and 8 localities in the Republic of Macedonia (Supplementary Table 1). Sample collection was performed by skin-swabbing live amphibians using sterile Dryswab® (MW100, Medical Wire & Equipment Co, UK) or by collecting toe clips (Hyatt et al. 2007). A new pair of disposable gloves was used for each specimen to prevent disease transmission between animals (Forzán et al. 2008). Each sampled individual was recorded, photographed, and then returned to the place of its capture.

The swab samples were stored dry and refrigerated after returning from the field. The tissue samples were pre-

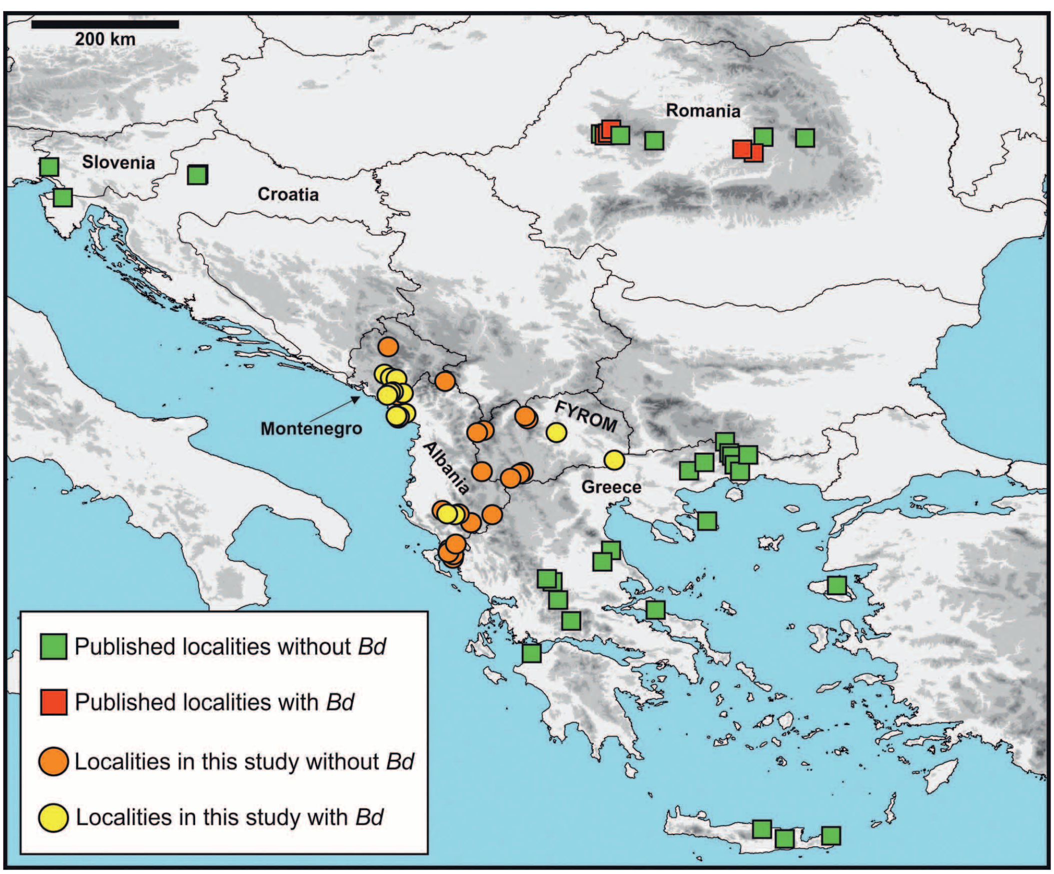

Figure 1. Current distribution of *Batrachochytrium dendrobatidis* (*Bd*) in the Balkan Peninsula. Published localities according to Garner et al. (2005), Vörös & Jelić (2011) and Vörös et al. (2013).

Table 1. Overview of amphibian taxonomic groups examined for the presence of *Batrachochytrium dendrobatidis* (*Bd*) in Albania, Montenegro, and the Republic of Macedonia. Total – overall number of sampled animals; *Bd*+ – number of *Bd*-positive animals; Prevalence – proportions of *Bd*-positive samples in percent with 95% confidence intervals (CI).

Taxa	Total/*Bd*+	Prevalence [%] (95% CI)
Anura		
Bombinatoridae		
Bombina variegata	20/5	25 (10.4–47.5)
Bufonidae		
Bufo bufo	45/0	0 (0–8.3)
Bufotes viridis	7/0	0 (0–37.7)
Hylidae		
Hyla arborea	54/1	1.9 (0.1–9.8)
Ranidae		
Pelophylax sp.	218/57	26.1 (20.6–32.5)
Rana dalmatina	17/0	0 (0–19.6)
Rana graeca	4/0	0 (0–52.7)
Caudata		
Salamandridae		
Ichthyosaura alpestris	17/0	0 (0–19.6)
Lissotriton vulgaris	73/1	1.4 (0.1–7.3)
Salamandra salamandra	1/0	0 (0–95)
Triturus macedonicus	10/1	10 (0.5–44.6)
Total	466/65	13.9 (11–17.5)

served in pure ethanol. Swab tips or small pieces of skin tissue were treated with PrepMan® Ultra Sample Preparation Reagent (Life Technologies), homogenised in MagNA Lyser (Roche Diagnostics), and boiled for 10 minutes. The prepared DNA samples were then diluted 10 times and examined for the presence of *Bd* by the TaqMan® probe-based qPCR-assay developed by Boyle et al. (2004) with bovine serum albumin (BSA) added to limit PCR inhibition (Garland et al. 2010). The addition of BSA prevents bias caused by PCR inhibition amongst taxa and improves the overall accuracy of the detection method (Baláž et al. 2014a). No additional means to control for false negative results, like internal positive controls, were used due to budget limitations. The detection procedure was performed in LightCycler 480 II (Roche Diagnostics) using the LightCycler Probes Master in a manufacturer-recommended total volume of 20 µl. Each sample was run in duplicates, and the samples were re-tested in cases of ambiguous results. Aliquots of known concentration of *Bd* DNA were provided by the Institute of Zoology, Zoological Society of London, and served as quantification standards in the reaction, allowing quantification of the pathogen load in genomic equivalents of a zoospore (GE). A sample was considered positive if it produced a typical fluorescence growth curve and the resulting quantity was > 0.1 GE.

The prevalence and its confidence intervals (95%, Sterne's exact method) between species were computed using the software Quantitative Parasitology (Rozsa et al. 2000). The occurrence of *Bd* in the three countries was compared using Generalized Linear Models (GLMs) within R statistical software, version 3.0.2 (R Core Team 2013). Within each GLM, we used log-linear modelling with Poisson distribution of the response variable (frequencies of *Bd*-positive and *Bd*-negative samples in each country).

Subsequent analyses were performed only for the data set derived from our surveys in Montenegro during 2014. That data set, originally consisting of 350 samples from 8 species at 13 localities, was further restricted to species with at least 10 sampled animals per species and locality. Thus, the final data set consisted of 278 samples from 5 species (*Bufo bufo, Hyla arborea, Ichthyosaura alpestris, Lissotriton vulgaris, Pelophylax* sp.) at 10 localities. *Pelophylax* sp. was considered as a single taxon for reasons of difficult identification. By using a GLM, we compared the occurrence of *Bd* – expressed as a binary response variable (*Bd*-positive vs. *Bd*-negative) – between species and areas. Within the data subset consisting of only positive *Pelophylax* samples, we compared the mean GE values between areas (Supplementary Table 1), as well as between adult and subadult animals. The response variable GE had a normal distribution following logarithmic transformation, and therefore a Linear Model (LM) was used. Quantitative comparison of GE was restricted to *Pelophylax* sp., because the numbers of *Bd*-positive samples within other species were very low.

To test the significance of each explanatory variable in the model, we used Chi-squared deletion tests in the case of GLM and an F-test for the LM (Crawley 2007). Each minimal adequate model was checked in the end with standard statistical diagnostics, i.e., by residuals and standardized residuals versus fitted and predicted values, as well as by Cook's distances (Crawley 2007).

Results

Of our 454 samples, 65 were *Bd*-positive (14.3%), and *Bd* was detected in 5 (*Bombina variegata*, *H. arborea*, *L. vulgaris*, *Pelophylax* sp., and *Triturus macedonicus*) out of 11 sampled species (45.5%) at 13 out of 38 localities (34.2%) across the three surveyed countries (Supplementary Table 1, Table 1). We found no differences between countries in the proportions of *Bd*-positive samples (df = 2, p = 0.24); Montenegro: 55 *Bd*-positive samples out of the total 382 samples (i.e., 14.4%), Albania: 6/59 (10.2%), the Republic of Macedonia: 4/13 (30.8%), probably due to the low numbers of sampled animals in Albania and the Republic of Macedonia.

During the 2013 pilot study, 32 individuals of *Pelophylax* sp. were sampled in Montenegro at one locality. Four of these (12.5%) were *Bd*-positive. During the more intensive sampling in the following year, we sampled 350 animals and found 51 of these (14.6%) to be infected. Of the 13 Montenegro localities examined in 2014, *Bd* was detected in 8 (Supplementary Table 1). Fifty of the 267 anuran samples (18.7%) and 1 of 84 urodelan samples (1.2%) tested in 2014 were positive for *Bd.* We recorded 8 amphibian species, three of which were *Bd*-positive (*H. arborea*, *L. vulgaris*, and *Pelophylax* sp.) and 5 of which were *Bd*-negative (Supplementary Table 1). The fungus occurred mainly at low infection intensity, with the GE values ranging from 0.29 to 212.0, and no apparent behavioural or macroscopic symptoms of disease were observed.

Across the 5 selected species with sufficient numbers of samples (at least 10 samples per species and locality in 2014) – *B. bufo*, *H. arborea*, *I. alpestris*, *L. vulgaris*, *Pelophylax* sp. – we found marginally significant difference in the proportions of *Bd*-positive and *Bd*-negative samples between five areas (df = 4, p = 0.038). Whereas the proportions of *Bd*-positive samples were about 20–25% in the areas of Lake Šas (7 out of 33 samples), Lake Skadar (24/94) and Danilovgrad (19/92), we found only one and no positive samples in the areas Štoj and Durmitor, respectively. The proportions of *Bd*-positive and *Bd*-negative samples differed greatly between species (df = 4, $p < 10^{-6}$), and the highest infection rate occurred in *Pelophylax* sp. (49 out of the 160 sampled animals, 30.6%). In another two species with proven *Bd* occurrence (*H. arborea* and *L. vulgaris*), we found only one positive sample per species (Supplementary Table 1). Within the data set consisting solely of positive *Pelophylax* samples, we found no differences in GE values either between adults and subadults (F = 0.77, p = 0.38) or between the four areas with *Bd* presence (F = 0.94, p = 0.40). Furthermore, the interaction between these two variables was not significant (F = 0.33, p = 0.57), meaning that GE values did not differ within age groups across areas.

Discussion

The overall risk posed by the amphibian chytrid fungus to European amphibians appears to be heterogeneous in both geography and host taxonomy. While the Mediterranean peninsulas contain several well-studied sites where chytridiomycosis has affected populations and caused mortalities (Bosch et al. 2001, Stagni et al. 2004, Walker et al. 2008, Bielby et al. 2009, Rosa et al. 2013), no such sites are known from central parts of Europe in spite of the wide distribution of the fungus in various environments (e.g., Ohst et al. 2011, Sztatecsny & Glaser 2011). This geographic variability is boosted further by differences within taxonomic groups in the probability of carrying the infection (Baláž et al. 2014a).

The highest infection rate in Balkan species (30.6%) was observed in *Pelophylax* sp. This result is particularly important with regard to the endemic water frog *Pelophylax shqipericus* (an endangered species according to the IUCN Red List of Threatened Species) whose distribution is limited to western Albania and southern Montenegro at altitudes below 500 m a.s.l. and whose populations are diminishing due to a variety of factors. The northernmost limits of its range are at Lake Skadar where its continued existence is significantly threatened by aquatic pollution and overcollection for commercial purpose (pet trade and food industry; Uzzel & Crnobrnja-Isailović 2009). Therefore, additional information about the role of chytridiomycosis in the decline of *P. shqipericus* is urgently needed.

Data supporting the universal role of *Pelophylax* frogs as *Bd* reservoirs are on the increase. *Bd* infections in this genus have been recorded across most of Europe (summary in Baláž et al. 2014b), as well as in Turkey (Göçmen et al. 2013). Showing low variability of *Bd* prevalence in *Pelophylax* frogs between different sites, our results imply that the water frog is itself the most important factor in the occurrence of *Bd* while environmental factors have secondary effects. Combined with the realisation that these frogs are immune to the disease itself (Woodhams et al. 2012), this information points to the water frogs as an important object of study for understanding the dynamics of *Bd* infection. The detection of *Bd* infections in amphibians from our dataset adds evidence to the presence of this pathogen in multiple areas of the northern Balkans (Carpathians; see Vörös et al. 2013), and improves our knowledge of its current distribution. We detected *Bd* in several species (*B. variegata*, *H. arborea*, *L. vulgaris*, *Pelophylax* spp., and *T. macedonicus*) that express high genetic variability and contain lineages endemic to the area. The identified positive areas (central and southern Montenegro, southern Albania and eastern Macedonia) are known as a Plio-Pleistocene refuge for many vertebrate taxa. As for amphibians, four Balkans-endemic species occur (*P. epeiroticus, P. shqipericus*, *Rana graeca,* and *T. macedonicus*) and unique haplotypes of *B. variegata* and *L. vulgaris* were detected here (Hofman et al. 2007, Fijarzcyk et al. 2011, Pabijan et al. 2015).

Based on the published works on the historical biogeography of amphibians in the Balkans and our current results, we suggest the following areas as important for study of *Bd* (besides the "Adriatic triangle"): Carpathians (*B. variegata*, *I. alpestris*; Hofman et al. 2007, Fijarzcyk et al. 2011), northern Balkan Mts. (*I. alpestris*), northwestern Adriatic (*H. arborea*, *L. vulgaris*; Dufresnes et al. 2013, Pabijan et al. 2015), Pindos Mts. (*B. variegata*, *H. arborea*, *L. vulgaris, T. macedonicus*, *Pelophylax ridibundus*; Lymberakis et al. 2007, Wielstra et al. 2013), Peloponnese (*L. vulgaris*), and Crete (*Pelophylax cretensis*). However, there are many "white" areas on the map where further sampling is required to assess the actual prevalence of *Bd* in local amphibians. Furthermore, sampling should cover multiple seasons, because the prevalence and intensity of infection are strongly affected by climatic conditions and may vary seasonally (Savage et al. 2011) and chytrid prevalence could be higher than suggested by our data. Badly chosen seasonality and poor sampling is probably the reason why *Bd* has yet not been found in, and published for, other regions of the Balkans (e.g., in Greece). It may be assumed that *Bd* is broadly distributed in the Balkans for which reason surveys at local and regional scales could provide valuable information about the spread and impact of the pathogen and help optimise potential conservation strategies.

In this study, we present the first regular survey of the occurrence of *Bd* in the Balkan Peninsula with the main focus on Montenegro. As the Mediterranean peninsulas are hotspots of diversity richness and endemism (Cox et al. 2006), it is important to conduct intensive research there as to the presence, prevalence, and infection trends of *Bd*. Our results support the importance of the *Pelophylax* species group as a *Bd* reservoir host. In the evolutionary past of the European fauna, the Mediterranean peninsulas have played roles as safe havens for biodiversity, including the batrachofauna. Today, these regions deserve to be the subjects of intense scientific and conservation measures in order to describe, understand, and save what is left before some of the unique species and lineages are lost to the pressure of invasive organisms, including pathogens.

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Supplementary material

Supplementary Table 1. Summary of a *Batrachochytrium dendrobatidis* (*Bd*) survey in Albania, Montenegro and the Republic of Macedonia during 2013 and 2014.