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Infectious disease threats to amphibians in Greece: new localities positive for *Batrachochytrium dendrobatidis*

Ilias Strachinis[1,*], Rachel E. Marschang[2], Petros Lymberakis[3], Korina M. Karagianni[1], Panagiotis Azmanis[4]

[1]Department of Genetics, Development and Molecular Biology, School of Biology, Faculty of Natural Sciences, Aristotle University of Thessaloniki, 54636, Greece

[2]LABOKLIN GmbH & Co. KG, Steubenstraße 4, 97688 Bad Kissingen, Germany

[3]National History Museum of Crete, University of Crete, 71409 Greece

[4]Dubai Falcon Hospital, 676R+46J Dubai, UAE

ABSTRACT: In the early 2000s, numerous cases of European amphibian population declines and mass die-offs started to emerge. Investigating those events led to the discovery that wild European amphibians were confronted with grave disease threats caused by introduced pathogens, namely the amphibian and the salamander chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) and ranaviruses. In Greece, *Bd* was previously documented among wild amphibian populations in 2 different locations and 3 different species. However, no disease-related mass declines or mortality events have been reported. In this work, we build upon previous findings with new, subsequently obtained data, resulting in a 225-sample dataset of 14 species from 17 different locations throughout Greece, in order to examine the occurrence status of all 3 pathogens responsible for emerging infectious diseases in European amphibians. No positive samples for *Bsal* or ranavirus were recorded in any location. We confirmed the presence of *Bd* in 4 more localities and in 4 more species, including 1 urodelan (Macedonian crested newt *Triturus macedonicus*) and 1 introduced anuran (American bullfrog *Lithobates catesbeianus*). All insular localities were negative for *Bd*, except for Crete, where *Bd* was identified in 2 different locations. Again, no mass declines or die-offs were recorded in any *Bd*-positive area or elsewhere. However, given the persistence of *Bd* across Greece over the past ~20 yr, monitoring efforts should continue, and ideally be further expanded.

KEY WORDS: Chytrid fungus · Ranavirus · *Batrachochytrium dendrobatidis* · *Bd* · *Batrachochytrium salamandrivorans* · *Bsal* · Frog · Salamander

1. INTRODUCTION

Emerging infectious diseases constitute a serious threat to wildlife, as well as to human health (Daszak et al. 2000, Acevedo-Whitehouse & Duffus 2009) and, together with other mostly anthropogenic causes, they are presently responsible for major animal population declines that can lead to extinctions, at both a local and global scale (de Castro & Bolker 2005). Whereas disease-related declines are likely to have a more severe impact in small wildlife populations, large-scale shrinkages can even take place in sizeable and apparently less fragile ones (Daszak et al. 2003). Amphibians are among the groups of animals that currently face serious population declines globally, mostly driven by human-related causes (Stuart

*Corresponding author: strachin@bio.auth.gr

et al. 2004, Sodhi et al. 2008). It is only in the last few decades that infectious diseases in amphibians were proven to be associated with numerous cases of population declines and extinctions globally (Berger et al. 1998, Daszak et al. 1999, Allain & Duffus 2019). To date, 3 major emerging disease threats to amphibians have been identified in Europe caused by introduced pathogens, namely *Batrachochytrium dendrobatidis*, *B. salamandrivorans* and ranaviruses, and a potentially emerging disease caused by amphibian herpesviruses (Allain & Duffus 2019 and references therein).

B. dendrobatidis (*Bd*) is a non-hyphal zoosporic fungus that causes chytridiomycosis, a lethal skin disease in amphibians (Berger et al. 1998). *Bd* colonizes and affects only keratinized tissue, namely the mouthparts of amphibians during their larval development and the epidermis (stratum corneum) of the metamorphosed animals (Berger et al. 1998, Pessier et al. 1999, Marantelli et al. 2004). Clinical signs of infected amphibians at the larval stage are usually limited to depigmentation of mouthparts. Rarer clinical signs include lethargy and poor swimming abilities, which can lead to low foraging efficiency (Berger et al. 1998, Rachowicz & Vredenburg 2004, Hanlon et al. 2015). In adults, clinical signs may be evident, mild, or completely lacking, depending upon the progression of the infection, environmental conditions, species, etc. (Allain & Duffus 2019). Among metamorphosed animals with clinical signs, *Bd* infection of the skin may be characterized by anorexia, lethargy, epidermal hyperplasia, hyperkeratosis, skin reddening, excessive shedding of the epidermis, convulsions and loss of the righting reflex (Berger et al. 1998, Nichols et al. 2001, Voyles et al. 2007, 2009, Marcum et al. 2010, Campbell et al. 2012). Extensive infection may lead to a cascade of physiological malfunctions such as disruption of the skin's osmoregulation leading to dehydration, electrolyte imbalance and cardiac arrest resulting in death (Berger et al. 1998, Nichols et al. 2001, Voyles et al. 2007, 2009, Marcum et al. 2010, Campbell et al. 2012). However, certain groups of amphibians can be tolerant to *Bd* infection and show no clinical signs associated with the disease (Woodhams et al. 2012).

Currently, *Bd* is associated with serious population declines in more than 500 species of amphibians (6.5% of the currently described amphibian species) on all continents on which amphibians are found (Scheele et al. 2019). Furthermore, *Bd* is considered responsible for the extinction of more than 90 amphibian species throughout the world so far (Wake & Vredenburg 2008, Scheele et al. 2019).

The presence of *Bd* in European wild anurans was first confirmed in Spain in 2001, where it affected populations of the common midwife toad *Alytes obstetricans* (Laurenti, 1768) (Bosch et al. 2001). Since then, *Bd* has been detected in most European countries and in a wide range of amphibian species (reviewed by Allain & Duffus 2019). Recent studies have shown that *Bd* is of East Asian origin (O'Hanlon et al. 2018). Increasing evidence suggests that the global spread of the *Bd* pathogen and its introduction in Europe may have been facilitated by the international pet trade, through the importation of *Bd*-infected amphibians (Wombwell et al. 2016). Once the pathogen infects wild populations, further spread may occur via transportation of moist soil (such as river sand) and possibly through migrating birds (Johnson & Speare 2005), as well as through accidental transferal of *Bd* zoospores on footwear and equipment of fieldworkers when moving between waterbodies and amphibian habitats in general (DAPTF 1998). In addition, tolerant host species with *Bd* infections can act as reservoir species that inadvertently shed *Bd* zoospores into the environment. The presence of those *Bd*-positive animals could also increase the spread of *Bd* (Daum et al. 2012, Woodhams et al. 2012).

B. salamandrivorans (*Bsal*) is closely related to *Bd* and, together with *Bd*, it is responsible for amphibian chytridiomycosis (Kelly et al. 2021). *Bsal* is the predominant chytrid fungus in Vietnamese native urodelans (Laking et al. 2017). It follows a dual transmission strategy with both motile and non-motile environmentally resistant spores (Stegen et al. 2017). The gross clinical signs of *Bsal* are anorexia, lethargy, ataxia and skin lesions (Martel et al. 2013).

Bsal was first identified in Europe in 2013 (Martel et al. 2013), when it was responsible for a severe decline in European fire salamanders *Salamandra salamandra* (Linnaeus, 1758) in the Netherlands (Spitzen-van der Sluijs et al. 2013). Since then, *Bsal* has been detected in 4 more European countries, causing serious outbreaks, namely in Belgium (Martel et al. 2014), the UK (Cunningham et al. 2015), Germany (Spitzen-van der Sluijs et al. 2016) and Spain (Martel et al. 2020). Evidence indicates that *Bsal* was also introduced into Europe via the pet trade, either through small-webbed bell toads *Bombina microdeladigitora* Liu, Hu & Yang, 1960 or Asian newts (Laking et al. 2017, Nguyen et al. 2017). While *Bsal*-related disease is mostly limited to salamanders, some anuran species and some less susceptible urodelans can function as disease reservoirs (Stegen et al. 2017).

Ranaviruses are large icosahedral cytoplasmic double-stranded DNA viruses belonging to the family *Iridoviridae* (Sharifian-Fard et al. 2011). Ranaviruses can infect amphibians, reptiles and fish (Campbell et al. 2020). In amphibians, the clinical signs of ranavirosis include lethargy, skin ulcerations, skin haemorrhages, skin reddening, necrosis of the digits and internal haemorrhages of multiple organ systems; however, not all ranaviral infections cause clinical signs (reviewed by Allain & Duffus 2019). Amphibian ranavirosis has been spreading in Europe since mid-2000 (Campbell et al. 2020), and the presence of ranaviruses has been confirmed in more than 10 countries, affecting over 10 amphibian species so far (Allain & Duffus 2019). Ranavirus infections in wild amphibian populations can lead to mass mortality events (e.g. Cunningham et al. 1996, Ariel et al. 2009, Miaud et al. 2016), persistent population declines, or even local extirpations (Teacher et al. 2010, Price et al. 2014, Rijks et al. 2016, Rosa et al. 2017), and thus, they are considered to pose an emerging conservation threat of great significance. Two ranavirus species have been identified as the most common in European amphibians, *Frog virus 3* (FV3) and *Common midwife toad virus* (CMTV) (Allain & Duffus 2019, Campbell et al. 2020). Different strains of these viruses vary in pathogenicity (Saucedo et al. 2018, 2019), whereas studies have shown that when both FV3-like and CMTV-like viruses occur in sympatry, chimeric viruses can ensue by recombination, which may result in viruses with more severe virulence (Price et al. 2014, Price 2015, Claytor et al. 2017, Rosa et al. 2017).

Greece hosts 24 species of amphibians in total, of which 8 are urodelans and 16 are anurans (Speybroeck et al. 2020). Two of them are Greek endemics — the Karpathos salamander *Lyciasalamandra helverseni* (Pieper, 1963) and the Cretan water frog *Pelophylax cretensis* (Beerli, Hotz, Tunner, Heppich & Uzzell, 1994) — while there are also several endemic subspecies (see Section 4). Currently, there is only 1 anuran amphibian that is non-native and invasive, the American bullfrog *Lithobates catesbeianus* (Shaw, 1802), introduced in an artificial lake in West Crete. Population trends of amphibians in Greece are not well studied yet, although most of the species that occur in Greece have a decreasing trend on a global level according to IUCN estimates (IUCN 2022), including the 2 Greek endemics.

To date, only 2 surveys regarding infectious disease threats to amphibians have been conducted in Greece (Garner et al. 2005, Azmanis et al. 2016). Azmanis et al. (2016) reported the occurrence of *Bd* in Greece in 2 locations among 3 different anuran species (i.e. *Bufotes viridis*, *Pelophylax* cf. *ridibundus* and *P. epeiroticus*). The purpose of the present study was to expand our knowledge on the distribution of *Bd*, *Bsal* and ranaviruses in amphibians in Greece, adding both to the locations and the species studied, as well as to assess the potential risks that these pathogens pose to native amphibian populations.

2. MATERIALS AND METHODS

2.1. Taxonomy and species identification

In this work we follow the taxonomy as proposed by Speybroeck et al. (2020). All animals were identified by their distinctive morphological characters and by range, with one exception: sampled *Pelophylax* frogs from Thrace (sites B and C; Fig. 1) could not be distinguished between *P. ridibundus* and *P. bedriagae*, as these species are macroscopically identical. We were able to confirm the occurrence of both species living in sympatry, by identifying both of their distinctive advertising calls (see Strachinis & Roussos 2016) at sampling sites B and C and in the wider area, and therefore, our samples may include both species or even hybrids. Hence, in order to overcome this taxonomic uncertainty, we assign all *Pelophylax* samples from areas B and C (Vistonida and Ismarida wetland, respectively) to *P. ridibundus*, following the current knowledge on the species' range.

2.2. Sample collection

Our research focussed on the presence of *Bd*, *Bsal* and ranaviruses in Greek amphibian populations. We collected 225 samples from 17 areas in Greece (8 on the mainland and 9 on islands; Fig. 1, Table 1) and among 14 species of amphibians: 3 urodelan and 11 anuran species (Table 2; Table S1 in the Supplement at www.int-res.com/articles/supp/d152p127_supp.xlsx). All samples were taken from adult individuals and tested for all 3 pathogens (*Bd*, *Bsal* and ranavirus). Of these 225 samples, 59 were collected during the period 2014–2015 from the areas B, J, L and O (see Fig. 1), and the results were published by Azmanis et al. (2016). The remaining 166 samples were primarily composed of samples collected from live animals between 2015 and 2018, but also 13 ethanol-fixed *L. catesbeianus* specimens deposited in the Natural History Museum of Crete (collected in 2003 and 2013; Table S1). The sampling was mostly

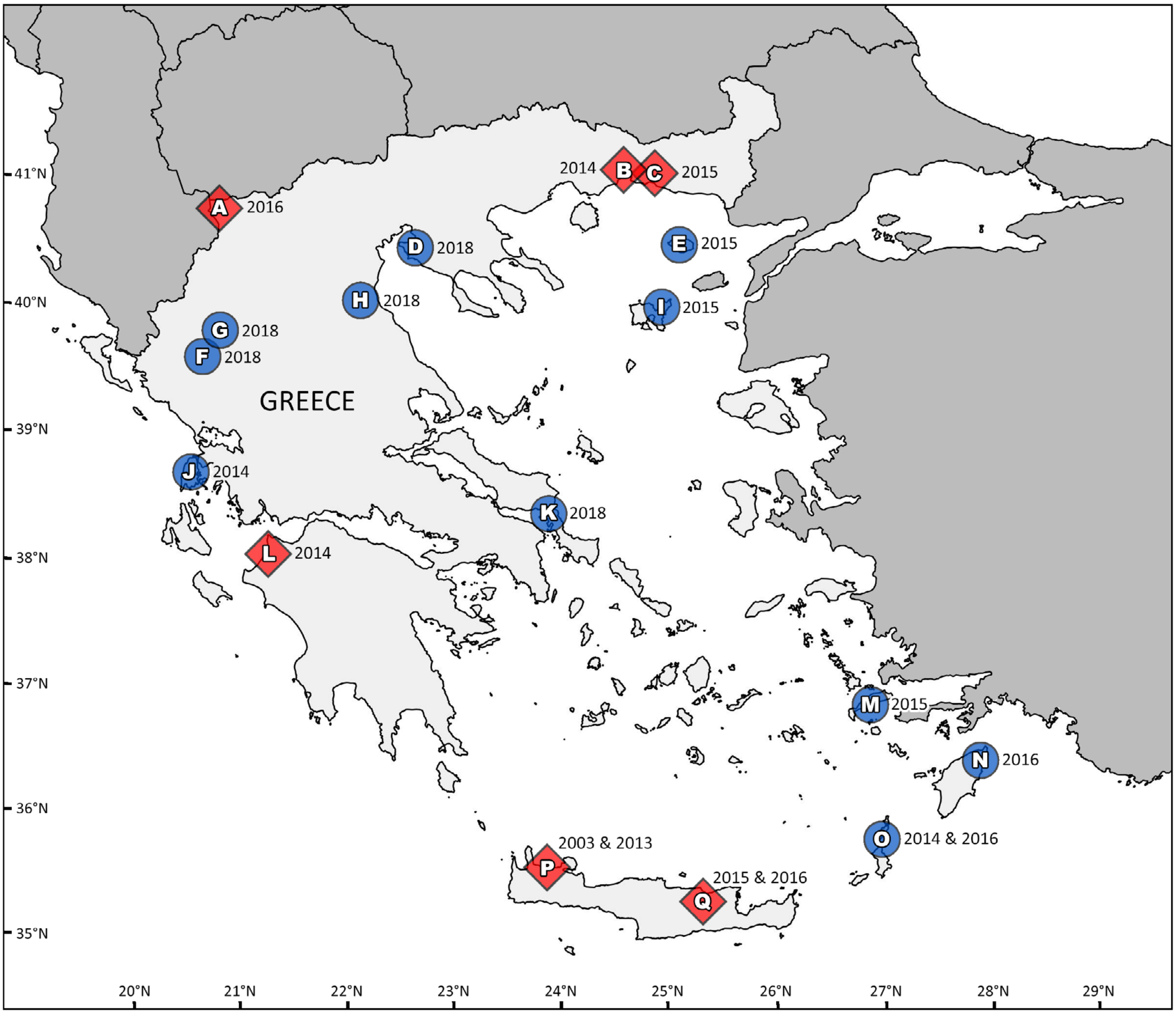

Fig. 1. All localities in Greece examined for *Batrachochytrium dendrobatidis* (*Bd*), *Batrachochytrium salamandrivorans* (*Bsal*) and ranaviruses, showing year of sampling (more details in Table S1 in the Supplement). Locations in red are those in which *Bd* was detected. (A) Prespes, Florina, (B) Vistonida Lake, Xanthi, (C) Ismarida Lake, Komotini, (D) Aggelochori-Scholari area, Thessaloniki, (E) Samothrace Island, (F) Pamvotida Lake, Ioannina, (G) Aoos Lake, Metsovo, Ioannina, (H) Olympus Mt. Pieria, (I) Lemnos Island, (J) Lefkada Island, (K) Evia Island, (L) Strofylia wetland, Achaia, Peloponnese, (M) Plaka, Kos Island, (N) Rhodes, (O) Karpathos, (P) Agia Lake, Chania, Crete, (Q) Thrapsano area, Heraklion, Crete. The map was generated using QGIS v.3.22.9 'Białowieża'

opportunistic (e.g. during field work for herpetofaunal monitoring projects). Most samplings took place during spring, summer and autumn, except for only a few cases (see Table S1 for dates of sampling). In the majority of cases, sampling took place at temperatures between 17 and 25°C, which is within the optimal temperature range for the growth of *Bd* (Hudson et al. 2019) and the amphibians were already highly active. Only a few samplings occurred during lower temperatures, between 10 and 17°C. All samples were obtained noninvasively using sterile cotton-tipped swabs. For *Bd* and *Bsal* detection, we used skin swabs from live animals and museum vouchers, as described by Hyatt et al. (2007) and Cheng et al. (2011). Oral and cloacal swabbing was conducted for ranavirus detection. To prevent cross-contamination between animals and sampling sites, a new pair of disposable, non-powdered vinyl gloves was used for the handling of each animal. Between sampling sites, all field equipment, as well as footwear, was disinfected with Virkon S® (Antec International) as described in the disinfection protocols of Phillott et al. (2010) and Van Rooij et al. (2017). All living animals were released back at the site of capture immediately after sampling, and dry samples were stored at −20°C a few hours after collection.

Table 1. Locations examined and species sampled in each location for *Batrachochytrium dendrobatidis* (*Bd*). Numbers of both total and *Bd*-positive samples per species for each location are shown. CI: confidence interval

Locality code	Location	Year of sampling	Species	No. of samples	*Bd*-positive samples	Total samples	Total *Bd*-positive samples	% *Bd*-positive samples	95% binomial CI
A	Prespes, Florina	2016	*Pelophylax ridibundus*	3	1	17	2	11.8	2.1–34.3
			Rana dalmatina	10	0				
			Triturus macedonicus	4	1				
B	Vistonida Lake, Xanthi	2014[a]	*Bufotes viridis*	10	2	19	4	21.1	8.5–43.3
			Pelophylax ridibundus	9	2				
C	Ismarida Lake, Komotini	2015	*Pelobates balcanicus*	9	0	10	1	10	0.5–40.4
			Pelophylax ridibundus	1	1				
D	Aggelochori-Scholari, Thessaloniki	2018	*Bufo bufo*	1	0	3	0	0	0–56.1
			Pelobates balcanicus	2	0				
E	Therma, Samothrace Isl.	2015	*Bufo bufo*	2	0	4	0	0	0–49
			Bufotes viridis	2	0				
F	Pamvotida Lake, Ioannina	2018	*Bufo bufo*	5	0	5	0	0	0–43.4
G	Aoos lake, Metsovo, Ioannina	2018	*Salamandra salamandra*	12	0	12	0	0	0–24.2
H	Olympos mt., Katerini	2018	*Bombina variegata*	15	0	19	0	0	0–16.8
			Salamandra salamandra	4	0				
I	Panagia, Lemnos Isl.	2015	*Bufotes viridis*	16	0	17	0	0	0–18.4
			Pelobates syriacus	1	0				
J	Lefkada Isl.	2014[a]	*Bufotes viridis*	10	0	10	0	0	0–27.8
K	Dystos Lake, Evia Isl.	2018	*Bufotes viridis*	9	0	10	0	0	0–27.8
			Pelobates balcanicus	1	0				
L	Strofylia Lake, Achaia, Peloponnese	2014[a]	*Bufotes viridis*	8	0	20	1	5	0.3–23.6
			Pelophylax epeiroticus	12	1				
M	Plaka, Kos Isl.	2015	*Bufo bufo*	3	0	7	0	0	0–35.4
			Pelophylax bedriagae	4	0				
N	Rhodes	2016	*Bufotes viridis*	5	0	5	0	0	0–43.4
O	Karpathos	2014[a] & 2016	*Lyciasalamandra helverseni*	19	0	29	0	0	0–11.7
			Pelophylax bedriagae	10	0				
P	Agia Lake, Chania, Crete	2003 & 2013	*Lithobates catesbeianus*	13	3	13	3	23.1	8.2–50.3
Q	Thrapsano area, Heraklion, Crete	2015 & 2016	*Pelophylax cretensis*	25	1	25	1	4	0.2–19.5
					Totals:	225	12	5.3	3.1–9.1

[a]Includes data from Azmanis et al. (2016)

2.3. Laboratory procedures

DNA was extracted from the swabs using the Roche MagNA Pure 96 system with the MagNA Pure 96 DNA and viral RNA Small Volume Kit according to the manufacturer's instructions. Detection of *Bd* and *Bsal* DNA was carried out in separate reactions using the methods described by Blooi et al. (2013). Each sample was tested once. Ranaviral DNA detection was carried out as described by Stilwell et al. (2018).

2.4. Statistical analyses

GraphPad Prism version 8.0.2 for Windows (GraphPad Software, www.graphpad.com, accessed on 29 April 2019) was used for all statistical analyses and

Table 2. All species examined for *Batrachochytrium dendrobatidis* (*Bd*) in both previous (Azmanis et al. 2016) and current work and the localities (locality codes as in Table 1 and Fig. 1) where each species was found and sampled. Numbers of both total and *Bd*-positive samples per species are shown. CI: confidence interval

Common name	Scientific name	Year of sampling	Locality code	No. of samples	*Bd*-positive samples	Total samples	Total *Bd*-positive samples	% *Bd*-positive samples	95% binomial CI
Yellow-bellied toad	*Bombina variegata*	2018	H	15	0	15	0	0	0–20.4
Common European toad	*Bufo bufo*	2018	D	1	0	11	0	0	0–25.9
		2015	E	2	0				
		2018	F	5	0				
		2015	M	3	0				
Green toad	*Bufotes viridis*	2014[a]	B	10	2	60	2	3.3	0.6–11.4
		2015	E	2	0				
		2015	I	16	0				
		2014[a]	J	10	0				
		2018	K	9	0				
		2014[a]	L	8	0				
		2016	N	5	0				
American bullfrog	*Lithobates catesbeianus*	2003 & 2013	P	13	3	13	3	23.1	8.2–50.3
Karpathos salamander	*Lyciasalamandra helverseni*	2016	O	19	0	19	0	0	0–16.8
Balkan spadefoot	*Pelobates balcanicus*	2015	C	9	0	12	0	0	0–24.2
		2018	D	2	0				
		2018	K	1	0				
Eastern spadefoot	*Pelobates syriacus*	2015	I	1	0	1	0	0	0–94.9
Bedriaga's frog	*Pelophylax bedriagae*	2015	M	4	0	14	0	0	0–21.5
		2014[a]	O	10	0				
Cretan water frog	*Pelophylax cretensis*	2015 & 2016	Q	25	1	25	1	4	0.2–19.5
Epeirus water frog	*Pelophylax epeiroticus*	2014[a]	L	12	1	12	1	8.3	0.4–35.4
Marsh frog	*Pelophylax ridibundus*	2016	A	3	1	13	4	30.8	12.7–57.6
		2014[a]	B	9	2				
		2015	C	1	1				
Agile frog	*Rana dalmatina*	2016	A	10	0	10	0	0	0–27.8
European fire salamander	*Salamandra salamandra*	2018	G	12	0	16	0	0	0–19.4
		2018	H	4	0				
Macedonian crested newt	*Triturus macedonicus*	2016	A	4	1	4	1	25	1.3–69.9
					Totals:	225	12	5.3	3.1–9.1

[a]Includes data from Azmanis et al. (2016)

for the generation of the graphs (see Fig. 2). The hybrid Wilson/Brown analysis (Brown et al. 2001) was used for the calculation of the 95% confidence interval (CI).

3. RESULTS

None of our samples was found to be positive for *Bsal* or ranavirus. Out of 225 samples in total, 12 were positive for *Bd* (5.3%) (Tables 1 & 2). Cycle-threshold-values of positive samples were relatively high and ranged from 32.57 to 38.14. However, qPCR was used for presence/absence only and not for quantification. *Bd*-positive animals were recorded in 6 out of 17 sampling sites (35.3%) throughout Greece (Fig. 1) and in 6 out of 14 sampled species (42.9%). Only 1 urodelan sample was *Bd*-positive (*Triturus macedonicus* from site A); all others (11) were anuran species (Table 2, Fig. 2). The locality presenting the highest infection rate was Agia Lake, Crete (Table 1). The species with the highest positivity rate was *Pelo-*

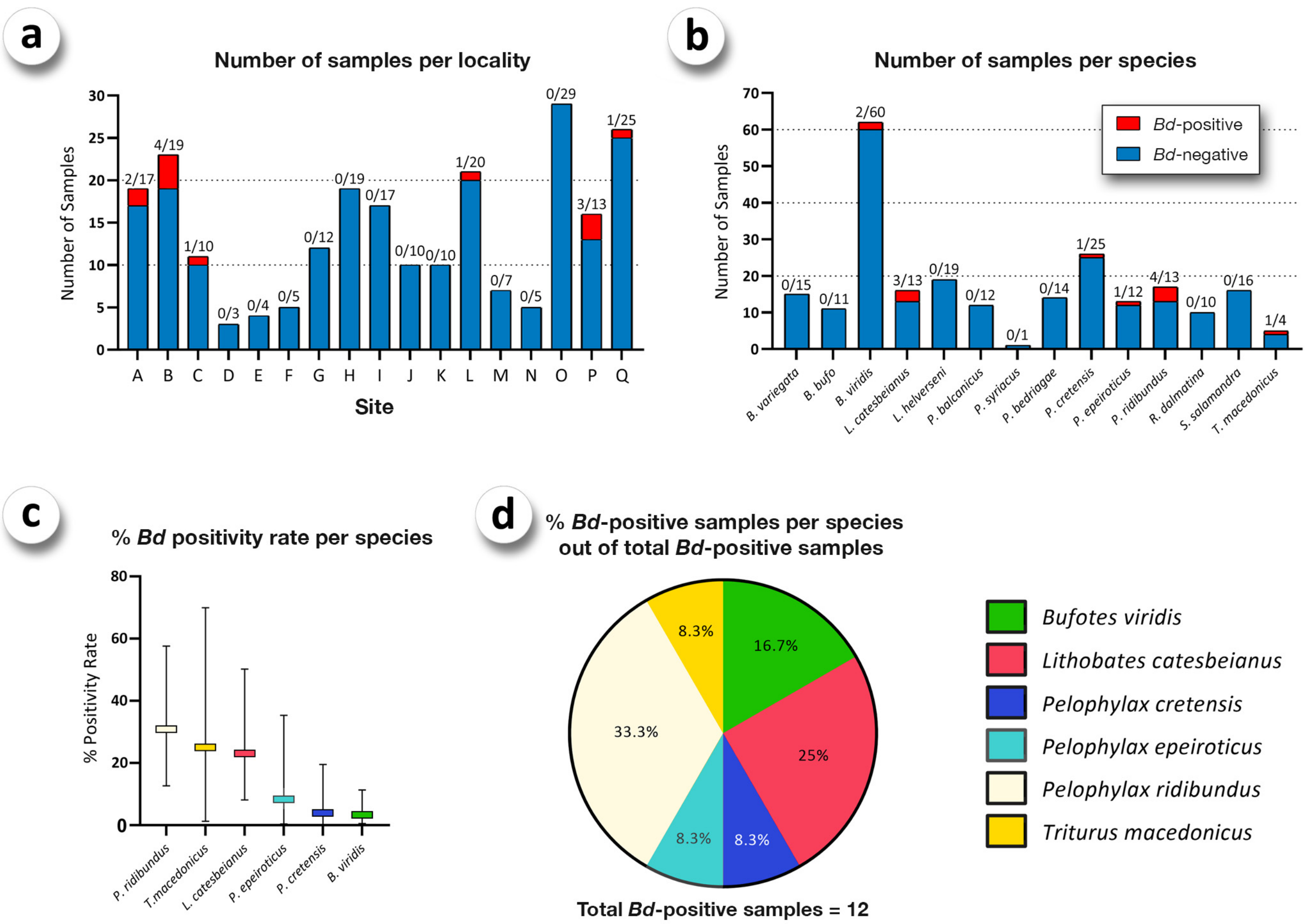

Fig. 2. (a) Number of samples collected per locality. (b) Number of samples per species (numbers above bars: positive samples/total samples). (c) Percentage of the *Batrachochytrium dendrobatidis* (*Bd*)-positivity rate per species, with 95% confidence interval. (d) Percentage of *Bd*-positive samples per species, out of the total 12 confirmed *Bd*-positive samples

phylax ridibundus (30.8%), followed by *T. macedonicus* (25%) and the introduced *Lithobates catesbeianus* from Crete (23.1%) (Table 2, Fig. 2). Given the small number of samples per site and the low spatial coverage of sampling, only descriptive statistics were done, and all percentage values used herein should be interpreted only as indicative.

Half of the mainland sampling sites (4/8) were positive for *Bd*, showing no particular distributional pattern. All samples from islands were negative, except for those collected on Crete, where *Bd* was detected in 2 areas, infecting the introduced American bullfrog and the endemic Cretan water frog *P. cretensis*.

No mass mortality events, nor any population or individual presenting any apparent disease-associated clinical symptoms, signs or lesions were detected during the sampling periods. All areas that were positive for *Bd* were revisited several times in the following years, and showed no signs of declines in densities or species diversity.

4. DISCUSSION

4.1. *Bd*-positive localities and species

We investigated the presence/absence in Greece of 3 pathogens that have been shown to cause major disease outbreaks in European amphibians. In combination with the previously reported dataset of 59 samples (Azmanis et al. 2016), a total of 225 samples from 17 areas throughout Greece have now been examined. Of the tested samples, 12 were positive for *Bd* (5.3% positivity), collected from 6 different sites. The highest positivity rate was recorded in *Pelophylax* from northern Greece (30.8%; Fig. 2). Of the total *Bd*-positive samples, 50% were obtained from *Pelophylax* species (Fig. 2), which were found in all *Bd*-positive localities, except Agia Lake, Crete, where the introduced American bullfrog has caused the decline of the endemic Cretan water frog *P. cretensis*. A high infection rate in *Pelophylax* spp. was also observed by Vojar et al. (2017), who investigated the occurrence of *Bd* in the Balkans. While some European anurans are susceptible to *Bd*, *Pelophylax* frogs seem to show high tolerance to the pathogen and are resistant to chytridiomycosis (Daum et al. 2012, Woodhams et al. 2012) and can act as *Bd* reservoirs. This, together with the fact that *Pelophylax* frogs have a wide spectrum of habitat preference (Speybroeck et al. 2016, Dufresnes 2019) and a high dispersal ability, could possibly enhance the spread of *Bd* in Greece.

We were able to confirm 4 new localities with *Bd*-infected amphibians since the work of Azmanis et al. (2016) (localities C, L, P and Q; Fig. 1). *Bd* had already been detected in amphibians in the Vistonida and Strofylia wetlands (localities B and L, respectively, in Fig. 1; see also Azmanis et al. 2016). Amongst the newly recorded *Bd*-positive localities, those from Crete (localities P and Q; Fig. 1) are the only insular ones. Regarding Agia Lake (site P), the only amphibian in the area that was sampled and found to be *Bd*-positive was the American bullfrog. Introduced populations of this species are known to consistently carry *Bd* (Garner et al. 2006). American bullfrogs were brought to Crete in 1994 for breeding and commercial use (Adamopoulou & Legakis 2016). They were introduced into Agia Lake in 2000 (Adriaens et al. 2013) after escaping from the breeding facilities nearby (Adamopoulou & Legakis 2016). Since then, they have established a reproducing population, posing a constant threat to native and endemic species. Our *Bd*-positive *L. catesbeianus* samples were taken from preserved specimens collected in 2003 and 2013 (Table S1). Given that the American bullfrog can act as a reservoir species for *Bd*, it is possible that the pathogen arrived on Crete as a stowaway on the introduced bullfrogs. To date, Agia Lake is the only known area in Greece where the American bullfrog can be found. Therefore, there cannot be a direct link between the distribution of the invasive bullfrog and the spread of *Bd* throughout the country; nevertheless, one cannot exclude the spread of the pathogen via indirect means (see Section 4.3).

The new *Bd*-positive site at Ismarida Lake (locality C) was not surprising, as it is in close proximity to Vistonida Lake. Both are frequently visited and used by the same researchers, fishermen, hunters and farmers. It is therefore possible that non-disinfected footwear and field equipment could have transferred *Bd* zoospores between areas. In fact, excluding the Thrapsano area in Crete (locality Q), all areas found to be *Bd*-positive are popular wetland destinations for local and foreign researchers, environmentalists, birdwatchers, herpetologists, fishermen, hunters and tourists, all of whom could potentially carry the pathogen via contaminated field gear.

The first detection of *Bd* in amphibians in Greece raised serious concerns for the implications of this pathogen for amphibian populations within the country. However, after monitoring the areas found positive for *Bd* for the last 6 yr, we have not noticed any apparent declines in population numbers or species diversity, nor mass mortality events associated with disease in general. In fact, the 4 mainland areas that were confirmed as positive for *Bd* were, and still are, considered hotspots for amphibian diversity and population densities. This is not the first indication that *Bd* infection could result in a wide heterogeneity of outcomes in different hosts or areas (see Baláž et al. 2014, Allain & Duffus 2019). While severe amphibian population declines caused by *Bd* have been witnessed on the Iberian Peninsula (Bosch et al. 2001, Bosch & Martínez-Solano 2006), it seems that elsewhere in Europe *Bd* infection does not necessarily cause disease and/or mass mortality (Allain & Duffus 2019) but can occur as an enzootic. This variety of infection responses could be due to different factors, such as the presence of multiple *Bd* strains of varying virulence (Fisher et al. 2009), the susceptibility level of different species to infection and disease (Bosch & Martínez-Solano 2006, Bielby et al. 2009, Sztatecsny & Glaser 2011) and environmental factors (Allain & Duffus 2019).

4.2. No *Bsal*- or ranavirus-positive samples

None of our samples was positive for *Bsal* and ranaviruses. This of course does not necessarily mean that those pathogens are absent from Greece; detecting ranavirus infection in non-clinically affected individuals is rare. All of our samples were from apparently healthy individuals that showed no lesions or other clinical signs, and thus it is possible that our sampling missed the presence of ranaviruses. On the other hand, it has been shown that not all ranavirus infections result in disease, so the absence of clinical signs does not always indicate absence of the virus (Rijks et al. 2016). In addition, no evidently disease-affected individuals were found during the samplings (or during the periods in between) and also, no mass die-offs and/or amphibian population declines were documented since the beginning of this survey.

4.3. Possible pathogen transmission pathways

It is yet unclear how *Bd* was introduced to Greece and spread throughout the *Bd*-positive areas. As mentioned, infected field equipment of wetland users could be a potential means for the introduction or/and dispersal of *Bd*. Other potential pathways could also be used by the pathogen to spread, both inter- and intranationally. Soil transportation, as well as the movement of birds and migration between wetlands are a possible transmission pathway for pathogens (Johnson & Speare 2005). Another possibility is via translocation of living amphibians. Cases of long-distance, human-related amphibian translocations that can lead to subsequent established introduced populations are feasible, as they have been reported numerous times in the literature (e.g. Dufresnes et al. 2018, Strachinis 2021). In addition, amphibian disease-related pathogens are known to occur and be widespread in private animal collections (Sabino-Pinto et al. 2015, Fitzpatrick et al. 2018) and could be easily transferred between European countries, or within a country, through the legal or illegal pet trade.

4.4. Safety concerns regarding amphibian diseases

The introduction of most invasive alien species (IAS) into the EU has been made through escapes from animal exhibition facilities, as well as via escapes linked with the ornamental trade (Tsiamis et al. 2017). The trade of ornamental animals is responsible for international movement of multiple species, and the unintended introduction of pathogens (Lucy et al. 2021). In order to prevent the spread of IAS in Europe, the EU has applied regulations to list and ban numerous animal species from importation and trade. Additionally, the Irish Environmental Protection Agency suggests a series of strict measures to confront introduction events and transmissions of pathogens that could threaten wildlife, such as examination of exotic species for pathogens within the pet trade, strict quarantine protocols for imported animals, as well as outright ban on the trade and the transport or the advertisement of species that pose a high risk for invasion (Lucy et al. 2021).

Greece hosts about 24 species of amphibians (the taxonomy of some taxa still needs to be clarified) including several endemic taxa at both specific and subspecific levels (Sillero et al. 2014, Dufresnes et al. 2019a,b, 2021, Speybroeck et al. 2020). The advent of a serious amphibian disease in Greece could potentially result in total extinction of stenoendemic taxa, such as *Lyciasalamandra helverseni*, *Pelophylax cretensis*, *Pelobates balcanicus chloeae* Dufresnes, Strachinis, Tzoras, Litvinchuk, and Denoël, 2019, and *Bufotes viridis dionysi* Dufresnes, Probonas, & Strachinis, 2021. *L. helverseni* is distributed only on 3 close islands, with the main population occurring on Karpathos, while *P. cretensis* is endemic to Crete. *P. b. chloeae* is currently only confirmed from a single location (Strofylia area; location code A in Fig. 1), consisting of a very small population (only a few hundred adult individuals), showing very low levels of genetic diversity (Dufresnes et al. 2019a). The Cycladic endemic *B. v. dionysi* is also distributed in a small area, currently known only from the island of Naxos (Dufresnes et al. 2021). Due to small population sizes, limited distribution and low genetic diversity, these taxa are likely to be extremely vulnerable to any environmental change or emerging pathogens such as *Bd*, *Bsal* and ranaviruses. Thus, the seeming *Bd* tolerance in Greek amphibians and the apparent absence of *Bsal* and ranaviruses from Greece, should not allow complacency. Emerging pathogens are constantly spreading in Europe as long as biosecurity measures are loose.

We suggest that disinfection protocols should be communicated and applied by management bodies of protected areas, field researchers and all visitors and users of rural and wetland areas. Especially in areas confirmed as positive for pathogens, human activities in and near waterbodies should be limited, or at least be done with great caution, followed by proper gear disinfection. Methods and guidelines on disinfecting equipment against chytrid fungi and ranaviruses can be found in Van Rooij et al. (2017). In addition, efforts should be made by the authorities to properly inform citizens and raise awareness on the major threats and consequences wildlife faces due to the introduction of non-native species, pet abandonment, translocations, capturing and trading wild animals and using non-disinfected gear between distant freshwater wetlands.

Finally, the data presented here should only be considered a preliminary examination of the distribution of *Bd*, *Bsal* and ranaviruses in Greece. It is vital for future studies to include a larger number of samples from a wider range of locations and species in order to better understand the threats posed by amphibian diseases for the future of amphibians in Greece.

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