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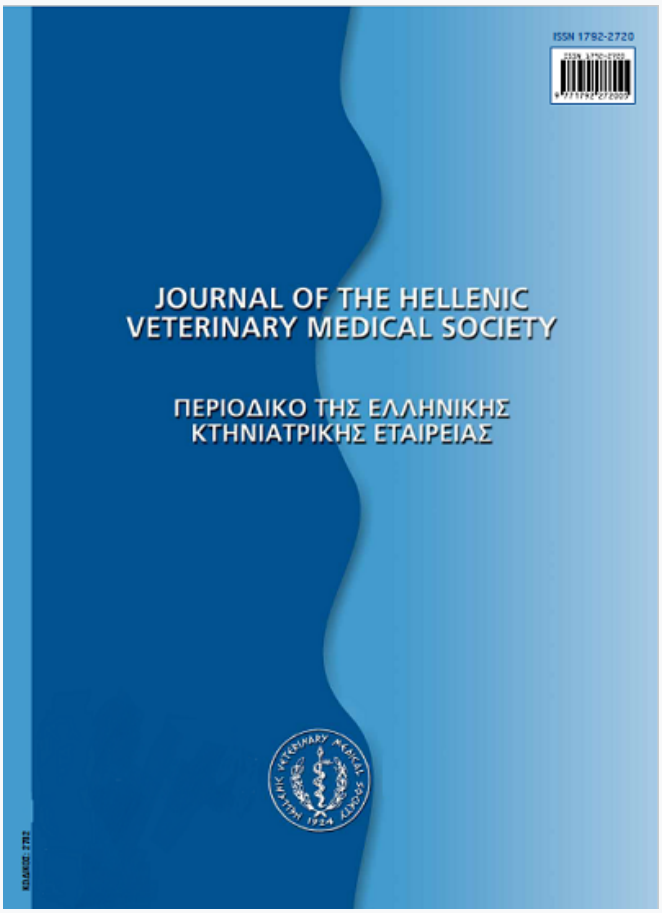

First detection of the amphibian chytrid fungus (Batrachochytridium dendrobatidis) in free- living anuran populations in Greece

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First detection of the amphibian chytrid fungus (*Batrachochytridium dendrobatidis*) in free- living anuran populations in Greece

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Πρώτη ανιχνεύση του χυτριδιομύκητα των αμφιβίων (*Batrachochytridium dendrobatidis*) από ελεύθερους πληθυσμούς ανούρων στην Ελλάδα

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ABSTRACT. The amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) is a widespread, cosmopolitan pathogen largely affecting free-living amphibian populations. So far there are no published studies for the presence of *B. dendrobatidis* in Greece. In this preliminary study we sampled 59 metamorphosed anurans from four Greek wetlands. Five samples were positive for the fungus by real-time PCR. *B. dendrobatidis* was detected in three species (*Bufo viridis, Pelophylax epeiroticus, Pelophylax ridibundus*) but not in endangered endemic Karpathos water frogs (*Pelophylax cerigensis*). This is the first report of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in free-living anuran populations from Greece..

Keywords: *Bufo sp, Pelophylax sp., Vistonida lake, real-time PCR*

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ΠΕΡΙΛΗΨΗ. Ο χυτριδιομύκητας των αμφιβίων (*Batrachochytrium dendrobatidis*) αποτελεί έναν ευρείας εξάπλωσης, κοσμοπολίτικο μικροοργανισμό, που επηρεάζει τους άγριους πληθυσμούς των αμφιβίων. Μέχρι στιγμής δεν έχει δημοσιοποιηθεί κάποια έρευνα για την ύπαρξη του μύκητα στην Ελλάδα. Σε αυτή την προκαταρκτική έρευνα δειγματίσαμε 59 μεταμορφωμένα άνουρα από τέσσερις ελληνικούς υγροτόπους. Πέντε δείγματα διαγνώστηκαν θετικά με την μέθοδο της αλυσιδωτής αντίδρασης της πολυμεράσης σε πραγματικό χρόνο (real-time PCR). Το Βατραχοχυτρίδιο (*Batrachochytrium dendrobatidis*) εντοπίστηκε σε τρία είδη βατράχων (*Bufo viridis, Pelophylax epeiroticus, Pelophylax ridibundus*) όχι όμως και στο απειλούμενο ενδημικό είδος του βατράχου της Καρπάθου (*Pelophylax cerigensis*). Αυτή αποτελεί την πρώτη αναφορά ανίχνευσης του βατραχοχυτριδίου (*Batrachochytrium dendrobatidis*) από άγριους πληθυσμούς αμφιβίων από την Ελλάδα.
Λέξεις ευρετηρίασης: *Bufo sp, Βιστωνίδα λίμνη, Pelophylax sp, real-time PCR*

INTRODUCTION

Chytridiomycosis is a lethal skin disease in amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Bd is an aquatic organism with two life stages: a sessile, reproductive zoosporangium and an amotile, uniflagellated zoospore released from the zoosporangium. Bd colonizes the keratinized layers (*stratum corneum*) of amphibian skin or larval mouthparts (Berger et al. 1998, Pessier et al. 1999). Clinical infection is characterized by epidermal hyperplasia, hyperkeratosis and excessive shedding of the epidermis. Extensive colonization gives rise to a series of physiological effects such as disruption of the osmoregulatory function of the skin, leading to dehydration, electrolyte imbalance and death due to cardiac arrest (Berger et al. 1998, Voyles et al. 2007, Voyles et al. 2009, Marcum et al. 2010, Campbell et al. 2012). The fungus can survive in sterile water for 3 months and may be transferred through moist river sand, infected amphibians and possibly birds (Johnson and Speare, 2005). Safe treatment of infected amphibians consists of terrarium disinfection, heat, chloramphenicol (Young et al. 2012) and voriconazole (Martel et al. 2010, Brannelly et al.2015)

The international presence of the amphibian chytrid fungus (Bd) as well as its importance and impact in amphibian populations is well documented and described in recent global reports (Olson et al. 2013). Bd has been detected in 350 amphibian species on all continents except Antarctica and has been implicated in driving the decline of 200 of these species. (Fisher et al. 2009). Moreover, it was linked with the reduction in monthly survival causing long-term pressure on the amphibian population (Murray et al. 2011). Despite public and scientific awareness this emerging pathogen has gained since its first description, there is little data from the Balkan Peninsula countries (including Greece), in contrast with Western and Central European countries, where extensive research and surveillance projects have been implemented. A previous large- scale European survey (1.664 samples from archived dead specimens in different collections) included samples from Croatia (n= 8) and Greece (n=88), all of which were tested negative (Garner et al. 2004). Recently, the chytrid fungus was detected in Romania (Vörös et al. 2013), and a small-scale survey in Croatia (Vörös and Jelic, 2011) showed again negative results.

Greece is well-known for its mosaic of habitats, which support a great biodiversity. A variety of different habitats can be found throughout the mainland and on the islands of the Ionian and Aegean seas. The main amphibian habitats include large lakes, river deltas, dam lakes, alpine lakes and streams, temporary insular wetlands, lagoons and sand dunes (Valakos et al. 2008). These habitats host 23 amphibian species, 16 of which are anurans (Valakos et al. 2008). The occurring anurans belong to the following taxonomic families: Bombinatoridae (2 species), Bufonidae (2 species), Hylidae (1 species), Pelobatidae (1 species), and Ranidae (10 species). The last family includes two endemic species (*Pelophylax cerigensis, Pelophylax cretensis*) and one alien species (*Lithobates catesbeianus*). These three species have limited distribution

on Karpathos and Rhodes islands (*P. cerigensis*), Crete (*P. cretensis*) and Agya lake in Crete (*Lithobates catesbeianus*) (Valakos et al. 2008).

The purpose of this study was to investigate the possible presence of *B. dendrobatidis* in various habitats in Greece and assess the necessity of broader scale research on the presence of *B. dendrobatidis.*

MATERIALS AND METHODS

For this study, anurans in four representative locations were sampled during spring months (March-May): an insular wetland on the touristic Lefkada island (Ionian Sea), an insular wetland of a relatively less touristic island (Karpathos island, Dodecanese, Aegean Sea), a large lake in North-eastern Greece (Vistonida lake, Thrace) and a lagoon with sand dunes in South-western Greece (Strofylia/Kotychi lagoon, Peloponnese). Additionally, the locations have different protection status: Vistonida lake and Strofylia/Kotychi lagoon are designated as National Parks and RAMSAR Convention wetlands, Karpathos island is an Special Protection Area (SPA)/Site of Special Interest (SCI) of the Natura 2000 Network, while Lefkada island, which is connected by mainland Greece with a traffic bridge, is an SCI of the Natura 2000 Network. The areas are depicted in Figure 1.

All capture activities were part of a broader national amphibian monitoring project and the field researchers had the required permits to enter protected areas and sample specimens. From each wetland, an effort was made to include as many species as possible, and to sample at least 10 individuals per species per location. Amphibians were caught by hand or by net during the evening hours. To prevent cross-contamination between animals, a new pair of disposable, non-powdered vinyl gloves was worn for the capture and handling of each animal from each site and the net and field equipment was disinfected with Virkon® (Antec International) and rinsed with fresh water between each capture site. Each animal caught was immediately sampled noninvasively with a sterile cotton-tipped metal swab (Böttger, Bodenmais, Germany). Each flank, inner thigh, web of hind legs and ventral

Figure 1. Sampling sites of this study for the detection of *Batrachochytrium dendrobatidis* in freeliving anurans in Greece. 1) Lake Vistonida 2) Lefkada island 3) Strofylia/Kotychi lagoon 4) Karpathos island

surface (drink patch) of metamorphosed amphibians were swabbed five times, as described by Hyatt et al. (2007). The swabbed animals were released back into the wild immediately after sampling. All samples were kept away from sunlight and stored frozen (−12 to –16°C) between 1 and 6 h after collection for 1 to 10 weeks before shipping to the laboratory.

Total DNA was extracted using the Roche MagNA Pure 96 system with the MagNA Pure 96 DNA and viral RNA small Volume Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. A real-time PCR was used to assess the presence/absence of *B. dendrobatidis* DNA within the extracted DNA as described previously (Boyle et al. 2004).

RESULTS

None of the adult, metamorphosed anurans collected had clinical lesions of *B. dendrobatidis* or other clinical disease. All anurans sampled were successfully released back in to their habitats. From 59 samples collected in total from the four areas, and four different species, only five samples were positive. Four positive samples were found in Vistonida lake and

Sites	Latitude	Longitude	Species	No. of samples	No. of positive samples
Vistonida lake	41.03°	25.11°	*Bufo viridis*	10	2
Vistonida lake	41.03°	25.11°	*Pelophylax ridibundus*	9	2
Lefkada Island	38.71°	20.64°	*Bufo viridis*	10	0
Strofylia/Kotychi lagoon	38.14°	21.39°	*Bufo viridis*	8	0
Strofylia/Kotychi lagoon	38.14°	21.39°	*Pelophylax epeiroticus*	12	1
Karpathos Island	35.68°	27.16°	*Pelophylax cerigensis*	10	0
				59	**5**

Table 1. Overview of the preliminary results on the detection of *Batrachochytrium dentrobatidis* in free-living anurans from Greece

one in Strofylia/Kotychi lagoon. No positive samples were detected in the insular populations of Lefkada and Karpathos islands. *B. dendrobatidis* was detected in three amphibian species (*B. viridis, P. epeiroticus, P. ridibundus*) but not in the endangered endemic *P. cerigensis*. The summarized results are shown in Table 1.

DISCUSSION

The results of this study verified our suspicion that the amphibian chytrid fungus (*B. dendrobatidis*) is present in Greek anuran amphibian populations, especially in mainland Greece, despite the negative results from a previous study a decade ago (Garner et al. 2004). In that study samples from Greece included the species *Bombina variegata, Bufo viridis, Hyla arborea, Pelophylax ridibundus, Pelophylax cretensis* and *Rana graeca*. The sampling sites included various upland streams of the Rhodope Mts (Thrace), Nestos river (Thrace), Ossa Mt (Thessaly), Plastira damlake (Thessaly), Velouchi Mt, and Vardousia Mt (Sterea Hellas), Strofylia/Kotychi lagoon (Peloponnese), Lesvos island (East Aegean Sea) and Crete. Since the fungus was not detected in any of these samples, which were collected between 1994-2004, it can be assumed that at that time point the fungus was not present in these populations. Of course, it cannot be excluded that a low prevalence of the chytrid fungus remained undetected due to low sample submission or sampling of unaffected remote upland or insular populations. Additionally, surveillance on amphibian disease is not well established among field researchers and could go easily unnoticed. Population estimates or trends are not available for the Greek amphibian populations, so a slow decline could not have been estimated and therefore cannot be excluded. Vistonida lake holds abundant amphibian populations but no signs of massive die-offs or increased mortality was noticed during the field study. This could be attributed to the different virulence of the fungal lineages (Farrer et al. 2011, Rosenblum et al. 2013) or the high ecological evolution of *B. dendrobatidis* strains with frog populations (Rodriguez et al. 2013). To the authors' knowledge, massive die-offs of amphibians have not been reported or when reported, have been attributed to environmental causes (e.g local poisoning by waste waters, contamination, habitat defragmentation and destruction). Nevertheless, dead specimens have never been previously submitted for standard veterinary diagnostic investigation (i.e. necropsy, histopathology, bacteriology, mycology, virology etc).

The role of anthropogenic transmission, through trade of bullfrogs, of the chytrid fungus has been discussed (Schloegel et al. 2012, Rodriguez et al. 2013). American bullfrogs are not considered a delicacy in

Greece and therefore Bullfrogs are not bred in captivity. The presence of American bullfrogs is isolated in Agya lake in Crete (Mantziou et al. 1999, Valakos et al. 2008). Since this study was limited to only a few sites, we cannot extrapolate any assumptions on the role of touristic activity (or general human activity), protection status of the area or the type of habitat/water consistency/temperature in the propagation and distribution of the fungus in Greece. Vistonida lake with the relatively higher occurrence of positive samples (4/5) is often visited by birdwatchers and hikers while a small fishing community is active between the lake and the nearby harbor, using the lake as a natural fish nursery. These two user communities might have transferred the fungus mechanically, but so far no proof has been found to support this scenario. Strofylia/Kotychi lagoon is also a birdwatcher hotspot and near the Patras harbor, from where many tourists, as well as refugees from African and Asian countries depart to reach Italy and Western Europe. Another possible source of *B. dendrobatidis* in these two wetlands might be the high presence of migrating and wintering waterfowl (Garmyn et al. 2012). Nevertheless, the findings might be only accidental to these wetlands and further research is needed.

The fact that three common widely distributed species are affected, indicates that individuals from these species in other wetlands throughout their distribution range could be also infected. Further research is needed from various amphibian species and wetlands in Greece to draw statistically valuable data on the distribution of *B. dendrobatidis* and attempt a first risk assessment and analysis for the Greek and Balkan amphibian populations. Forthcoming field research should include a larger number of wetlands with variable habitats, a larger number of samples and amphibian anuran species-especially endemic species. The role of the alien American bullfrog (*L. catesbeianus*), a known carrier of the amphibian chytrid fungus (Daszak et al. 2004, Schloegel et al. 2010, Schloegel et al. 2012), should be evaluated in the local decrease of the population of the endemic Cretan water frog (*P. cretensis*) (Mantziou et al. 1999).

Last, this pathogen and its effects on Greek amphibians should be further studied with the help of the public authorities, naturalist associations, and local communities in order to prevent the further spread of the fungus in other wetlands. Biosecurity measures should be communicated to the local and central authorities (national park management bodies, ministry directions) and published in the mass media to increase vigilance on amphibian mass mortality events. Our team as part of the Hellenic Herpetological Society (Societas Herpetologica Hellenica) has already addressed the previously mentioned issues. Experienced field herpetologists have been adequately informed to report any sighting of suspected amphibian disease or mass mortality in order to create a long-term database on suspected amphibian diseases for future research analysis.

CONCLUDING REMARKS

The chytrid fungus is reported for the first time in Greek anurans (OIE notifiable disease in amphibians). At the moment its distribution in the Greek amphibian populations and potential impact is not well–known. Of special interest are the insular and endemic amphibian populations due to their fragility. A vigilance project on mass mortality of amphibians and further research has been initiated. The competent authorities and the public have been informed of these first results.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

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