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MONITORING *BATRACHOCHYTRIUM DENDROBATIDIS* IN ITALY

SUMMARY

Amphibian declines is a paradigmatic example in the context of the current sixth mass extinction. Drivers of these declines include the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). The skin disease caused by this fungus is named chytridiomycosis and affects the vital function of amphibian skin. In this study we analysed by means of real-time PCR the abundance of *Bd* in more than 1300 Italian amphibian swabs. Preliminary results show a diffusion of *Bd* in 11 out of the 17 species tested and a prevalence of the fungus in 6% of the individual infestation rate was relatively low, not overpassing 150 genome equivalents of *Bd*.

Key words: amphibian decline, chytridiomycosis, *Bd* individual load.

RIASSUNTO

Il Monitoraggio di Batrachochytrium dendrobatidis *in Italia*. La diffusione del fungo patogeno *Batrachochytrium dendrobatidis* (*Bd*) è una delle cause del declino delle popolazioni delle popolazioni di anfibi. Questo fungo causa una infezione della pelle degli anfibi chiamata chitridiomicosi. In questo studio abbiamo analizzato tramite PCR quantitativa oltre 1300 tamponi di pelle di anfibio appartenenti a 17 specie. La percentuale di individui infetti è risultata del 6% a livello nazionale, mentre il tasso di prevalenza individuale è risultato relativamente basso, non superando i 150 equivalenti genomici di *Bd*.

Parole chiave: declino degli anfibi, chitridiomicosi, carico individuale di Bd.

Introduction

The widespread chytridiomycosis is caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) that affects, often with lethal consequences,

the skin of many amphibian species. Its origin is still unknown, but recent hypotheses suggest that it could come from Asia (SCHEELE *et al.*, 2019). This fungus is dangerous for amphibians because it is able to colonize host's epidermis, compromising the integrity of the keratin of the skin. Skin infection occurs in the water, through the mobile zoospore penetrating the amphibian skin and forming the sessile, reproductive zoosporangium, from where, the uniflagellated zoospore are released to reinfect the same individual or others nearby. It has been documented that amphibian skin is able to exert a sort of chemotaxis, thus attracting *Bd* zoospores thanks to the presence of specific molecules such as sugars, proteins and amino acids (MOSS *et al.*, 2008). Amphibians affected by the pathogen can be lethargic and the most obvious clinical signs, including ulcers and small hemorrhages, are observed on the epidermis.

These lesions have consequences on skin respiration and osmoregulation. Thus, leading to loss of muscle contraction force and eventually to death by heart failure. However, the sensitivity to this fungal agent in amphibian species may vary: while some die quickly as a result of infection, others manage to survive, contributing to the spread of the agent through water contamination. Italy is the European country with the highest number of endemic amphibian species, thus monitoring *Bd* spread in Italy can be a useful tool to preserve amphibian diversity. A previous study by COSTA *et al.* (2021), focused on the modeling the spread of *Bd* on the basis of presence/absence data, whereas in this study we aimed to determine the species-associated individual load of *Bd* in amphibians all over peninsular Italy.

MATERIALS AND METHODS

Starting from 2013, we analyzed more than 1000 amphibian swabs from several Italian national parks and nearby regions, in the framework of the national project "Monitoraggio delle specie di ambiente umido acquatico" coordinated by Cinque Terre National Park. Sampling permits were issued by the Italian Ministry of Environment (PNM-2013-00424466; PNM-2015-0016824; PNM-2016-0013862; PNM-2017-005370; PNM-2019-00004038). The ITS region of *Bd* DNA was quantified by Real-Time PCR (RT-PCR) (Fig. 1).

DNA was extracted from the swabs minced in PrepMan Ultra extractant solution then were subjected to mechanic shock by using microspheres of silica and a bead beater ant then centrifuged at 13'000xg for 3 minutes. The steps above were performed twice. The samples were then incubated at 100°C for 10 minutes. After centrifugation at 13'000xg for 3 minutes, samples were diluted in molecular biology grade water 1:10 and 1:100 for further

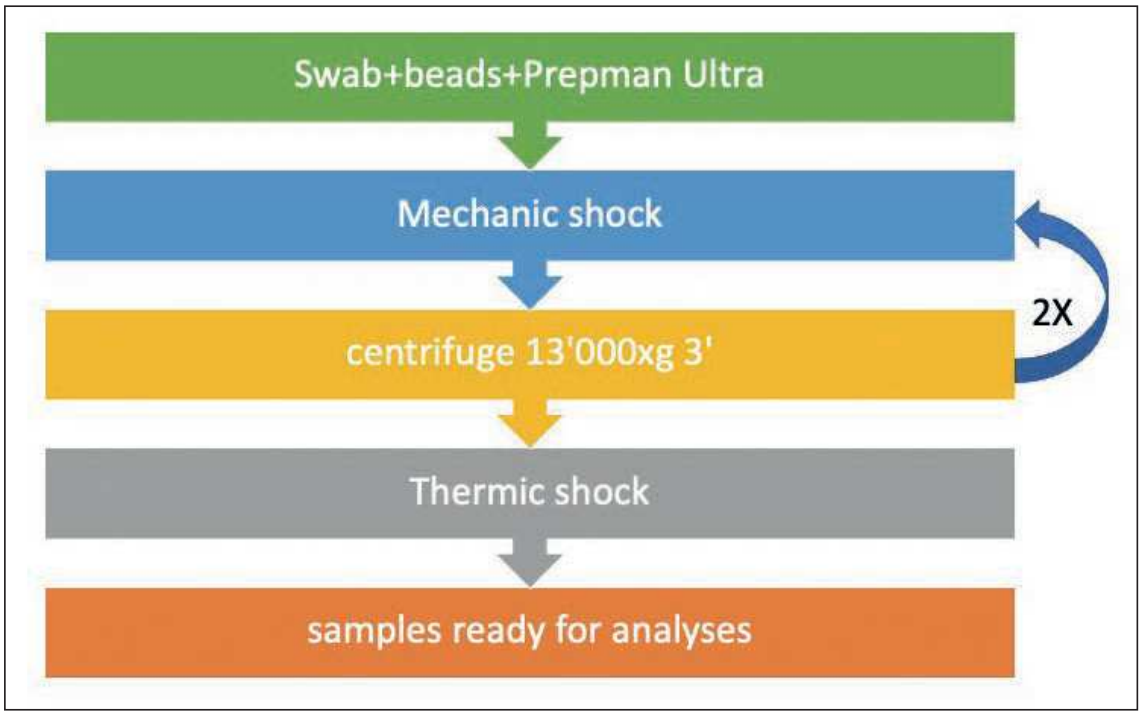

Fig. 1 — Steps performed for DNA extraction from swabs as published online (https://savethefrogs.com/chytrid-fungus-detection-qpcr/)

analyses. Samples were analyzed through RT-PCR, using SYBR green as a method for detecting the presence of *Bd*. A standard curve was also performed by running samples with known amount of *Bd* genome zoospores: 100, 10, 1 and 0.1 (Fig. 2A). The resulting standard curve (Fig. 2B) allowed us to determine the amount of *Bd* genome equivalents (*Bd* GE) in the analysed samples CANESSA *et al.* (2017).

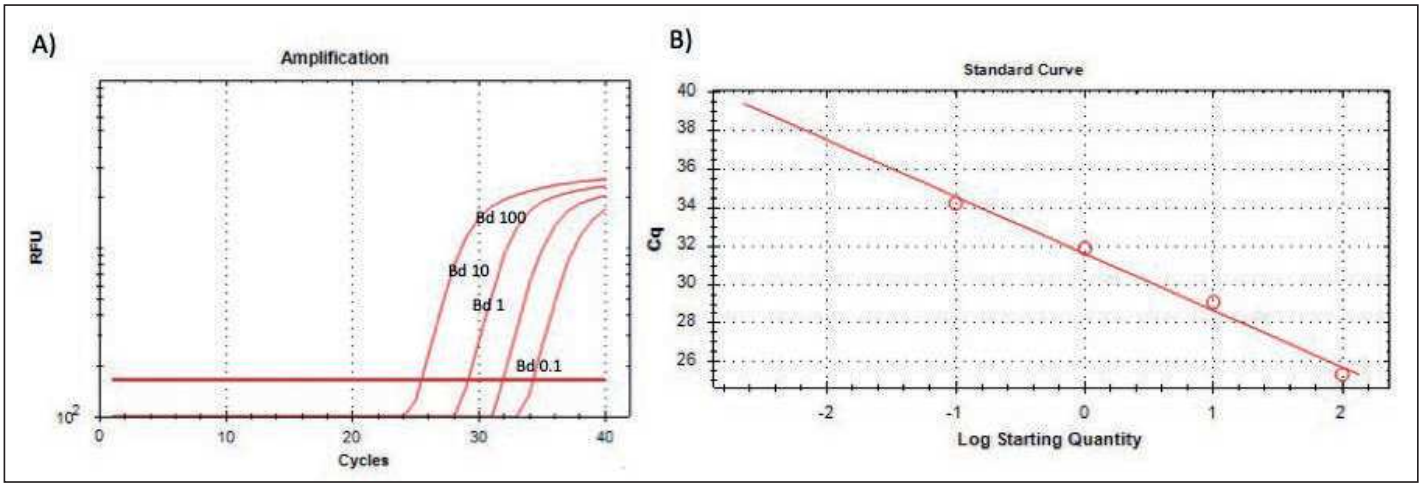

Fig. 2 — A) Amplification curves of *Bd* standards and B) standard curve.

RESULTS AND CONCLUSIONS

The mean individual *Bd* load per species expressed in GE is given in Table 1. Infected individuals were found in 11 out of 17 species and all over the Apennines, from Liguria to Calabria, where *Bd* seems relatively frequent (see Fig. 2 in COSTA *et al.*, 2021). We did not observe any associated mass mortality and GE were generally low, ranging from 1 to 148, in comparison with BALÁZ *et al.* (2013), who reported up to 4000 GEs. The overall *Bd* prevalence in Italian amphibian species was 6%. Further sampling in particular in the Alps should clarify the distribution of *Bd* prevalence and the ecological correlates that may concur in spreading this pathogen in Italian amphibian populations.

Table 1
Prevalence of *Batrachochytrium dendrobatidis* (*Bd*) in Italian amphibians.
The mean infestation load and the range are provided in genome equivalents (GE).

Species	**N**	***Bd*+**	***Bd* Prevalence (%)**	**GE Mean**	**GE Range**
Bombina pachypus	410	9	0.02	64	21 - 148
Bufo bufo	84	8	10	9	3 - 21
Bufotes balearicus	46	0	0	/	/
Hyla intermedia	1	1	100	/	30
Hyla meridionalis	5	0	0	/	/
Pelophylax sp.	159	9	6	23	1-127
Rana italic	97	13	13	9	3-25
Rana dalmatina	74	0	0	/	/
Euproctus platycephalus	3	0	0	/	/
Ichthyosaura alpestris	124	4	3	2	2-3
Lissotriton italicus	77	15	19	26	2-57
Lissotriton vulgaris	53	5	9	14	3-30
Salamandra atra aurorae	3	0	0	/	/
Salamandra salamandra	50	1	2	/	6
Salamandra lanzai	1	0	0	/	/
Salamandrina terdigitata	31	7	23	12	4-20
Triturus carnifex	90	2	2	22	15-29

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