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# *Batrachochytrium dendrobatidis* in amphibians from the Po River Delta, Northern Italy

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**Abstract**. *Batrachochytrium dendrobatidis* is a pathogen infecting amphibians at the global scale and causing their decline, but knowledge of the distribution of this pathogen is far from complete. We sampled amphibians from three species (*Hyla intermedia*, *Rana dalmatina* and *Pelophylax* synklepton *esculentus*) to evaluate whether *B. dendrobatidis* infects amphibians in the Po River Delta Natural Park, Northern Italy. We detected the pathogen in one population of *P.* sk. *esculentus* (prevalence: 0.33). These findings expand the known distribution of *B. dendrobatidis* in Italy and add further concern to the conservation of amphibians in this area.

**Keywords**. Amphibian decline, Chytridiomycosis, Emerging infectious disease, Management, Prevalence, Pool frogs.

The chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), is the agent of chytridiomycosis, an emerging infectious disease causing amphibian decline and extinctions at the global scale (e.g., Berger et al., 1998; Fisher et al., 2009). The study of distribution, prevalence and impact of *Bd* is becoming a central theme of amphibian conservation: information on *Bd* distribution can be used to better understand the causes of amphibian decline and eventually to set up management and prevention protocols (Andreone et al., 2008; Fisher et al., 2009). However, in several areas of the world the knowledge of *Bd* distribution remains limited. For instance, only a few studies investigated the occurrence and the impact of *Bd* in Italy. *Bd* has been detected in populations of the yellow-bellied toad (*Bombina pachypus*) in the northern Apennines (Stagni et al., 2004), in pool frogs (*Pelophylax* synklepton *esculentus*) from Piedmont and central Italy

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(Simoncelli et al., 2005; Di Rosa et al., 2007), in populations of the frogs *Rana latastei* and *Lithobates catesbeianus* from Piedmont (Garner et al., 2004; Garner et al., 2006) and in the endemic painted frog (*Discoglossus sardus*) and Sardinian newt (*Euproctus platicephalus*) in Sardinia (Bovero et al., 2008; Bielby et al., 2009). Furthermore, *Bd* is probably a cause of the decline of *B. pachypus*, *E. platicephalus* and *D. sardus* (Stagni et al., 2004; Bovero et al., 2008; Bielby et al., 2009). Nevertheless, information on the presence (or absence) of *Bd* from large portions of Italy is extremely limited, and further data are required for a more complete assessment of the distribution of *Bd*; these data may also have important consequences for the conservation and management of amphibian populations.

The aim of this study was to evaluate whether *Bd* infects amphibians in the Po River Delta. This area is particularly interesting for the study of *Bd* because it includes some of the most important wetland systems of Italy and hosts a rich herpetofauna (Mazzotti, 2007; Mazzotti et al., 2007). On the other hand, the Po River Delta is one of the first areas of Italy that has been invaded the American Bullfrog *L. catesbeianus* (Albertini and Lanza, 1987; Ficetola et al., 2010), and this alien species is probably implicated in the global spread of *Bd* (Garner et al., 2006).

In May 2010, we sampled amphibians in three areas of the Po River Delta Regional Park (Table 1). One area (Bosco Mesola integral Natural State Reserve) is located in the north of the park, while the other two are nearby the town of Ravenna, in the central part of the park. Amphibians where captured by hand or using small nets. The complete body was comprehensively swabbed using fine-tip swabs (Medical Wire & Equipment Co. MW 100–100). The underside of the legs, feet and drink patch was swabbed 3 to 5 five time following the protocol of Hyatt et al. (2007). All individuals were released in the environment immediately after swabbing.

Swab samples were stored frozen until DNA extraction. DNA extraction and real-time PCR were performed using the protocol by Boyle et al. (2004)slightly modified. Swab was cut and a 2 mm portion was put in 60 µl of PrepMan Ultra (Applied Biosystem) with 30 to 40 mg of 0.5 mm diameter glass beads (Scientific Industries, Inc.). The samples were homogenised for 45 s at 30 m/s in a QIAGEN *TissueLyser* II (*Retsch* Technology GmbH) and centrifuged for 30 s at 14000 g. The homogenisation and centrifugation was repeated. Samples were then incubated at 100°C for 10 min, cooled for 2 min, then centrifuged at 14000 g for 3 min. All the liquid was collected and stored at -20°C. The DNA extracted was diluted 10 times for subsequent real-time quantitative PCR assay. Real-time quantitative PCR reactions were performed in a iQ5 system (BioRad) following the protocol described by Boyle et al. (2004), but using iQ Supermix (BioRad) instead of Taqman Master Mix. Both samples and the negative controls were run in duplicates. PCR stand-

**Table 1.** Sampling locations, amphibian species and prevalence of *B. dendrobatidis*.

| Locality | Lat. | Long. | Species | n | Prevalence | 95%CI |
|---|---|---|---|---|---|---|
| Bosco Mesola (FE) | 44.83°-44.85°N | 12.25°-12.26°E | *Rana dalmatina* | 2 | 0 | 0-0.667 |
| Bosco Mesola (FE) | 44.83°N | 12.26°E | *Pelophylax* sk. *esculentus* | 3 | 0.33 | 0.039-0.823 |
| Ravenna | 44.58°N | 12.27°E | *Pelophylax* sk. *esculentus* | 27 | 0 | 0-0.088 |
| Bardello (RA) | 44.53°N | 12.24°E | *Hyla intermedia* | 28 | 0 | 0-0.085 |

ards were obtained using the protocol described by Boyle et al. (2004). The isolates were acquired from infected *Alytes obstetricans* collected in Spain and cultured at the Imperial College of London (Fisher et al., 2009). Infection data were submitted to the *Bd* Global Mapping Project (www.bd-maps.net)

We then used the Bayesian equal-tailed Jeffreys prior intervals to estimate 95% confidence intervals (CI) of *Bd* prevalence in collected samples (Brown et al., 2001). Jeffreys intervals have been shown to perform well in estimating binomial CI under a variety of conditions (Brown et al., 2001).

We obtained samples from 60 individuals from 3 species: Italian treefrog (*Hyla intermedia*), agile frog (*Rana dalmatina*) and pool frogs (*P.* sk. *esculentus*) (Table 1). One adult pool frog from Bosco Mesola was positive to *Bd*; the amount of *Bd* DNA in the positive frog was 0.24 genome equivalents.

The detection of *Bd* infection in amphibians from the Po River Delta adds evidences to the presence of this pathogen in multiple areas of mainland Italy, and improves our knowledge of its distribution. *Bd* prevalence in our populations was probably not high, as we detected only one positive sample. On the other hand, in the Bosco Mesola area we captured a small number of individuals, therefore the confidence intervals associated to the prevalence in this area remain wide (Table 1). In this area, further sampling is required to assess the actual prevalence of *Bd* in pool frogs and in other amphibians. Furthermore, sampling should cover multiple seasons, because the prevalence and intensity of infection are strongly affected by climatic conditions and may vary seasonally (Pullen et al., 2010; Savage et al., 2011).

It should be noted that the only positive sample was from *P.* sk. *esculentus.* This result was analogous to the findings of Federici et al. (2008) from Piedmont (north-western Italy): out of 10 species analyzed, they detected *Bd* in pool frogs only. As this species may coexist with *Bd* without suffering a direct decline, it might act as a reservoir for the pathogen, spreading it to other species (Simoncelli et al., 2005; Di Rosa et al., 2007).

The Po River Delta hosts important populations of amphibians (Mazzotti et al., 2007), but unfortunately these are threatened by multiple factors, including the salinization of breeding wetlands, the isolation of wetlands, and alien invasive species (Ficetola et al., 2004; Mazzotti, 2007). As a consequences, some threatened amphibians, such as the spadefoot toad *Pelobates fuscus* and the Italian agile frog *R. latastei* are nearly extinct in the area (Mazzotti, 2007). The presence of *Bd* is a further threat to amphibians, and increases the complexity of management. For instance, captive breeding and translocation programs are ongoing in the study area for the conservation of several amphibians (Costa and Gattelli, in press). The potential impact of *Bd* should be taken into account during these actions, because translocating animals may increase the spread of the pathogen. It would be particularly interesting testing whether the declining *P. fuscus* and *R. latastei* are infected by *Bd*. Furthermore, a more systematic application of precautionary measures to avoid the dissemination of these emerging diseases through human activities is highly desirable (Speare et al., 2004; Dejan et al., 2010; Phillott et al., 2010). To date, information on the spread of *Bd* in Italian amphibians remains limited. It is thus important to expand the sampling of this pathogen and to evaluate whether it is implicated in the decline of other Italian amphibians (Bonardi et al., 2011).

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