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# Identification of the pathogen *Batrachochytrium dendrobatidis* in amphibian populations of a plain area in the Northwest of Italy

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**Abstract.** In Italy, *Batrachochytrium dendrobatidis* has been firstly reported in 2001. Here, we present the results of a study planned to obtain an overview of its distribution in two areas of Turin province (Northwest Italy). Samples from the Pianalto of Poirino were collected in 2008, and tissue samples from the area surrounding Ivrea were collected in 2003. The occurrence of *B. dendrobatidis* zoospores, tested using a highly sensitive PCR reaction, has been confirmed in four wild populations from one of the two tested areas. To prevent the dispersion of pathogen zoospores in the whole area, precautionary measures are strongly encouraged.

**Key words.** *Batrachochytrium dendrobatidis*, Pianalto of Poirino, *Pelophylax* kl. *esculentus*, Conservation

## Introduction

*Batrachochytrium dendrobatidis* (from here onwards Bd) is largely implicated in global amphibians declines (Berger et al., 1998). After the first report of chytridiomycosis infection in amphibian wild populations, dating back to the late nineties (Berger et al., 1998; Pessier et al., 1999), the disease has been detected in numerous localities around the world (Skerratt et al., 2007), and currently 365 globally distributed *B. dendrobatidis* presence points exist (http://www.spatialepidemiology.net/bd/).

In Europe, the first documented infections in wild populations of *Rana arvalis* (Germany) and *Alytes obstetricans* (Spain) caused by Bd date to 2000 (Mutschmann et al., 2000; Bosch, Martínez-Solano and Garcia-Paris, 2001), while the first record in Italian wild populations has been reported in 2001 (Stagni et al., 2004). Later, this disease has been reported in Umbrian populations (Trasimeno Lake) of the *Pelophylax esculentus* complex (Simoncelli et al., 2005; Di Rosa et al., 2007). In the meantime, Bd has been detected in *Rana latastei* (Garner et al., unpublished data), *Lithobates catesbeianus* (Garner et al., 2006, Adams et al., in press) and *P.* kl. *esculentus* (Adams et al., in press) of northern Italy (Piedmont), and finally, in Sardinian populations of the endemic *Euproctus platycephalus* (Bovero et al., in press).

The aim of the present work is to evaluate the presence and distribution of Bd in two areas of the Northwest Italy: (A) the "Pianalto of Poirino" (from here onwards Pianalto), in the South of Turin province (Fig. 1, Tab. 1); (B) the area close to Ivrea, in the North of Turin province (Fig. 2, Tab. 1). In both these areas, the presence of some residual populations of *Pelobates fuscus* makes this survey of particular interest (Andreone, Gentilli and Scali, 2007).

## Material and Methods

The Pianalto is a plateau area extending for 400 km$^2$, about 20 km from Turin metropolitan area. Two Communitary Important Site (CIS) are included in the Pianalto: (*i*) IT1110035 "Stagni di Poirino-Favari" and (*ii*) IT1110051 "Peschiere e laghi di Pralormo". The CIS "Stagni di Poirino-Favari" lays in the western part of the Pianalto, and extends for 1844 ha in the area between Poirino, Santena and Villastellone. This CIS hosts some of the most abundant Italian populations of *Pelobates fuscus* (Andreone, Gentilli and Scali, 2007), and numerous individuals of the North American species *L. catesbeianus*, introduced in Italy more than 50 years ago (Lanza et al., 2007). This species is known to act as a vector for Bd dispersion, and chytrid infection has already been detected in *L. catesbeianus* samples from Valfenera (Turin) (Garner et al., 2006), a locality close to the Pianalto area

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that we sampled. Together with samples collected in the Pianalto area during 2008, we processed samples collected during 2003 in the area close to Ivrea, in the North of Turin province (Fig. 2). Although there are no reports about chytridiomycosis infection in this area, the choice to screen these sites is due to the presence of other abundant relict populations of *Pelobates fuscus* (Andreone et al., 2004).

We report the results of the survey for the pathogen in ten amphibian species from 12 wild populations in the Northwest Italy (see Tab.1; Fig. 1). The Pianalto area hosts nine analysed populations (Tab. 1, A; Fig. 1), three of which are included in the CIS "Stagni di Poirino-Favari" (see Tab. 1). The remaining three sampled populations come from the area close to Ivrea (see Tab. 1, B; Fig. 2). Totally, 153 samples were tested for Bd presence. Ethanol-stored tissue samples were obtained in several different ways: from previous field collections, from adults killed by road traffic, from swabbed and toe clipped individuals, subsequently released. Indeed, toe clipping is a method that in amphibians is known to allow high survival rates for the released individuals (Hott and Scott, 1999).

In 2008, we captured individual amphibians and sampled them for Bd on swabs and toe-clips. To avoid Bd zoospores dispersion we used different nets and disinfected them between each sampling location, following Societas Herpetologica Italica hygienic guidelines. Frogs were kept separately prior to swabbing and we wore new pair of latex gloves for each sampled individual. While handling the frog, the swabbing procedure was carried on by strongly rubbing back and forth ca. 30 times the underside of adult, preferably the drink patch, the thighs and the webbing between toes. Each swab and toe-clip was analyzed for the presence of Bd DNA using PCR.

Total genomic DNA was extracted using proteinase K digestion (10 mg/ml concentration) following the protocol of Bandi et al. (1994). The presence of pathogen was detected using Bd-specific primers: Bd1a (5'-CAGTGTGCCATATGTCACG-3') and Bd2a (5'-CATGGTTCATATCTGTCCAG-3') (Annis et al., 2004), designed from the ITS1 and ITS2 regions, respectively. PCR reactions were performed in 20µl reactions using ca. 1 ng of genomic DNA, 0.2µl of Bd1a (0.2 mM), 0.2µl of Bd2a (0.2 mM), 2µl of total dNTPs (0.2 mM), 0.1µl of (0.5U) of MasterTaq Eppendorf®, 2µl 1x Buffer including $MgCl_2$ at 1.5 mM and 14.5µl of water. PCR conditions were: an initial denaturation step at 93°C for 600s, 30 cycles of denaturation at 93°C (45s), annealing at 58°C (45s) and extension at 72°C (60s), followed by 10 minutes

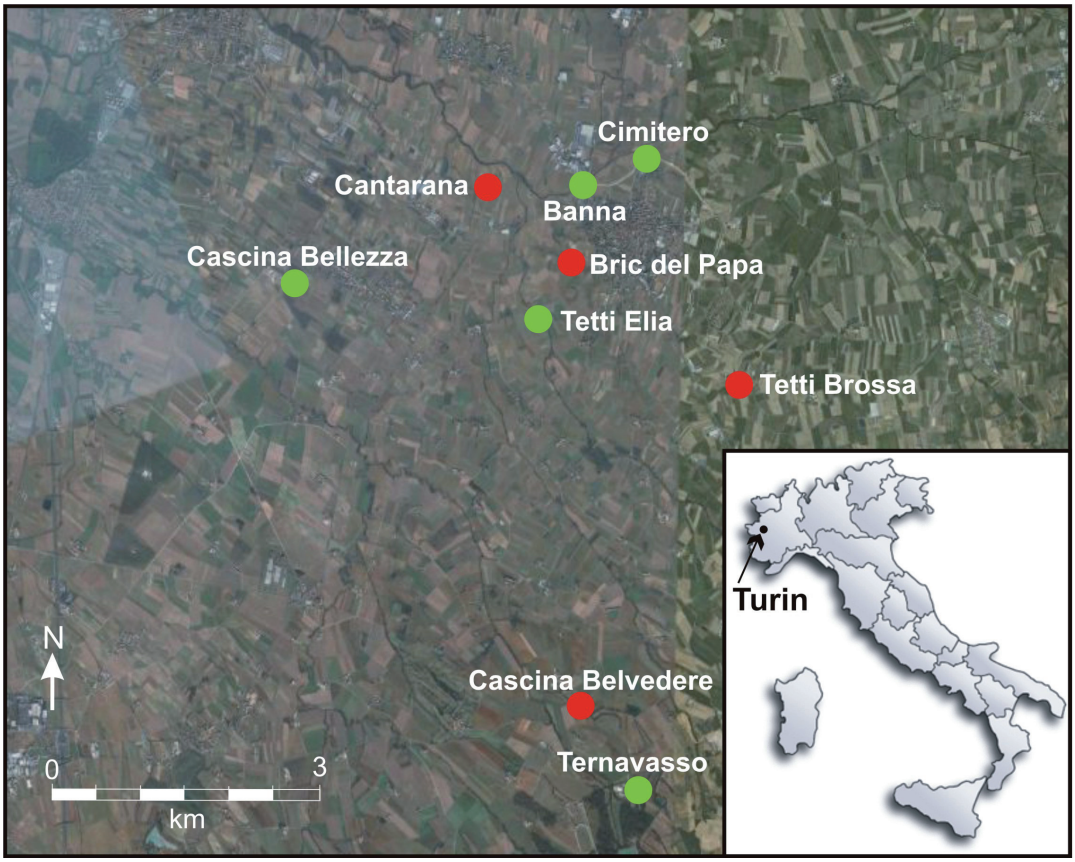


**Figure 1.** Map of the Pianalto of Poirino, with the nine sample localities tested for Bd. The inset map shows the geographic location of Turin in Italy. Green circle, site where no positive samples were found; Red circle, site where Bd infection was detected.

**Table 1.** List of localities sampled for this study and information concerning their geographical area, geographical coordinates, sample size (*n*); number of individuals sampled for each species and information about the presence of Bd. A, Pianalto of Poirino; B, the area close to Ivrea; *, sites included in the CIS IT1110035 "Stagni di Poirino-Favari"; #P, number of individuals positive to Bd infection and percentage; #N, number of individuals negative to Bd infection; Pe, *Pelophylax* kl. *esculentus*; Rd, *Rana dalmatina*; Lv, *Lissotriton vulgaris*; Pf, *Pelobates fuscus*; Pv, *Pseudopidalea viridis*; Tc, *Triturus carnifex*; Hi, *Hyla intermedia*; Bb, *Bufo bufo*; Ss, *Salamandra salamandra*; Rl, *Rana latastei*.

| Area | Site | Latitude | Longitude | *n* | Species | #P | #N |
|---|---|---|---|---|---|---|---|
| A | Cascina Bellezza* | 44°54'44" | 7°47'19" | 12 | Pe (5); Rd (1); Lv (1); Pf (1); Pv (3); Tc (2) | 0 | 12 |
| A | Ternavasso | 44°51'01" | 7°51'02" | 8 | Pe (8) | 0 | 8 |
| A | Cimitero | 44°55'42" | 7°50'60" | 7 | Pe (1); Pv (3); Hi (3) | 0 | 7 |
| A | Cantarana* | 44°55'28" | 7°49'20" | 10 | Hi (5); Tc (1); Pv (2); Pe (2) | Pe (1) (10%) | 9 |
| A | Cascina Belvedere | 44°51'37" | 7°50'25" | 7 | Pe (7) | Pe (3) (42.8%) | 4 |
| A | Bric del Papa | 44°54'55" | 7°50'14" | 12 | Pv (5); Tc (1); Pe (6) | Pe (2) (16.7%) | 10 |
| A | Banna | 44°55'30" | 7°50'19" | 1 | Pe (1) | 0 | 1 |
| A | Tetti Brossa | 44°54'02" | 7°52'00" | 16 | Pe (14); Pv (2) | Pe (7) (43.7%) | 9 |
| A | Tetti Elia* | 44°54'29" | 7°49'53" | 12 | Pf (22); Hi (1); Rd (1); Bb (7); Pe (2); Tc (1) | 0 | 12 |
| B | Cascinette | 45°28'53" | 7°54'24" | 11 | Pf (11) | 0 | 11 |
| B | Maceratoio Rettore | 45°29'28" | 7°54'44" | 35 | Ss (5); Rl (5); Pe (5); Rd (5); Tc (5); Bb (5); Hi (5) | 0 | 35 |
| B | La Sneira | 45°29'49" | 7°53'52" | 22 | Lv (1); Tc (5); Pe (5); Rd (5); Ss (1); Bb (5) | 0 | 22 |
| TOTAL | 12 | - | - | 153 | 10 | 13 (8.5%) | 140 |

of elongation at 72°C. PCR products were loaded onto 1.5% agarose gels, stained with ethidium bromide, and visualised under ultraviolet light (365 nm). Samples were scored as positive for Bd infection based on the presence of the approximately 300 bp band (see Fig.3). Known Bd was used as positive control in all PCR runs (Dall'Olio, pers. comm.). Positive products were purified using QIAquick spin columns (Qiagen). The light strands were sequenced using an ABI3730XL by Macrogen Inc. Sequences were manually edited and aligned using the BioEdit sequence alignment editor (version 7.0.5.3; Hall, 1999), and were submitted to Genbank (FJ010547-FJ010560). Blast analysis of the fragment revealed the highest match with sequences of Bd.

## Results and discussion

We tested 153 amphibians, 85 from the Pianalto and 68 from Ivrea surroundings. Of these, only samples coming from the Pianalto were positive for Bd, while none of the individuals from Ivrea was positive (Tab. 1). Thirteen individuals of *P.* kl. *esculentus* (8.5%), were positive to Bd. Four different ponds hosted positive individuals: Cantarana, Cascina Belvedere, Bric bel Papa and Tetti Brossa (Tab. 1; Fig. 1, A). Swabs and toe-clips agreed in showing positivity or negativity to Bd. Given the small dataset, in sites where we detected its presence, Bd occurrence was relatively high, with values ranging between the 10% to the 43.7% of the sampled individuals at each locality. Curiously, the occurrence of Bd has been detected only in water frogs (*Pelophylax*), while the other species of urodeles and anurans resulted as negative. Anyhow, according to our opinion, the presence of Bd in water frogs is of special concern because, as for *L. catesbeianus* (Daszak et al., 2004), the infected frogs could act as vectors and contribute to the spreading of the infection on less resistant species (Simoncelli et al., 2005).

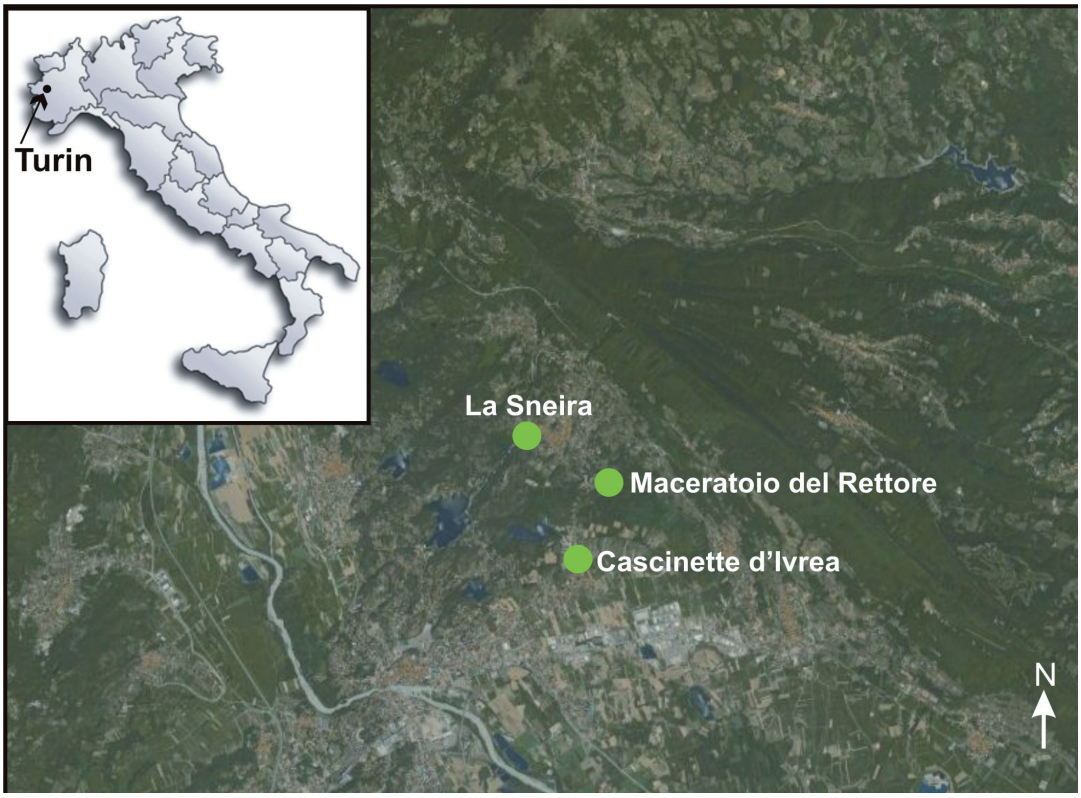


**Figure 2.** Map of the area close to Ivrea, with the three sample localities tested for Bd. The inset map shows the geographic location of Turin in Italy. Green circle, site where no positive samples for Bd were found.

Our results stress the necessity of additional survey in the area of Pianalto, in order to depict a detailed map of occurrence for Bd. In sites where positive samples were found it is compulsory to take the necessary precautions to avoid the diffusion of this dangerous pathogen. In particular, people taking part in amphibian monitoring should pay special attention not to transfer the zoospores from site to site, using different tools or disinfecting them accurately before moving between sites. Moreover, seen the presence of Bd, it is also recommended that any action dealing with pond creation and land displacement is made with a special caution, taken into consideration that the spread of Bd could result in alarming declines of populations reproducing in ponds isolated by the intense agricultural matrix. Further environmental monitoring, combined with proper methods of quarantine and pathogen elimination are badly needed to guarantee amphibian survival and to prevent the spread of chytridiomycosis in this area.

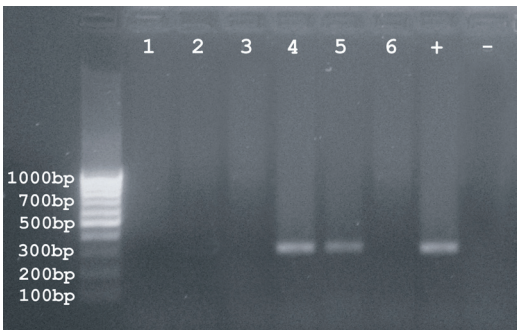


**Figure 3.** Agarose gel electrophoresis of amplification products. Samples are scored as negative or positive for B. dendrobatidis based, respectively, on the absence (samples 1, 2, 3, 6) or presence (samples 4 and 5) of the approximately 300 bp band. +, known Bd DNA used as positive control; -, negative control.

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