Vol. 107: 61–68, 2013
doi: 10.3354/dao02655

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published November 25

# Geographic distribution of the chytrid pathogen *Batrachochytrium dendrobatidis* among mountain amphibians along the Italian peninsula

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ABSTRACT: The amphibian chytrid pathogen *Batrachochytrium dendrobatidis* (*Bd*) is considered a major cause of amphibian population declines, particularly in montane areas. Here, we investigated the presence and distribution of *Bd* among populations of 3 mid- to high-altitude species spanning the entire Italian peninsula (486 individuals from 39 sites overall): the stream frog *Rana italica*, the fire salamander *Salamandra salamandra gigliolii*, and the alpine newt *Mesotriton alpestris apuanus*. We found *Bd* in all of the analyzed species. Despite the widespread distribution of the pathogen, its overall prevalence (6, 9 and 19%, respectively) was lower than previously reported for the endangered Apennine yellow-bellied toad *Bombina pachypus* (62.5%). Moreover, several populations of the species studied here were not infected, even at sites where *Bd* has been detected in other host species. When coupled with the lack of evidence for *Bd*-related mortalities in these species in peninsular Italy, these results suggest that mechanisms of resistance and/or tolerance are protecting populations of these species from the pathogenic activity of *Bd.* Nevertheless, in light of the dynamic pattern of *Bd*–host interactions reported in other studies, of *Bd*-related mortalities in at least 1 study species (*S. s. salamandra*) in other areas, and the ongoing climate changes in montane environments, we suggest that the occurrence of *Bd* should be considered a potential threat to the long-term persistence of these species, and urge the implementation of monitoring and conservation plans.

KEY WORDS: *Batrachochytrium dendrobatidis* · *Bd* · Italian peninsula · *Salamandra salamandra* · *Rana italica* · *Mesotriton alpestris* · Amphibian conservation



## INTRODUCTION

Amphibians are declining worldwide, and chytridiomycosis is among the main causes (Berger et al. 1998, Daszak et al. 1999, 2003, Fisher et al. 2009, Kilpatrick et al. 2010). This emerging infectious disease is caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which affects the keratinized epidermal cells of its amphibian hosts (Longcore et al. 1999, Pessier et al. 1999). In recent years, many studies have been devoted to assessing the global distribution of *Bd* and the susceptibility of different amphibian species and populations to decline following infection (e.g. Berger et al. 1998, Bosch et al. 2001, Muths et al. 2003, Schloegel et al. 2006, Goka et al. 2009).

Despite these efforts, many aspects of the epidemiology of *Bd* are still poorly understood (McCallum 2005, Rachowicz et al. 2005, Fisher et al. 2009). The geographic origin of *Bd*, its spread dynamics, interactions with other factors, and the variability in the outcome of infection are among the main issues still unclear (Weldon et al. 2004, Rachowicz et al. 2005, Pounds et al. 2006, Blaustein et al. 2011). Indeed, field observations show that, despite its wide distribution and the many species it infects, the outcome of

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the infection varies not only between different species, but also between populations within species and between geographic areas (e.g. Tobler & Schmidt 2010, Walker et al. 2010).

Altitude is one of the geographic factors apparently related to the severity of disease; the most severe mass mortalities have been observed in mountain species (Berger et al. 1998, Bosch et al. 2001, Fellers et al. 2001). Early observations of chytridiomycosis have been reported from montane rain forests in Australia and Central America (Berger et al. 1998). Severe die-offs at high altitudes have also been observed in temperate regions in North America and Europe (Bosch et al. 2001, Fellers et al. 2001). In Europe, mass mortalities of 3 common species (*Alytes obstetricans*, *Salamandra salamandra* and *Bufo bufo*) have been observed at a high altitude in a protected area (Bosch et al. 2001, Bosch & Martínez-Solano 2006), and a strong association between altitude and fatal chytridiomycosis has been detected (Walker et al. 2010).

In a previous assessment of *Bd* occurrence along the Apennine Mountains (Italy), we found evidence of this pathogen's widespread presence in the endangered yellow-bellied toad *Bombina pachypus*, a species with a mid- to high-altitude range (Canestrelli et al. 2013). This Italian endemic species has experienced severe population declines in most of its range, with the exception of the southernmost area, over the last 15 yr (Barbieri et al. 2004, Andreone et al. 2009), and it has been proposed that *Bd*-related mortality has played a role in this (Stagni et al. 2004). Interestingly, we found a delay of at least 1 decade between the first evidence of *Bd* occurrence and the beginning of the *B. pachypus* decline. Moreover, we observed heterogeneity in the spatial patterns; while *Bd* was widespread throughout the study area, *B. pachypus* population declines varied by geographic region (Canestrelli et al. 2013). On the whole, these observations suggests that (1) *Bd* did not act as a 'lone killer', (2) *Bd* can occur in susceptible host species without signs of decline until triggered by other factors, and (3) other factors can influence the outcome of the host–pathogen interaction, resulting in a temporally and spatially heterogeneous pattern (see Rosenblum et al. 2013). Since this pattern could apply to other amphibian species, we stress the importance of assessing the infection status of potential host species in areas of *Bd* occurrence, even in the absence of pathological signs or evidence of population declines.

In this study, we extended the assessment of *Bd* occurrence along the Italian peninsula to 3 species with a mid- to high-altitude range, which are sympatric and frequently syntopic with *Bombina pachypus* along the Apennine Mountains. We carried out diagnostic tests using a nested PCR approach to check for the presence of *Bd* on individuals of the Italian fire salamander *Salamandra salamandra gigliolii*, the Italian endemic stream frog *Rana italica* and the Italian alpine newt *Mesotriton alpestris apuanus*. These species are not considered to be as severely endangered as *B. pachypus*, although population declines have been recently reported (e.g. Ambrogio & Gilli 1998, Bologna et al. 2000, Canestrelli et al. 2006, Lanza et al. 2007). In light of the occurrence of the pathogen along their range and their frequent syntopy with a highly infected species, our aim was to assess the *Bd* infection status for these species in the wild and so, to evaluate if *Bd* could be considered a potential risk factor for these species from a conservation perspective.

## MATERIALS AND METHODS

We analyzed a total of 486 individuals: 77 *Salamandra salamandra gigliolii*, 136 *Rana italica* and 273 *Mesotriton alpestris apuanus*. Sampling locations, as well as the number of individuals sampled in each site and year are presented in Table 1 and Fig. 1. All of the samples were from adult individuals, collected from April to June. *S. s. gigliolii* individuals were captured on the ground, within 50 m from the nearest stream; *R. italica* individuals were captured near the banks of streams; and *M. a. apuanus* were captured in a variety of standing-water habitats, during their aquatic phase. Sampling was carried out using fine-tipped swabs (Medical Wire & Equipment Co. MW 113) for samples collected from 2010 onwards and toe clipping for samples collected before 2010.

Genomic DNA was extracted from swabs following the protocol of Boyle et al. (2004), with some modifications. We placed a single swab in a 2 ml tube, adding 30 to 40 mg of glass beads measuring 0.4 to 0.6 mm in diameter (Sartorius) and 70 µl of PrepMan Ultra (Applied Biosystems). Tubes were vortexed for 1 min at 2400 rpm in a vortex and centrifuged for 30 s at 13 000 × *g* in a microfuge (Eppendorf Centrifuge 5415 D). The vortex and centrifugation procedures were done twice. Next, samples were boiled for 10 min, cooled at room temperature for 2 min and centrifuged at 13 000 × *g* for 3 min; 20 µl of supernatant were retained and stored at –20°C until further analysis.

Genomic DNA was extracted from tissue samples following the standard cetyltrimethyl ammonium bromide (CTAB) protocol (Sambrook et al. 1989) with

Table 1. Geographic location, altitude and sampling year of the sampled populations of *Salamandra salamandra gigliolii*, *Rana italica* and *Mesotriton alpestris apuanus*. Number of individuals analyzed (n), and number of individuals testing positive for the presence of *Batrachochytrium dendrobatidis* (P) are also reported. m a.s.l.: meters above sea level; ID: identification numbers refer to locations in Fig. 1A

| ID | Site | Latitude (N) | Longitude (E) | Altitude (m a.s.l.) | *S. s. gigliolii* | | | *R. italica* | | | *M. a. apuanus* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Year | n | P | Year | n | P | Year | n | P |
| 1 | Pianpaludo | 44°26′ | 8°35′ | 800 | | — | | | — | | 2010 | 10 | 0 |
| 2 | Rossiglione | 44°32′ | 8°37′ | 480 | | — | | | — | | 2010 | 16 | 1 |
| 3 | Capanne di Marcarolo | 44°33′ | 8°46′ | 780 | | — | | | — | | 2009 | 18 | 0 |
| 4 | Vallecalda | 44°32′ | 8°57′ | 580 | 2007 | 6 | 0 | | — | | | — | |
| 5 | Monte Penna | 44°29′ | 9°29′ | 1460 | | — | | | — | | 2007 | 16 | 0 |
| 6 | Lago di Bargone | 44°19′ | 9°29′ | 850 | | — | | | — | | 2009 | 18 | 0 |
| 7 | Minucciano | 44°09′ | 10°14′ | 800 | | — | | | — | | 2009 | 20 | 5 |
| 8 | Cipollaio | 44°03′ | 10°15′ | 820 | 1981 | 3 | 0 | | — | | | — | |
| 9 | Stazzema | 44°01′ | 10°18′ | 890 | | — | | | — | | 1984 | 5 | 0 |
| 10 | Abetone | 44°07′ | 10°37′ | 1800 | | — | | | — | | 2009 | 24 | 0 |
| 11 | Monghidoro | 44°13′ | 11°17′ | 830 | | — | | | — | | 2009 | 16 | 4 |
| 12 | Greve in Chianti | 43°33′ | 11°23′ | 500 | | — | | | — | | 1983 | 4 | 0 |
| | | | | | | — | | | — | | 2009 | 14 | 2 |
| 13 | Camaldoli | 43°48′ | 11°49′ | 1000 | | — | | | — | | 2001 | 5 | 0 |
| | | | | | | — | | | — | | 2004 | 12 | 12 |
| | | | | | 2007 | 10 | 0 | | — | | | — | |
| | | | | | 2012 | 4 | 0 | 2012 | 1 | 0 | 2012 | 18 | 17 |
| 14 | Bagno di Romagna | 43°50′ | 11°57′ | 460 | | — | | 2007 | 7 | 0 | | — | |
| | | | | | | — | | 2012 | 5 | 0 | | — | |
| 15 | Lama | 43°05′ | 11°14′ | 260 | | — | | | — | | 2009 | 7 | 0 |
| 16 | Monte Rufeno | 42°48′ | 11°53′ | 500 | | — | | 2007 | 1 | 0 | | — | |
| 17 | Blera | 42°15′ | 12°03′ | 270 | | — | | 2007 | 2 | 0 | | — | |
| 18 | Tolfa | 42°07′ | 11°56′ | 400 | | — | | 2007 | 7 | 0 | | — | |
| 19 | Monti della Laga | 42°42′ | 13°19′ | 1500 | | — | | | — | | 2000 | 3 | 0 |
| | | | | | | — | | | — | | 2003 | 23 | 5 |
| | | | | | | — | | | — | | 2008 | 18 | 1 |
| | | | | | | — | | | — | | 2012 | 2 | 0 |
| 20 | Percile | 42°04′ | 12°54′ | 580 | | — | | 2003 | 6 | 1 | | — | |
| 21 | Patrica | 41°35′ | 13°14′ | 400 | | — | | 2007 | 2 | 0 | | — | |
| 22 | Palena | 41°59′ | 14°08′ | 770 | | — | | 2003 | 1 | 0 | | — | |
| 23 | Pescolanciano | 41°43′ | 14°20′ | 945 | 2003 | 2 | 1 | | — | | | — | |
| 24 | Mercogliano | 40°54′ | 14°44′ | 600 | 2004 | 1 | 0 | | — | | | — | |
| 25 | Serino | 40°50′ | 15°51′ | 420 | | — | | 2005 | 5 | 0 | | — | |
| 26 | Oppido | 40°51′ | 15°10′ | 600 | | — | | 2009 | 2 | 1 | | — | |
| 27 | S. Angelo a Fasanella | 40°27′ | 15°20′ | 520 | | — | | 2003 | 10 | 2 | | — | |
| 28 | Laurino | 40°19′ | 15°20′ | 900 | | — | | 2006 | 3 | 1 | | — | |
| 29 | S. Severino Lucano | 40°03′ | 16°07′ | 680 | 2003 | 6 | 2 | | — | | | — | |
| | | | | | | — | | 2012 | 5 | 0 | | — | |
| 30 | Viggianello | 39°58′ | 16°05′ | 550 | | — | | 2003 | 4 | 1 | | — | |
| 31 | S. Lorenzo Bellizzi | 39°53′ | 16°20′ | 830 | | — | | 2003 | 6 | 0 | | — | |
| 32 | Fagnano Castello | 39°33′ | 16°01′ | 1090 | 2012 | 6 | 0 | 2012 | 2 | 0 | 2010 | 19 | 2 |
| | | | | | | — | | | — | | 2012 | 5 | 2 |
| 33 | Villaggio Mancuso | 39°04′ | 16°33′ | 1200 | 2003 | 10 | 2 | 2003 | 2 | 1 | | — | |
| 34 | Serra S. Bruno | 38°35′ | 16°20′ | 805 | 1998 | 5 | 0 | | — | | | — | |
| | | | | | | — | | 2003 | 7 | 0 | | — | |
| | | | | | 2010 | 3 | 0 | 2010 | 10 | 0 | | — | |
| | | | | | 2012 | 1 | 0 | 2012 | 1 | 0 | | — | |
| 35 | Stilo | 38°29′ | 16°28′ | 290 | | — | | 2003 | 4 | 0 | | — | |
| | | | | | | — | | 2010 | 12 | 0 | | — | |
| | | | | | | — | | 2012 | 9 | 0 | | — | |
| 36 | Zomaro | 38°20′ | 16°09′ | 960 | | — | | 2006 | 9 | 0 | | — | |
| 37 | Carmelia | 38°14′ | 15°55′ | 1220 | 2006 | 6 | 2 | 2006 | 3 | 0 | | — | |
| 38 | Gambarie | 38°10′ | 15°50′ | 1310 | 2003 | 14 | 0 | 2003 | 1 | 1 | | — | |
| | | | | | | — | | 2012 | 7 | 0 | | — | |
| 39 | Cardeto | 38°05′ | 15°46′ | 680 | | — | | 2006 | 2 | 0 | | — | |

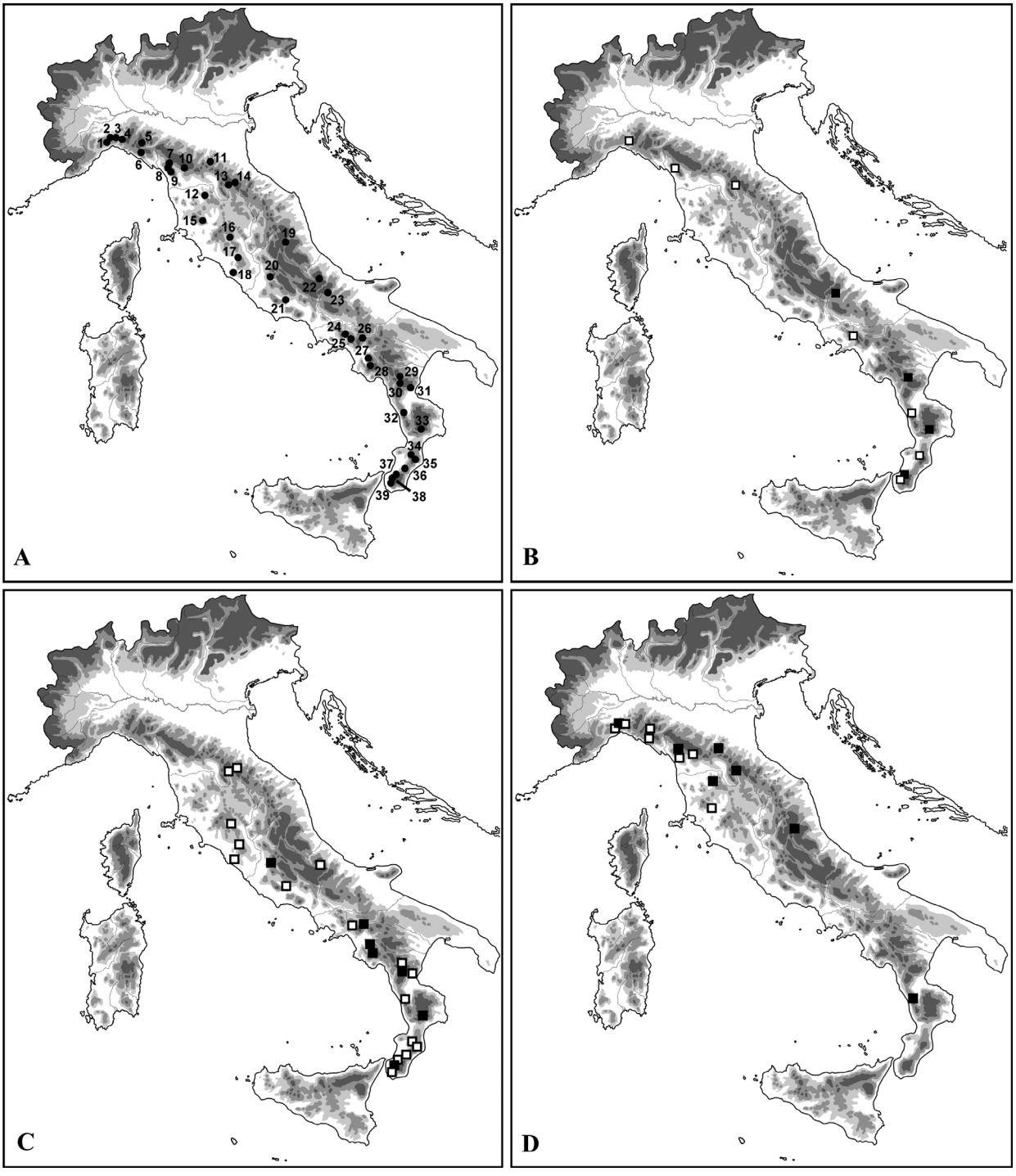


Fig. 1. Geographic distribution in Italy of (A) the 39 sampling sites and (B–D) sites of presence (■) and absence (□) of individuals testing positive for *Batrachochytrium dendrobatidis*: (B) *Salamandra salamandra gigliolii*, (C) *Rana italica* and (D) *Mesotriton alpestris apuanus*

final elution in 30 µl in order to gain a concentrated DNA solution.

The molecular diagnostic screening to test for the presence of *Bd* DNA within the pool of extracted DNA was conducted using the nested-PCR protocol developed by Goka et al. (2009), with some modifications. The target DNA was amplified twice using 2 different pairs of primers.

The first PCR was performed using the primer pair Bd18SF1 (5′-TTT GTA CAC ACC GCC CGT CGC-3′) and Bd28SR1 (5′-ATA TGC TTA AGT TCA GCG GG-3′). The reaction mix (25 µl) contained: 2 µl of template DNA (DNA extracted from swabs using PrepMan Ultra was diluted 1:10 with distilled water), 0.5 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, Colorless GoTaq reaction buffer 1× and 1 U of GoTaq Polymerase (Promega). The PCR cycling process was as follows: an initial denaturation step for 9 min at 95°C, 30 cycles of 30 s at 94°C, 30 s at 50°C and 2 min at 72°C, and a final extension step of 7 min at 72°C (Goka et al. 2009).

The second PCR was performed using the primer pair Bd1a (5′-CAG TGT GCC ATA TGT CAC G-3′) and Bd2a (5′-CAT GGT TCA TAT CTG TCC AG-3′) (Annis et al. 2004). The reaction mix (25 µl) contained: 2 µl of the first PCR product used as template, 0.5 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, Colorless GoTaq reaction buffer 1× and 1 U of GoTaq Polymerase (Promega). The PCR cycling was conducted with a touchdown program. After an initial denaturation for 5 min at 95°C, 35 cycles were performed as follows: 30 s at 94°C, 30 s at 65°C with a decrement of 0.2°C in each cycle and 30 s at 72°C. These cycles were followed by a final extension of 7 min at 72°C. Each assay included a positive control with DNA extracted from *Bd* zoospores (JEL423 kindly provided by Prof. Joyce Longcore, University of Maine) and a negative control of DNA-free distilled water.

PCR products (if any) were separated and visualized on a 1% agarose gel. We considered a positive result to occur if a sample returned an amplification product of the correct size (approximately 300 bp). To confirm that the amplification products were from the *Bd* genome, a randomly selected 18% of the PCR products were double-sequenced (n = 12) and compared with reference sequences in GenBank. Sequencing were carried out by Macrogen Inc. (www.macrogen.com) using an automated capillary electrophoresis system (ABI 3730XL).

## RESULTS

From 486 total screened samples the diagnostic nested PCR assay yielded 66 positive results, i.e. showing a PCR band of the expected size (approximately 300 bp). Among these, the highest *Bd* prevalence was observed in *Mesotriton alpestris apuanus*, in which 51 out of 273 samples (19%; 95% confidence interval: 15–24%) tested positive, while the observed prevalence in other analyzed species was 7 out of 77 (9%; 95% confidence interval: 5–18%) in *Salamandra salamandra gigliolii* and 8 out of 136 (6%; 95% confidence interval: 3–11%) in *Rana italica*. All the sequenced PCR products showed 100% identity with the *Bd* sequences available from GenBank.

Numbers of positive and tested individuals in each population, along with geographic information on the sampling site and year are reported in Table 1. The pathogen was widespread geographically among the analyzed species (Fig. 1). Among them, *Mesotriton alpestris apuanus* showed a wider *Bd* distribution, with positive individuals detected in 50% of the sampling sites, distributed throughout its entire range. *Salamandra salamandra gigliolii* and *Rana italica* showed *Bd* occurrence in 36 and 30% of the sampling sites, respectively.

A significant increase in *Bd* prevalence with increasing altitude was observed in *Mesotriton alpestris apuanus*, in which 22% individuals tested positive from populations sampled at sites above 800 m a.s.l., while 5% individuals tested positive from populations sampled at sites below 800 m a.s.l. (Fisher exact test, p = 0.002). In *Salamandra salamandra gigliolii* and *Rana italica* we did not observed significant differences in *Bd* prevalence with altitude. The prevalence of *Bd* occurrence in individuals from *S. s. gigliolii* populations sampled above and below 800 m a.s.l was 8 and 15%, respectively (Fisher exact test, p = 0.336), and 6% of individuals tested positive from *R. italica* populations sampled both above and below 800 m a.s.l. (Fisher exact test, p = 1).

Finally, contrary to what was previously observed for *Bombina pachypus* (Canestrelli et al. 2013), we did not find significant differences in *Bd* prevalence according to latitude. The prevalence of *Bd* in *Salamandra salamandra gigliolii* was 9% among both northern and central populations and populations from Calabria (Fisher exact test, p = 1), while its prevalence in *Rana italica* was 11% among northern and central populations and 3% in populations from Calabria (Fisher exact test, p = 0.139).

## DISCUSSION

Our results are in agreement with previous findings in showing a wide distribution of *Bd* along the Apennine Mountains (see Canestrelli et al. 2013). Nevertheless, both the infection prevalence and the proportion of the affected populations in each species were lower in the 3 species analyzed here than

in *Bombina pachypus*. Moreover, several of the populations testing negative were sampled at sites where individuals from other species tested positive (Sites 13, 29, 32, 37 and 38) or at sites where *B. pachypus* populations were previously observed to be highly infected (Sites 14, 29, 35, 38 and 39). Furthermore, despite some reports of population declines (e.g. for *Salamandra salamandra gigliolii* in the north-central Apennines and for *Mesotriton alpestris apuanus* in the southern areas; see Ambrogio & Gilli 1998, Bologna et al. 2000, Canestrelli et al. 2006, Lanza et al. 2007), no evidence of *Bd*-related die-offs have been reported for these species in Italy, and other factors have been indicated as the main drivers, such as the introduction of fishes, eutrophication due to intensive farming, water catchment, climate changes, geographic isolation and reduced genetic variability. On the other hand, some populations testing positive did not appear demographically impoverished or unstable over multiple years of observation (e.g. *M. a. apuanus* at Site 13; D. Canestrelli pers. obs.).

On the whole, contrary to what has previously been suggested for *Bombina pachypus*, and even if we cannot exclude a role for *Bd* in past population disappearances on the basis of our data, it does not seem that *Bd* is currently threatening populations of *Salamandra salamandra gigliolii*, *Rana italica*, or *Mesotriton alpestris apuanus* in Italy. In turn, this suggests that mechanisms of resistance or tolerance are protecting populations of these species from the pathogenic activity of *Bd*.

Nevertheless, we suggest that the widespread distribution of *Bd* along the Italian peninsula and its occurrence in the studied populations should be regarded as a serious threat for the long-term survival of *Salamandra salamandra gigliolii*, *Rana italica*, and *Mesotriton alpestris apuanus* in the area, for at least 2 reasons. First, a time lag between the earliest evidence of *Bd* occurrence and host population declines has been observed in other studies (Puschendorf et al. 2006, Canestrelli et al. 2013), indicating that currently resistant/tolerant populations can eventually become threatened in the future, when altered environmental conditions shift host–pathogen interactions toward the pathogen's optimum or increase host susceptibility. For instance, within Peñalara Natural Park in central Spain the fire salamander *Salamandra salamandra* and the common toad *Bufo bufo* underwent mass mortalities 4 yr after chytridiomycosis almost extirpated the midwife toad *Alytes obstetricans* from the same area (Bosch et al. 2001, Bosch & Martínez-Solano 2006). In addition, in the case of *Bombina pachypus*, population declines were observed more than a decade after the first evidence of *Bd* occurrence in the area (Canestrelli et al. 2013). Second, most of the reported events of *Bd*–related mass mortality are from montane areas, both in tropical and temperate regions, suggesting that the outcome of host–pathogen interactions could be more affected by environmental changes at high altitude (Berger et al. 1998, Bosch et al. 2001, Fellers et al. 2001). On the one hand, most montane amphibians show prolonged aquatic life stages, giving them a prolonged exposure to *Bd* zoospores (Catenazzi et al. 2013). On the other hand, the particularly severe impact of ongoing climate change on montane biodiversity (Diaz et al. 2003, Nogués-Bravo et al. 2007; Raxworthy et al. 2008) could lead to a combination of suboptimal environmental conditions for host species due to the upward shift of the climatic niche and increased pathogenic activity as the 'chytrid thermal optimum' is approached (Pounds et al. 2006). Interestingly, we found a significantly higher prevalence of *Bd* among high-altitude populations of *M. a. apuanus*, the species most strictly confined to montane habitats. Moreover, as mentioned above, at least one study species (*S. salamandra*) has already been observed to undergo *Bd*–related mortalities in montane areas in other geographic regions (Bosch & Martínez-Solano 2006).

Unravelling the regional distribution of *Bd* is a first, highly necessary but insufficient step toward the appreciation of disease dynamics in the area and the implementation of management and conservation plans for its amphibian hosts (Woodhams et al. 2011). Here we have shown that this pathogen is widespread among montane amphibians throughout the Italian peninsula. Monitoring plans to assess *Bd* prevalence and virulence and their variation over time among multiple host populations should be carried out in the future, in order to identify possible shifts in host–pathogen interactions toward increased pathogenicity. Moreover, it will be of the utmost importance to extend the study to the whole amphibian fauna in the area, to better understand disease ecology and dynamics among different species and populations, and to fully appreciate the potential threats posed by *Bd* to the amphibians in the area. Finally, in light of recent advances in the development of strategies to mitigate chytridiomycosis (Woodhams et al. 2011, Bletz et al. 2013), it now appears increasingly reasonable to develop management plans that include *ex situ* conservation programs for host populations.

*Acknowledgements*. This work was funded by the Agenzia Regionale dei Parchi (ARP) Lazio and the Italian Ministry of Environment. We are grateful to Roberta Bisconti for kind help with sampling and manuscript preparation, and to Pietro Bagalà, Claudio Bagnoli, Francesco Paolo Caputo, Florinda Sacco and Francesco Spallone for assistance during sampling.

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*Submitted: May 22, 2013; Accepted: July 30, 2013*
*Proofs received from author(s): October 30, 2013*