

Threats of the emerging pathogen *Batrachochytrium salamandrivorans* (*Bsal*) to Italian wild salamander populations

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Abstract. The salamander killing fungus *Batrachochytrium salamandrivorans* (*Bsal*), recently introduced from Asia, is threatening salamander populations in different parts of Europe. In fact, this pathogen is rapidly spreading in Central Europe and has been also introduced into NE Spain. Of special concern are those regions with an exceptionally high salamander diversity such as Italy, where 19 salamander species are present most of which are strictly endemic. In this study, we update the information on the presence of *Bsal* in Italian wild salamanders, by adding samples from two presumptive outbreak sites, one on the island of Sardinia and one on continental Italy (Liguria). In addition, we reviewed the potential susceptibility of all the Italian salamander species on the basis of laboratory experimental infection trials, or from the tested susceptibility of the phylogenetically nearest species, according to the literature. Overall, 15 skin swabs from three species (*Speleomantes sarrabusensis*, *Speleomantes strinatii* and *Salamandra salamandra*) collected in the two presumptive *Bsal* outbreak sites were analysed by quantitative molecular methods, but none gave positive results. The majority of Italian salamander species and almost all of the endemic ones showed a high susceptibility to *Bsal* infection. Therefore, even if the presence of *Bsal* in Italian salamander populations has not been proven yet, the entire salamander fauna is highly threatened and preventive management actions should be taken. The need for strict biosecurity protocols on the international trade of captive or wild amphibians and for the implementation of preventive measures during field activities to limit the introduction and spread of the *Bsal* pathogen is again stressed.

Keywords. Amphibians, biosecurity, chytridiomycosis, conservation, emerging infectious diseases, mitigation, real-time PCR.

INTRODUCTION

The chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) are amphibian pathogens that were introduced in Europe from East Asia, probably through the international trade (O'Hanlon et al., 2018; Laking et al., 2019). The continuous spreading of these emerging pathogens is now threatening wild amphibian populations in different

parts of Europe (Scheele et al., 2019; Bosch et al., 2021). In particular, the salamander killing fungus *Bsal* is a real threat to European salamanders, because many species are extremely susceptible to this infection and, therefore, highly vulnerable (Martel et al., 2014). The pathogenic effect of *Bsal* is caused by its zoospores that penetrate the skin cells of adult salamanders, breaking innate immunological defences, and producing tissue erosions and deep ulcerations that may disrupt the host infected

skin respiratory and rehydration functions (Martel et al., 2013; Grogan et al., 2020). In this way, *Bsal* infections are capable of causing severe illness or even death of the infected amphibian hosts (Martel et al., 2013, 2014). In fact, *Bsal* is constantly expanding from the Netherlands, where it was first detected and where it caused a population collapse in the local fire salamander *Salamandra salamandra* population (Martel et al., 2013). Currently, *Bsal* has been confirmed in about 80 localities surrounding Bunderbos, in the Netherlands, where it was first detected (Martel et al., 2013), and also in Belgium and Germany (e.g., Spitzen-van der Sluijs, 2016; Lötters et al., 2020; Schmeller et al., 2020; Thein et al., 2020). *Bsal* has also been detected in 2018 in Catalonia (Northeastern Spain), where it has infected the marbled newt *Triturus marmoratus* and caused a local mass mortality event of this species (Martel et al., 2020), while its presence in North Central Spain has not been yet confirmed (Bosch et al., 2021). Therefore, to date, the presence of *Bsal* in Europe has been confirmed in the wild in four countries: The Netherlands, Belgium, Germany and Spain. The emergence and the diffusion of *B. salamandrivorans* through Europe, apparently facilitated by humans transferring and introducing in the wild environment captive amphibians, is worrying and requests urgent prevention measures and monitoring actions to prevent further loss of amphibian diversity (Thomas et al., 2019). In particular, the high salamander diversity of the southern European peninsulas, such as Italy and Spain, appears at risk. Italy alone hosts 19 species of urodeles (Sindaco and Razzetti, 2021), many of which are endemic, such as the entire genus *Salamandrina*, the Italian newt *Lissotriton italicus*, the Sardinian brook newt *Euproctus platycephalus*, and seven species of cave salamanders belonging to the genus *Speleomantes* (Sindaco and Razzetti, 2021). However, to date, only two studies screened *Bsal* in Italian salamanders (Grasselli et al., 2019, 2021). These studies analysed by means of real-time PCR (qPCR) 136 skin swabs from 6 species of Italian wild salamanders and 53 from non-native individuals that were bred in private collections (Grasselli et al., 2019). The results from these studies were that none of the 189 Italian salamander swabs gave positive results. However, recent events raised concerns on the possibility of *Bsal* outbreaks in Italian populations. In the summer of 2021, a mass mortality of *Speleomantes sarrabusensis* was observed in Sardinia by the wildlife photographer Emanuele Biggi while, more recently (in spring 2022), a living fire salamander bearing skin lesions from Liguria was photographed by Michael Fahrback and reported to the authors by Frank Pasmans from Ghent University. These observations were compatible with the suspected infection of *Bsal* on

Italian amphibians but needed robust confirmation by molecular, and histological methods or by both. Therefore, the aim of this study was twofold: i) to expand the current knowledge about the presence of *Bsal* in wild salamander populations in Italy, adding the molecular data obtained from new skin samples collected from the two *Bsal*-suspected outbreaks and ii) to assess the susceptibility to *Bsal* of all Italian salamanders, on the basis of published experimental studies or, in the absence of experimental evidence, from their phylogenetic affinities. This information will be needed to better plan *Bsal* mitigation actions and also to guide strategic conservation and management efforts on the national territory of Italy.

MATERIALS AND METHODS

Origin of skin swab samples, DNA extraction and quantitative PCR

We obtained skin swabs from two areas, one in Southern Sardinia and one in NW Italy. In Southern Sardinia (Monte Sette Fratelli), we sampled a protected underground site that hosts the largest known population of *Speleomantes sarrabusensis*. This site is closed by a gate and only an authorised person has access, however, in this location several dead cave salamanders were observed from July to August 2021, by different herpetologists. Skin swabs were collected a few days after the first mortality record (August 2021) from all the living *S. sarrabusensis* individuals (two individuals), plus one recently dead individual; a fourth swab was obtained from a *Hyla sarda* corpse found at the same site (Supplementary material, Fig. S1).

In the province of Genova (Liguria, NW Italy), we collected twelve swabs in April and May 2022 along a small stream or in an adjacent artificial cave from living fire salamanders (*Salamandra salamandra*) and cave salamanders (*Speleomantes strinatii*) where an individual of *Salamandra salamandra* with suspect skin lesions was photographed (Supplementary material, Fig. S1). In both sites, all skin swabs were obtained with a standardised protocol used in previous *Bd* and *Bsal* studies on Italian salamanders (Costa et al., 2021; Grasselli et al., 2019, 2021). Sterile cotton swabs were rubbed 30 times on the skin of different parts of the salamander's body and were preserved in individual sterile plastic tubes at 4 °C until extraction (Spitzen-van der Sluijs et al., 2016). DNA was extracted in 200 µl of Prepman ULTRA (Thermo Fisher Scientific Technologies, Monza, Italy). Samples were then analysed for *Bd* and *Bsal* DNA using a duplex qPCR, targeting the ITS1 rRNA gene of *Bd* and 5.8S rRNA gene of *Bsal*, as described by Blooi et al. (2013). All samples

were run in duplicate, together with standard curves obtained from suspensions of known numbers of *Bd* and *Bsal* zoospores (kindly provided by An Martel and Frank Pasmans) in the same plate, and results were expressed in genome equivalents (GEs) according to Thomas et al. (2018).

Assessing Italian salamanders' *Bsal* susceptibility

The susceptibility of Italian salamanders to *Bsal* infection was estimated by collecting all the published data on laboratory experimental infection trials on the same salamander species. In absence of such experimental evidence, the susceptibility was inferred from the phylogenetically nearest species, adopting a precautionary approach and thus selecting the more threatening outcome. Species susceptibility was estimated as “High” if the experimental infection had a lethal outcome, “Moderate” if the gravity of infection was dose-dependent and “Low” if the infected salamander was able to clear infection or remained asymptomatic.

Moreover, we used the results of Beukema et al. (2018, Table 2) as an index of potential niche overlap between *Bsal* and Italian salamanders. Beukema et al. (2018) estimated both native and invasive ecological niches of *Bsal* and of all native European salamanders using three different ordination methods (see Material and Methods in Beukema et al., 2018). The statistical overlap between both *Bsal* native and invasive ecological niches and the current niche of Italian salamanders was then calculated (Beukema et al., 2018). In the present study, we counted all significant overlaps between Italian salamanders and *Bsal* native and invasive niche. Therefore, there were several possible outcomes per species, ranging from 0/6 if all overlaps were non-significant, to 6/6 if all the six niches overlapped significantly. This “overlap score” may be used as a gross indication of the environmental compatibility between each Italian species and the salamander killing fungus *Bsal*.

In this study, we used species nomenclature following the most recent checklist of Italian Herpetofauna (Sindaco and Razzetti, 2021), and we revised the available data on the 19 native Italian species. We also report information for one subspecies of alpine salamander (*Salamandra atra aurorae*), that is listed as a priority taxon in annex II of the European Union (EU) Directive “Habitats” 92/43/CEE. According to Art. 1 letter (h) of this Directive, Italy has a particular responsibility for the conservation of this endemic taxon that shows an extremely small distributional range limited to the Eastern Alps.

RESULTS

DNA testing

We analysed one skin swab from *Hyla sarda*, three *Speleomantes sarrabusensis*, five *Speleomantes strinatii* and seven *Salamandra salamandra*. None of these swabs gave *Bd* or *Bsal*-positive results. Therefore, the possible *Bsal* infection in the Sardinian (prevalence 0; 95% confident limits 0.00-0.60), and Ligurian (prevalence 0; 95% confident limits 0.00-0.30) sites were not confirmed, but given the very small samples analysed, a high level of uncertainty concerning these results remains. To date, the number of Italian wild salamanders screened for *Bsal* is 151, obtained from nine species (Table 1).

Bsal susceptibility

Ten of the nineteen (53%) salamander species found in Italy were infected experimentally in laboratory trials with *Bsal*, thus providing direct evidence of their potential susceptibility to the pathogen (Table 2). In the case of the cave salamanders (genus *Speleomantes*), three species were infected in laboratory experiments by Martel et al. (2014): *S. genei*, *S. strinatii* and *S. imperialis*. The two former species resulted highly susceptible (i.e., *Bsal* infection was lethal), while the latter was able to clear the infection, thus showing low susceptibility. In this study, following the precautionary approach, we considered all non-tested *Speleomantes* species to be highly susceptible, as already done by Gilbert et al. (2020).

Overall, the majority of Italian salamander species, 15 out of 19 (79%), were shown or inferred to possess

Table 1. Italian salamanders tested for *Batrachochytrium salamandrivorans*. ^a and ^b indicate presence of samples from the non-confirmed *Bsal* outbreaks in Liguria (2022) and Sardinia (2021), respectively. This table is an expanded version of Grasselli et al. (2019).

Species or subspecies	Sample (N)	<i>Bd</i> positive	<i>Bsal</i> positive
<i>Euproctus platycephalus</i>	3	0	0
<i>Ichthyosaura alpestris</i>	76	1	0
<i>Lissotriton italicus</i>	22	0	0
<i>Salamandra atra aurorae</i>	3	0	0
<i>Salamandra salamandra</i> ^a	11	0	0
<i>Salamandrina terdigitata</i>	14	0	0
<i>Speleomantes sarrabusensis</i> ^b	3	0	0
<i>Speleomantes strinatii</i> ^a	5	0	0
<i>Triturus carnifex</i>	14	3	0
Total	151	4	0

Table 2. Susceptibility of Italian salamander species to *Bsal* infection, according to the literature or inferred from phylogenetic related species. *Bsal* susceptibility was updated from Gilbert et al. (2020). The overlap score was calculated from Table 2 of Beukema et al. (2018). * not reported in Beukema et al. (2018).

Species	Italian endemic	IUCN Status	Habitat Directive	<i>Bsal</i> susceptibility	Comments	Reference	Overlap score
<i>Euproctus platycephalus</i>	yes	EN	IV	High	Lethal, laboratory tested	Martel et al., 2014	1/6
<i>Ichthyosaura alpestris</i>	no	LC	--	Moderate	Dose-dependent laboratory tested	Martel et al., 2014	3/6
<i>Lissotriton italicus</i>	yes	LC	IV	High	Lethal, laboratory tested	Martel et al., 2014	0/6
<i>Lissotriton vulgaris</i>	no	LC	--	Moderate	Dose-dependent, laboratory tested	Bates et al., 2019	1/6
<i>Proteus anguinus</i>	no	VU	II/IV	Low	Asymptomatic, laboratory tested	Li et al., 2020	2/6
<i>Salamandra atra</i>	no	LC	IV	High	Inferred from congeneric species		2/6
<i>Salamandra atra aurorae</i>	yes		II*/IV	High	Inferred from congeneric species		-*
<i>Salamandra lanzai</i>	no	VU	IV	High	Inferred from congeneric species		0/6
<i>Salamandra salamandra</i>	no	LC	--	High	Lethal, laboratory tested	Martel et al., 2013, 2014	2/6
<i>Salamandrina perspicillata</i>	yes	LC	II/IV	High	Lethal, laboratory tested	Martel et al., 2014	1/6
<i>Salamandrina terdigitata</i>	yes	LC	II/IV	High	Inferred from congeneric species		1/6
<i>Speleomantes ambrosi</i>	yes	NT	II/IV	High	Inferred from congeneric species		3/6
<i>Speleomantes flavus</i>	yes	VU	II/IV	High	Inferred from congeneric species		0/6
<i>Speleomantes genei</i>	yes	VU	II/IV	High	Lethal, laboratory tested	Martel et al., 2014	1/6
<i>Speleomantes imperialis</i>	yes	NT	II/IV	Low	Clears infection, laboratory tested	Martel et al., 2014	1/6
<i>Speleomantes italicus</i>	yes	NT	II/IV	High	Inferred from congeneric species	Martel et al., 2014	1/6
<i>Speleomantes sarrabusensis</i>	yes	VU	II/IV	High	Inferred from congeneric species	Martel et al., 2014	1/6
<i>Speleomantes strinatii</i>	no	NT	II/IV	High	Lethal, laboratory tested	Martel et al., 2014	3/6
<i>Speleomantes supramontis</i>	yes	EN	II/IV	High	Inferred from congeneric species		0/6
<i>Triturus carnifex</i>	no	LC	II/IV	High	Inferred from congeneric species		3/6

high susceptibility to *Bsal* infection, while two (11%) had moderate and two (11%) had low susceptibility (Table 2). When focusing on endemic salamanders, 10 out of 11 (91%) have, or were inferred to have, high susceptibility, the only exception being *S. imperialis* (Table 2). Finally, only four species showed no niche overlap with *Bsal* pathogen. Three of them were salamanders found exclusively in the Mediterranean bioclimatic region (i.e., *Lissotriton italicus*, *Speleomantes flavus* and *Speleomantes supramontis*), while the fourth one (*Salamandra lanzai*) lives at high altitudes in the Alps. On the other hand, four species showed a relatively high overall niche overlap score with *Bsal*: *Ichthyosaura alpestris*, *Speleomantes ambrosii*, *Speleomantes strinatii* and *Triturus carnifex*. The two latter also had a high experimental susceptibility towards the infection with the pathogen (Martel et al., 2014).

DISCUSSION

The diffusion of *Bsal* infection is a dramatic threat to salamander diversity in Central Europe and Spain (Martel et al., 2014; Bosch et al., 2021), and recently two potential outbreak sites have been recorded in Italy. How-

ever, none of our qPCR samples from these sites provided positive results. Clearly, these partial results cannot exclude the presence of *Bsal*-infected individuals in the studied sites or elsewhere, because of the very small sample size tested. The mass mortality event reported in Sardinia is particularly concerning, because the cave salamander population lives in a high-altitude protected site, where environmental stressors should be absent. Therefore, local Authorities in charge of the site management were immediately alerted and the water quality inside the site is monitored to report any further critical condition and to report further changes as well as to mitigate a possible spread of any kind of pathogen, as indicated by Thomas et al. (2019). In the Ligurian site, no mass mortality was observed, and a monitoring programme is regularly taking place as a preventive mitigation measure (Salvidio, unpublished data).

Our review of species susceptibility already conducted by Beukema et al. (2018) and Gilbert et al. (2020) shows that the great majority of Italian salamanders, and in particular many endemic taxa, are potentially highly vulnerable to *Bsal* infection. Indeed, the endemic genus *Salamandrina* and the many species belonging to the genus *Speleomantes* have been experimentally proven highly vulnerable to this infection. In addition, all Ital-

ian species with few exceptions share a relatively high ecological niche overlap score with *Bsal* (Beukema et al., 2018 synthesized in Table 2 of this study), suggesting that, even in the Mediterranean region, salamanders inhabiting humid and cool microhabitats, such as *Speleomantes strinatii* and *Triturus carnifex* are exposed to a high risk. Concerning the EU priority micro-endemic subspecies of Alpine salamander, *Salamandra atra auro-rae*, Beukema et al. (2018) did not calculate niche overlap, while a high susceptibility to *Bsal* was inferred from the experimental data on other *Salamandra* subspecies (Gilbert et al., 2010). Given the high conservation concern of this taxon possessing a restricted range of occurrence (Romano et al., 2018), a demographic monitoring project has been implemented by the local province of Trento starting in 2017, while skin swabs have been collected from different sites in August 2022 (Romano, pers. com. September 2022).

Active disease surveillance and large-scale monitoring of populations of the most threatened species should be the ideal preventive strategy, but the costs associated with these activities would be clearly prohibitive (Bosch et al., 2021). Therefore, a more cost-effective policy should integrate passive reporting and intervention in the cases of confirmed mass mortality events combined with strong preventive measures, as already suggested by Thomas et al. (2019). These measures should include testing for *Bsal* throughout the amphibian international commerce to exclude the unintentional introduction of fungal pathogens from captive amphibians legally traded. Moreover, controlling and eradicating invasive populations of alien amphibians that could act as *Bsal* intermediate hosts should be considered and realised, when possible. These preventive eradications should be undertaken especially for species that act as intermediate hosts and are associated with the spread of amphibian chytrid pathogens, such as the African clawed frog *Xenopus laevis* and the North American bullfrog *Lithobates catesbeianus* (Pasmans et al., 2017). Moreover, it is pivotal that professional and amateur herpetologists always implement strict biosecurity measures during field activities, to avoid the spread of the pathogen across distant study areas, and the existence of online information about biosecurity precautions, such as <http://bsaleurope.com/>, should be better advertised.

Finally, herpetologists are not the only potential drivers of the spread of these pathogens, because unfortunately they can be spread by any kind of people performing outdoor activities. A broad dissemination of the issues of wildlife pathogens, and of the protocols to limit them, remains a fundamental preventive action (e.g., <http://www-9.unipv.it/webshi/images/files/All.%20II%20-%20CHITRIDOMICOSI.pdf>; <http://bsaleurope.com/>).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <<http://www-262.9.unipv.it/webshi/appendix/index.html>> manuscript number 13279

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