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Correspondence

First screening for *Batrachochytrium salamandrivorans* (*Bsal*) in wild and captive salamanders from Italy

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Amphibian populations are declining rapidly around the world. Emerging infectious diseases increasingly cause population declines, in particular two pathogenic chytrid fungi affecting the skin of their hosts (e.g., Skerratt et al. 2007). In Southern Europe, disease outbreaks in Spain and Sardinia have been attributed to the chytrid fungus *Batrachochytrium dendrobatidis*, *Bd* (Bosch et al. 2001, Bielby et al. 2009), while in Central Europe, the pathogen *Batrachochytrium salamandrivorans* (*Bsal*) has been causing local declines in populations of fire salamanders (*Salamandra salamandra*) in the Netherlands, Belgium and Germany (Spitzen-van der Sluijs et al. 2016, Lötters et al. 2018). The origin of this recently identified amphibian pathogen is suspected to be the Asian continent (Martel et al. 2014), where the fungus is found across large geographic areas and on different amphibian host species (Laking et al. 2017). The main diffusion pathway of *Bsal* in Europe is probably the incessant trade of thousands of Asian salamanders that are imported each year to be sold as pets (Stegen et al. 2017, Yuan et al. 2017). In fact, *Bsal* has already been reported from several private collections in different European countries including Germany, Netherlands, Spain, Sweden and the UK (Fitzpatrick et al. 2018, Sabino Pinto et al. 2018).

Recently, the European Union (EU) funded a multi-national project to counteract the spread of *Bsal* in wild amphibian populations and to set up an early warning system to identify *Bsal* outbreaks ("Mitigating a new infectious disease in salamanders to counteract a loss of European biodiversity"; http://bsaleurope.com).

Within the Mediterranean, Italy hosts one the most diverse amphibian faunae and many endemic salamanders, such as the endemic genus *Salamandrina*, the Sardinian brook newt, *Euproctus platycephalus*, the Italian newt, *Lissotriton italicus,* and several cave salamanders, genus *Speleomantes* (Sindaco et al. 2006). Therefore, the introduction of the highly pathogenic *Bsal* is expected to threaten Italian salamander diversity and to result in the loss of unique evolutionary amphibian lineages. Therefore, data on the presence of *Bsal* in Italy are of high interest. In this note, we report the results of the first molecular screening for *Bsal* in native Italian salamanders, along with samples of exotic salamanders, including Asian species, kept in private live collections.

Between 2015 and 2018, we sampled native salamanders during national *Bd* surveys across Italian national parks and on other occasions (Table 1). We collected skin swabs following a standardised protocol that consisted of rubbing a total of 30 times the venter, cloaca and inner faces of the legs of a salamander individual with a sterile cotton swab (cf. Spitzen-van der Sluijs et al. 2016). We preserved the swabs in the field in sterile plastic tubes at 4°C for subsequent DNA extraction. We used the same general procedure to obtain swabs from captive-bred salamanders, except that we shipped sterile swabs, tubes and illustrated sampling instructions to volunteering keepers. The latter were found by advertising our *Bsal* screening program through the Italian Herpetological Society web site (http://www-3.unipv.it/webshi/), its official mailing list, or the Italian Facebook page dedicated to the EU mitigation project

Table 1. List of wild and captive-kept salamanders screened for the chytrid pathogens *Batrachochytrium dendrobatidis* (*Bd*+ = positive) and *Batrachochytrium salamandrivorans* (*Bsal*+ = positive) in Italy. PNCT = Parco Nazionale delle Cinque Terre, PNP = Parco Nazionale della Majella, PNP = Parco Nazionale del Pollino.

Species	Region (Province)	National Park	Collection date	N	Bd+	Bsal+
Wild populations						
Euproctus platycephalus	Sardinia (Cagliari)		Jan 2015	3	0	0
Ichthyosaura alpestris apuana	Liguria (La Spezia)	PNCT	Apr 2015	35	1	0
Ichthyosaura alpestris apuana	Liguria (La Spezia)	PNCT	Sep 2015	17	0	0
Ichthyosaura alpestris apuana	Liguria (La Spezia)	PNCT	Oct 2015	24	0	0
Lissotriton italicus	Calabria (Cosenza)	PNP	Nov 2016	6	0	0
Lissotriton italicus	Calabria (Cosenza)	PNP	Mar–Jun 2018	10	0	0
Lissotriton italicus	Calabria (Cosenza)	PNP	Sep 2016	6	0	0
Salamandra atra aurorae	Trentino Alto Adige (Trento)		Jul–Aug 2018	3	0	0
Salamandra salamandra	Basilicata (Potenza)	PNP	Oct 2016	4	0	0
Salamandrina terdigitata	Basilicata (Potenza)	PNP	Oct 2016	6	0	0
Salamandrina terdigitata	Calabria (Cosenza)	PNP	Apr 2016	8	0	0
Triturus carnifex	Liguria (La Spezia)		Aug 2015	2	0	0
Triturus carnifex	Abruzzo (Aquila)	PNM	Jul 2017	9	3	0
Triturus carnifex	Calabria (Cosenza)	PNP	Mar–Apr 2018	3	0	0
Captive-bred individuals						
Ambystoma opacum	Keeper n 4		2018	2	0	0
Cynops ensicauda popei	Keepers n 1, 2, 4		2018	8	0	0
Cynops orientalis	Keepers n 1, 2, 4		2018	17	0	0
Cynops cyanurus	Keepers n 1, 4		2018	5	0	0
Hynobius dunni	Keeper n 1		2018	5	0	0
Laotriton laoensis	Keeper n 1		2018	5	0	0
Notophthalmus viridescens piaropicola	Keeper n 4		2018	1	0	0
Ommatotriton ophryticus	Keeper n 4		2018	5	0	0
Pachytriton labiatus	Keepers n 1, 4		2018	2	0	0
Triturus marmoratus	Keeper n 3		2018	3	0	0

(https://www.facebook.com/groups/134273207141409/). We granted anonymity to all keepers and informed them about the results along with treatment and clearance measures in the case of positive findings (Blooi et al. 2015).

We extracted DNA in 200 µl of Prepman ULTRA (Thermo Fisher Scientific Technologies, Monza, Italy). Samples were analysed for the presence of *Bd* and *Bsal* DNA using a duplex quantitative polymerase chain reaction (qPCR), targeting the ITS1 rRNA gene of *Bd* and 5.8S rRNA gene of *Bsal*, as described by Boyle et al. (2004) and Blooi et al. (2013). We ran each sample in duplicate; when a sample produced contradictory results, we repeated the qPCR until the duplicates produced consistent results. We ran standard curves in each plate from suspensions containing standardized numbers of *Bd* and *Bsal* zoospores, as described by Boyle et al. (2004). We prepared tenfold serial dilution series ranging from 1,000 to 0.01 genomic equivalents (GEs) of zoospores per real-time PCR mixture for both *Bd* and *Bsal* according to Thomas et al. (2018).

We analysed 189 salamander skin swabs by means of duplex q-PCR for both *Bd* and *Bsal.* Overall, we collected 136 swabs from seven wild native species, and 53 swabs from seven Asian, two North American, and one European species kept in private collections (Table 1). All individuals were negative for *Bsal* (Bayesian credible interval 0–2%), while four out of 136 wild salamanders were positive for *Bd* (prevalence 3%, Bayesian credible interval 1–7%); one Alpine newt (GE = 1) and three Italian crested newts (GEs = 55, 60 and 68, respectively).

Our screening for *Bsal* produced only negative results. These findings update the European *Bsal* dataset, increasing the number of countries where the standardized screening protocol based on qPCR has been used (Thomas et al. 2018). However, we consider our study a pilot survey, because even if we did not detect *Bsal*, it is known that *Bsal* infection may be present in both wild populations and captive collections of amphibians at extremely low prevalences (e.g., Sabino-Pinto et al. 2018, Yuang et al. 2018). Therefore, our findings are not yet sufficient to infer with strong confidence the presence or absence of this pathogen across the Italian territory. In this context it is interesting to link our data to those from other European countries in which surveys have been undertaken on wild and captive salamanders. In the UK, Czech Republic and Sweden, *Bsal* was detected in privately kept animals (Cunningham et al. 2015, Balaz et al. 2018, Sabino-Pinto et al. 2018), while

Bsal is unknown from the wild in these countries. Similarly, screening wild salamanders in southern Switzerland (Canton Ticino) produced all negative results (Parrott et al. 2017). These findings, along with ours, give hope that *Bsal,* at least in the wild, is generally absent from European countries other than Belgium, Germany and the Netherlands (cf. Spitzen-van der Sluijs et al. 2016). However, the presence of *Bsal* in captive collections at the same time poses an immense threat.

Our preliminary findings on *Bd* and *Bsal* in Italy will be useful to increase awareness not only among professional and amateur herpetologists, but also among amphibian private keepers. All these operators should be aware about the danger of spreading pathogens by facilitating direct or indirect contact between captive-kept and wild animals. The most effective strategy to prevent the diffusion of these devastating amphibian pathogens may be by guiding human behaviour, rather than imposing legal restrictions on animal trade or importation.

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