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Absence of the chytrid fungus *Batrachochytrium salamandrivorans* in populations of the Near Eastern Fire Salamander (*Salamandra infraimmaculata*) in Israel

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The Near Eastern Fire Salamander, *Salamandra infraimmaculata* von Martens, "1884" 1885, is an amphibian known from Iran, Iraq, Turkey, Syria, Jordan, Lebanon, and northern Israel in the Middle East. Across most of this geographic range, it is classified as 'Threatened', and 'Endangered' at the southern margin of its distribution range, in northern Israel (Blank et al. 2013). The IUCN Red List of Threatened Species considers *S. infraimmaculata* as globally 'Near Threatened' (www.redlist.org, accessed 10 July 2021).

The amphibian chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*) causes the infectious disease chytridiomycosis in salamanders and newts (Martel et al. 2013). Different species, populations and individuals show different symptoms. Cases range from asymptomatic individuals that spontaneously die to those exhibiting various symptoms before their demise. Members of the genus *Salamandra* apparently are among the most susceptible to *Bsal* infections, and individuals usually perish within two weeks after infection (Martel et al. 2013, 2014, van Rooij et al. 2015). Not much is known about the worldwide prevalence of *Bsal.* In Germany and Belgium, it has been found in the wild, while it seemed absent in multiple locations around Spain and Italy (Spitzen-van der Sluijs et al. 2016); however, it has recently been detected in The Netherlands and Spain (Lötters et al. 2020). In Germany, the United Kingdom and Spain it has also been found in salamanders living in private collections. Some of these collections are located in places where *Bsal* was not found in the wild (Spitzen-van der Sluijs et al. 2016), indicating that it is likely the fungus spreads through human trade in amphibians. *Bsal* has also been reported from Vietnam, but in contrast to European salamanders, Asian species seem to have greater immunity to the disease. It is likely that the fungus originates in Southeast Asia and that local amphibians have evolved to co-exist with it (Laking et al. 2017).

The aim of this research is to add to the global database of knowledge on the spread of *Bsal* by testing for the presence or absence of the pathogen in wild Israeli populations of *S. infraimmaculata*. We hypothesised that *Bsal* is absent in Israel as no decline has been reported in salamander populations in Israel; nonetheless, its close relative, *Batrachochytrium dendrobatides* (*Bd*), has been found in local amphibian species (Perl et al. 2017).

We sampled 11 sites within the salamander's national range from the most southerly populations in the Mt. Carmel area, to more northerly populations in the Upper Galilee (Fig. 1). Up to ten adult individuals of different sizes and sexes from each population (total N = 74 specimens) were swabbed using Dryswab™ fine tip swabs (MWE, UK). Extreme care was taken to ensure sterility and hygiene as not to inadvertently spread the fungus. Samples were stored in 95% ethanol at 4°C for later DNA extraction. Qiagen's DNeasy Blood and Tissue Kit was used to extract the DNA immediately following previously reported lysozyme-based lysis buffer treatment (Perl et al. 2017), according to the cell extraction protocol.

The qPCR was performed using Applied Biosystems Step One Plus. Forty amplification cycles at 95°C for 3 sec and 60°C for 30 sec, and a melting curve stage, were performed on each sample to facilitate comparison between different PCR products. The standard curve was built by applying triplicates of four different amounts (1 zeptogram, 0.1, 0.01 and 0.001 z per well) of a synthetic oligonucleotide representing the g-Block of *Bsal* 5.8srRNA partial sequence (NCBI Accession # NR_111867.1) and *Bsal* primers

Table 1. List of primers and positive-control oligonucleotide used in this paper.

Synthetic oligonucleotide sequence of the g-Block of *Bsal* 5.8srRNA partial sequence (NCBI Accession #NR_111867.1)	Ttatctgctccatctccccctcttcatccctaaccctatttttatatcacttttagatgatataaaaagacaaggaaatgaat-taaaaaaagaaaaatagaacaagaaaatactattgattctcaaacaggcatactctacaaagtagagtgcaatgtgcgttcaaa-gat
Locally designed *Bsal* primers	Forward: tctccccctcttcatccctaa Reverse: tttagatgatataaaaagacaaggaaatgaa
Bsal primers found in the literature (Martel et al. 2013 and others)	Forward: tgctccatctccccctcttca Reverse: tgaacgcacattgcactctac

(Table 1), diluted to 10 micro molars, were used. First, all samples were amplified using the primer pair locally designed by using Primer Express 2.0 software (Applied Biosystems); then, if any amplification was detected, the PCR was repeated with our laboratory primers as well as the primers previously reported (Martel et al. 2013).

The *Bsal* standard is identical to the expected PCR product of the fungus, thus the standard acted as a positive control for *Bsal*. Minimal detectable amounts of the standard were used, and the criteria set for *Bsal*-positive samples were an amplification within the standard curve or higher, and a similar peak melting temperature on the dissociation (melting) curve. No sample met the criteria of testing positive in multiple qPCR runs (Table 2). This alone is evidence of there being no positive cases of *Bsal* among the samples. To be extra rigorous and certain of this result, all samples that showed amplification in any of the qPCR runs were checked to see if the melting curves matched the standards and agarose gel electrophoreses were run to visualize any DNA amplification products. No sample matched

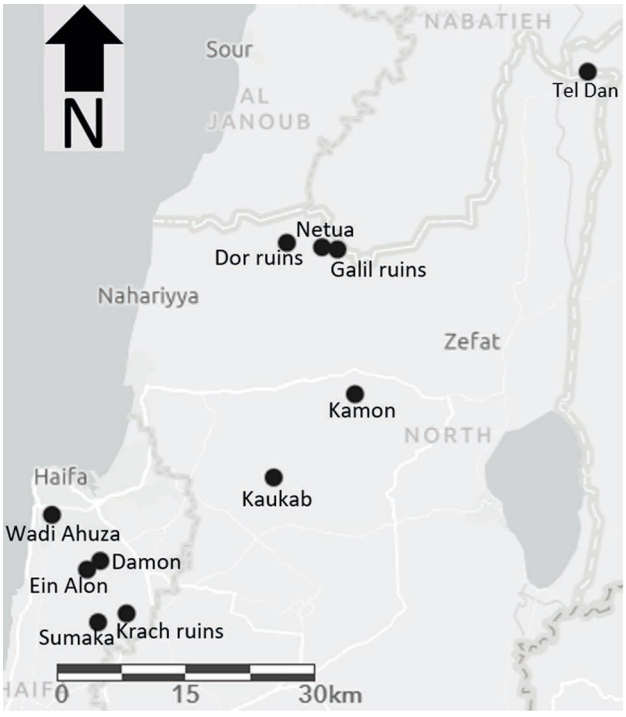

Figure 1. Salamander breeding sites sampled in northern Israel.

Table 2: Details of *Bsal* screening sites and specimens in northern Israel. All specimens were negative.

Site	Coordinates	Date	Number of specimens
Wadi Ahuza	32°46'59.7"N, 34°58'46.1"E	7 February 2020	1
Sumaka	32.671°N, 35.037°E	7 February 2020	5
Damon	32°44'03.8"N, 35°02'21.8"E	7 February 2020	1
Ein Alon	32.726°N, 35.022°E	7 and 8 February 2020	2
Krach ruins	32°40'44.1"N, 35°04'18.0"E	5 March 2020	6
Kaukab	32.823°N, 35.256°E	3 and 9 February 2020	5
Kamon	32.910°N, 35.350°E	3 February 2020	8
Netua	33°03'52.1"N, 35°18'56.2"E	8 and 12 February 2020	9
Galil ruins	33°03'46.4"N, 35°20'14.1"E	19 February 2020	7
Dor ruins	33°04'06.9"N, 35°16'22.8"E	9 March 2020	9
Tel Dan	33.249°N, 35.649°E	7 February 2020, 6 March 2020	12

the melting point of the standards. In line with this, visual examination of all salamanders sampled revealed no evidence of skin sloughing, ulcers, haemorrhaging, anorexia, lethargy, or any other symptoms of disease. Based on the rigorous and scientific methodology of this research, we conclude that to the best of our knowledge that Israeli salamanders do not carry the *Bsal* pathogen as of to-date.

The Middle East, and specifically Israel, is an important place to survey the spread of a pathogen, as it is land and habitat that connects Africa, Asia, and Europe. It is also situated along the major flyways of migratory birds, and *Bsal* has been found to be spread by the latter (O'Hanlon et al. 2018). If this pathogen were found in Israel, it would not only be a threat to the local Fire Salamander populations, but also a potential threat to all salamanders and newts inhabiting this region. Undertaking this survey in Israel, where the status of *Salamandra infraimmaculata* is endangered and it lives at the edge of its distribution range,

Figure 2. Examples of sampled salamander individuals showing no signs of disease.

was also extremely important for shedding light on the status of this pathogen in a species and area that is in need of heightened conservation efforts.

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