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Testing of Fire Salamanders around Salzburg for *Batrachochytrium salamandrivorans* within a school project

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Keywords: Salamandra salamandra, Batrachochytrium salamandrivorans, *chytridiomycosis, Red List, lethal fungus infection*

Abstract

Amphibians are declining worldwide. Recently an alarmingly steep decline in abundances of Fire Salamanders was reported in the Netherlands. In 2013 a unique fungus, *Batrachochytrium salamandrivorans,* was isolated and shown to be responsible for the observed mortality in the Fire Salamander populations. In a project funded by the relevant Austrian ministry, we are cooperating with schools to study the distribution and encourage the protection of Alpine and Fire Salamanders. Consequently we decided to test some of the Fire Salamander populations around Salzburg for the presence of *B. salamandrivorans* within the framework of this project. In addition, we involved the school children in collecting samples in order to introduce them to a current topic and to sensitize them to issues of amphibian protection. Of the 58 skin samples of Fire Salamanders from 8 different locations collected in autumn 2013, none tested positive for the presence of *B. salamandrivorans*. These results suggest that there is no evidence for infected Fire Salamanders in the study areas around Salzburg.

Figure 1 – A female Fire Salamander in a stream. © M. Meikl

Introduction

Amphibians are more threatened and are declining more rapidly in numbers than either birds or mammals. 43.2% of amphibian species are experiencing population decrease in some way, with 32.5% being globally threatened. Although many declines are due to habitat loss and overutilization, there are other unidentified processes, which are quickly driving species to extinction (Stuart et al. 2004).

One factor can be the disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (order Chytridiales, Longcore et al. 1999). This waterborne pathogen disperses zoospores and is able to survive in water bodies without hosts being present. Chytridomycosis leads to thickening of skin, anorexia and changes in behaviour and is fatal for the infected individuals because they are not able to feed, release toxins or breathe. The fungus causes sporadic deaths in some populations and 100% mortality in others (Whittaker & Vredenburg 2011).

In Austria all of the 20 autochthonous amphibian species described are registered on the Red List (Gollmann 2007), in which the Fire Salamander (*Salamandra salamandra salamandra*) is categorized as *near threatened.*

In a project funded by the Austrian ministry BMWFW we are cooperating with schools to study the distribution and encourage the protection of Alpine (*Salamandra atra*) and Fire Salamanders (Meikl et al. 2014) (Figure 1). The children get to know these species in the course of workshops and field trips. The main aim is gathering data about the distribution of both species in a publicly available database (www.alpensalamander.eu).

Recently an alarmingly steep decline in abundances of Fire Salamanders was reported in the Netherlands. Since 2008, dead animals have been found in the field without external signs of injury. A monitoring programme revealed a 96% decrease in the population between 1997 and 2012 (Spitzen-van der Sluijs et al. 2013). In 2013 a unique fungus, *Batrachochytrium salamandrivorans* sp. nov. was isolated and characterized from these salamander populations (Martel et al. 2013). It could be shown that the fungus causes erosive skin disease and experimentally infected salamanders died within seven days. Hence it was concluded that *B. salamandrivorans* is responsible for the observed mortality in the salamander population.

According to our present knowledge, chytridiomycosis can be caused by two different fungi, the well-known *Batrachochytrium dendrobatidis* and the newly discovered *Batrachochytrium salamandrivorans*. The origin of this new fungus is as yet unknown. Recently it was found in a dying salamander from Eupen, Belgium (Speybroeck 2014).

In Austria *B. dendrobatidis* was detected at 10 amphibian breeding sites. Nine amphibian species, not

Figure 2 – School children collecting skin samples from salamanders. © M. Hahn

including *S. atra* and *S. salamandra,* were infected. However, none of them showed obvious signs of the disease (Sztatecsny & Glaser 2011).

Therefore *B. salamandrivorans* may also be present in healthy-looking Fire Salamander populations, such as the ones we monitored in our study sites.

Consequently we decided to test some of the Fire Salamander populations present around Salzburg within our school project. Our first aim was to establish if there is any evidence for the presence of *B. salamandrivorans* by using the PCR method described by Martel et al. 2013. In addition, we involved the school children in collecting samples during excursions to salamander habitats in order to introduce them to a current topic and to sensitize them to issues of amphibian protection (Figure 2).

Material and methods

Sampling

In total, 58 skin samples of Fire Salamanders from 8 different locations (7 in Salzburg, 1 in Upper Austria) were taken (Figure 3). One sampling site (Site 1) is located in Aigner Park, a locally protected area (*Geschützter Landschaftsteil*) (22.2 ha). In addition it has been designated as Nature Park for its cultural-historical significance and the richness of its natural landscape elements. Samples were collected in autumn 2013, between 10.09.2013 and 19.10.2013. The school children were instructed in the appropriate methods (sterile skin swabbing and how to prevent the dispersion of chytrids from one location to another). From each individual, skin swabs were taken by gently rubbing sterile nylon flocked swabs (COPAN FLOQSwabs™) 10 to 15 times over the salamander's ventral surface, feet and tail, as suggested by Hyatt et al. 2007.

Exact locations of the sampling sites were recorded. Where no GPS data were available in the field, they were determined with Austria Map online (www.austriamap.at). The altitude data were calculated with www.gpsvisualizer.com. The map was created with DIVA-GIS (www.diva-gis.org).

DNA extraction

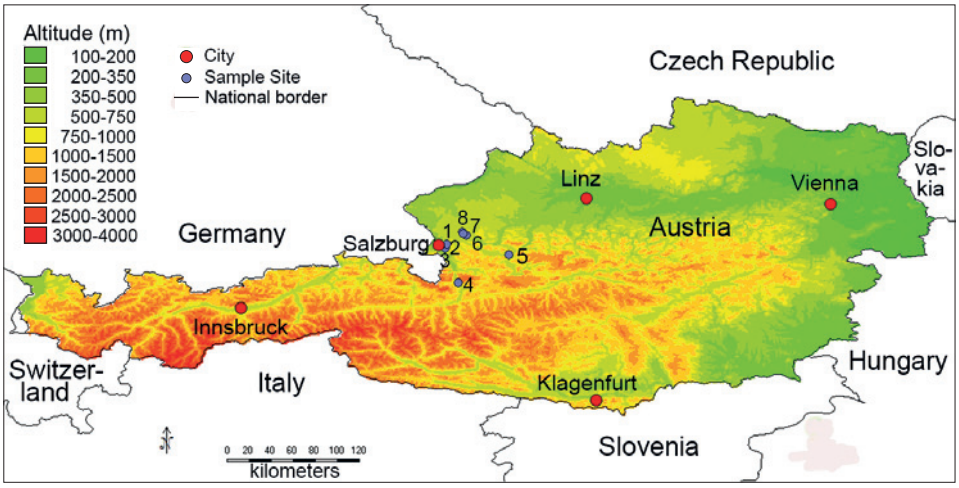
Air-dried skin swabs were stored at −20°C for at least 5 months. DNA was extracted with Applied Biosystems®PrepMan® Ultra Sample Preparation Reagent. Two different methods were used resolving the DNA from the swabs. 50 µl of the PrepMan® Ultra reagent were added to the swab and incubated for 5 minutes at room temperature. Then the moistened swab was inserted into a yellow sterile plastic pipette tip. The tip with the swab was put into a sterile Eppendorf tube and the swab was pushed firmly down the tip to press out the liquid. The tube containing tip and swab was centrifuged for 1 minute at 9 000 rpm. Afterwards, tip and swab were discarded. Samples were heated on a heat block for ten minutes at 100°C and afterwards cooled down to room temperature on crashed ice for 2 minutes. After centrifugation for 3 minutes at 13 000 rpm, the extracted DNA was stored at −20 °C. For the second method, Qiagen® – QIAshredderTM mini spin columns were used instead of the pipette tip.

Quantification of the DNA product was done with a Thermo Scientific-NANODROP 2000c Spectrophotometer.

PCR reaction

Fire Salamander skin samples were checked for the presence of DNA from the chytrid fungus *B. salamandrivorans* by means of conventional PCR.

The primer set (STerF 5'TGCTCCATCTCCCCCTCTTCA3' and STerR 5'TGAACGCACATTGCACTCTAC3'), developed by Martel et al. 2013, which amplifies the 5.8S rRNA gene, was used to detect the presence of the chytrid salamandrivorans in the Fire Salamander skin samples. The primers were synthesized by Microsynth and supplied at 10 µmol / L. The amplification reactions were performed in a total volume of 25 µl. Each consisted of 1 µl DNA template, 1 µM of each primer, 10x Coral Load Buffer (contains Tris.HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, gel loading reagent, orange dye, red dye; pH 8.7 (20 °C)), 0.2 mM of each dNTP, and 0.8 units of QiagenTaq Polymerase. The amplifications were performed in a geneAmp® PCR System 2700 (Applied Biosystems). PCR conditions were as follows: an initial denaturation at 93 °C for 10 min, 30 cycles of 45 s at 93 °C, 45 s at 59 °C, 60 s at 72 °C, to end with a final extension of 10 min at 72 °C. We performed a PCR sensitivity test using different concentrations of zoospores of a culture of *B. salamandrivorans* (50, 20, 10 zoospores / µl). A positive control consisting of a DNA template including 20 or 50 zoospores from a culture of *B. salamandrivorans* was added to each amplification. As negative control DEPC-treated water was used.



Site		Longitude	Latitude	Altitude [m]	Samples
1	Salzburg, Aigen, Glasbach	13.09324°	47.77921°	483	15
2	Salzburg, Gaisberg	13.10501°	47.79978°	904	3
3	Elsbethen, Schaferlberg	13.09186°	47.76192°	555	3
4	Werfen, Kalcherbauer	13.19751°	47.48147°	621	11
5	Bad Ischl, Reiterndorf	13.62102°	47.71237°	471	3
6	Thalgau, Kolomannsberg	13.26833°	47.87528°	993	10
7	Thalgau, Grosse Plaike	13.24111°	47.88389°	930	2
8	Henndorf, Lichtentann	13.23500°	47.89778°	740	11

Figure 3 – Sites for collecting salamander skin samples: 1–4 and 6–8 in Salzburg, 5 in Upper Austria. The map was created with DIVA-GIS (www.diva-gis.org), elevation data: CGIAR-SRTM (http://srtm.csi.cgiar.org/).

Results

The quantity of extracted DNA ranged between 3.4 ng / µl and 226.4 ng / µl.

The PCR returned a positive detection in the positive controls for *B. salamandrivorans,* starting at an amount of 10 zoospores / µl (see Figure 4). Gel detection showed a positive band for zoospores at 161-bp. None of the 58 Fire Salamander skin swabs tested positive for the presence of *B. salamandrivorans* (Figure 5).

Discussion

Our findings suggest that the newly discovered fungus *B. salamandrivorans* is not yet present in the study areas around Salzburg. During our excursions with the schools and our own observations at several sites we

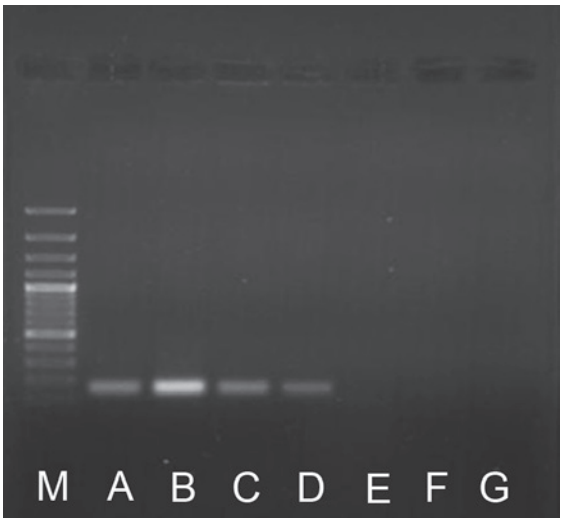


Figure 4 – Sensitivity test with zoospores of B. salamandrivorans *(courtesy of Prof. An Martel, University of Ghent). M: DNA standard (GeneRuler 100 bpPlus), A: 20 zoospores / µl culture, B: 50 zoospores / µl culture, C: 20 zoospores / µl culture, D: 10 zoospores / µl culture, E: skin swab 24, F: skin swab 50, G: negative control (DEPC-treated water).*

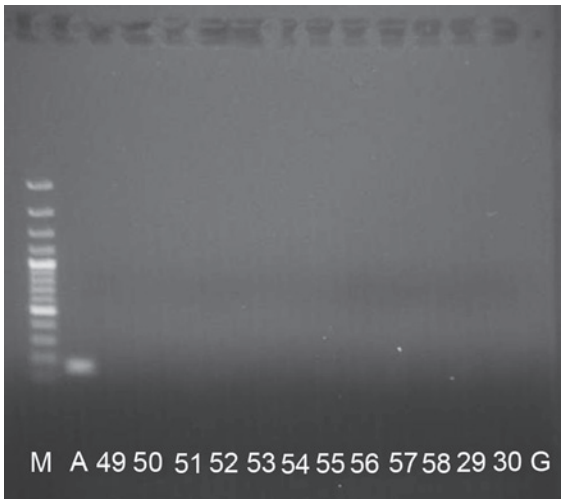


Figure 5 – Fire Salamander skin swab samples amplified with the Bs primers STer F and Ster R. M: DNA standard (GeneRuler 100bp Plus); A: 20 zoospores Bs; 49 to 58, 29 and 30: Fire Salamander samples; G: negative control (DEPC-treated water).

never found dead Fire Salamanders, except for those caused by road kills. The PCR results support our impression that there is no evidence for infected Fire Salamanders in the province of Salzburg and bordering areas of Upper Austria.

Under laboratory conditions *B. salamandrivorans* grew at temperatures as low as 5 °C, with optimal growth between 10 °C and 15 °C, and death temperatures above 25 °C (Martel et al. 2013). According to this, our sampling in autumn should be the ideal period to detect spores from the skin of infected salamanders, if they were present. Moreover, the positive controls in the PCRs indicate that even as little as 10 zoospores give a clear positive result.

A web-based dataset (www.Bd-maps.net) shows the worldwide distribution of *B. dendrobatidis* with the aim of providing new insights into occurrence patterns and the ecology and epidemiology of the fungus. It reveals that *B. dendrobatidis* has been detected on all continents, except for Antarctica. In 68% of the countries where data were collected the presence of the fungus was confirmed. (Olson et al. 2013)

A survey for *B. salamandrivorans* in Chinese amphibians was published this year (Zhu et al. 2013). The authors tested 665 individual amphibians of 30 species, spread across thirteen provinces and two municipalities in China. They used epithelial swabbing (260 samples) and histology of phalanges (165 samples) with the nested PCR technique. None of the tested samples were positive.

However, we cannot assume that the new fungus has not yet reached Austria or Salzburg. Additional investigations in other areas covering the whole country are required. Furthermore, it has to be clarified if other amphibian species are also endangered by this new chytrid.

The collaboration with the children involved in our project worked very well. They learned about sterile working while taking samples and prevention of dispersal of the fungus. We explained to them that the endangerment of species can be due to many different reasons. Often they asked us what we would do if we found infected salamanders. At present we do not have an answer. However an intensified monitoring of Fire Salamanders in future is very important in order to react immediately if *B. salamandrivorans* was found in a population. If the fungus was discovered in a salamander population, the most important thing to do would be to prevent the dispersal of the fungus to other, healthy populations. Depending on the size of the infected population and the connection or interaction with other populations, such prevention measurements might be difficult. If the population were small and isolated, not living in a highly connected forest system, the infection would be easier to control. In the Netherlands, fire salamanders from infected populations were collected and a successful captive breeding programme was started (Spitzen-van der Sluijs et al. 2013) (see also www.sosvuursalamander.nl). In infected populations, and especially those in danger of going extinct, such breeding programmes would be vital. The treatment of individuals with antifungal agents may also be a possibility. Indeed, this treatment has to be tested further and it seems to be difficult to apply on entire populations in the wild.

However, the more than 1 400 children, teachers and parents involved in our school project will continue reporting unusual findings of dead salamanders to us or to a responsible authority. Thus we can say that area-wide monitoring of an endangered species is more successful if the general public is included. To achieve this goal, the collaboration with schools, involving the children and their social circles, is a very effective way.

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