# Study of the microbial community present on eastern red backed salamanders (*Plethodon cinereus*)

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## Abstract

Amphibian populations around the world are in decline. One of the major causes of decline in amphibian populations is the chytrid fungus, *Batrachochytrium dendrobatidis*. The eastern red backed salamander (*Plethodon cinereus*) is an important amphibian in U.S. eastern forest. Bacterial species that produce a protein called violacein have been found on eastern red backed salamanders in other areas of its geographic range. Violacein has antifungal properties and prevents the deadly skin infection caused by the chytrid fungus. DNA extracted from red backed salamanders was amplified using PCR. PCR results show that chytrid was not present on salamanders sampled. The violacein gene was successfully amplified from salamanders. The presence of violacein may provide eastern red backed salamanders vital protection from chytrid if the fungus if the fungus enters New York State.

## Introduction

Amphibians around the world are experiencing drastic population declines. Over one third of amphibian species are in a state of decline (Stuart et al. 2004) and the current extinction rate of amphibians is over 200 times the background extinction rate (Collins 2013). There are many causes for amphibian decline including habitat loss, climate change, and pollution. In the late 1990's an emerging disease that infects the skin of amphibians caused by the *Batrachochytrium dendrobatidis*, more commonly referred to as the Chytrid fungus, was found to be a major cause of amphibian decline (Harris et al. 2009). The skin of amphibians is an

important organ responsible for respiration, electrolyte balance, hydration and other important physiological functions (Rosenblum et al. 2012).

The skin of amphibian species possesses unique microbial communities. The microbial community present on amphibian skin is part of the organisms' innate immune system (Woodhams et al. 2007). There are multiple bacterial species present on the skin of amphibians that produce a protein called violacein including; *J. lividum*, *Duganella* sp., *Chromobacterium violaceum*, *Iodobacter fluviatile*, *Pseudoalteromonas tunicata*, and *Pseudoalteromonas luteoviolacea* (Becker et al. 2009). Violacein has essential antifungal properties and has been shown to inhibit the skin disease caused by the chytrid fungus on both natural and inoculated hosts (Becker & Harris 2010; Harris et al. 2009). The chytrid fungus has been found on every continent, except Antarctica, and though it has not been found in New York State, it has been found in many neighboring states and sampling efforts in New York have been limited (Bd-maps). Violacein producing bacteria as well as the violacein protein have been found on the skin of eastern red backed salamanders (*Plethodon cinereus*) in other regions of their range (Becker et al. 2009; Harris et al. 2009) where the species' skin microbial interactions have been heavily researched. The goal of my research is to assay for the presence of the chytrid fungus (*Batrachochytrium dendrobatidis*) and identify the presence of the violacein gene on eastern red backed salamanders collected from New York City.

**Methods**

Samples were collected postmortem in November 2011 from outside George Washington High School in New York City (provided by Evon Hekkela at Fordham University). Cultures of *B. dendrobatidis*, the chytrid fungus, used for positive controls in the study were provided by Joyce Longcore at University of Maine Chytrid Laboratory.

DNA was extracted from salamander samples and chytrid cultures using FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). To ensure uniform cell lysing of all microbial species present on the salamanders, the salamander samples were homogenized using a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). Purified DNA was amplified using PCR. Fungal DNA was amplified using universal fungal primers were taken from Schoch et al. 2012, chytrid DNA was amplified using *Batrachochytrium dendrobatidis* specific primers were taken from Annis et al. 2004, and the violacein gene was amplified using original primers designed by Julie Lynn (Table 1). Violacein primers were designed to the overlapping regions of the VioA domain in *J. lividum* and *Duganella* sp. PCR products were ran on a 1% agarose gel and visualized on a and visualized using a BioRad UV trans-illuminator (Hercules, CA, USA). PCR products were purified using QIAquick PCR Purification Kit® (Qiagen, Venio, Netherlands) and then sent to Genewiz, Inc. (South Plainfield, NJ, USA) for sequencing. Sequencing results were inserted into Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information, Bethesda, MD, USA) to confirm appropriate species and genes were amplified.

**Results**

PCR with universal fungal primers successfully amplified fungal DNA (Figure 1). A BLAST search of sequencing information confirmed the DNA templates amplified were from fungal species. The PCR with chytrid specific primers amplified the positive control only, suggesting the salamander samples were negative for the chytrid fungus (Figure 2). A PCR using both chytrid specific primers and universal fungal primers combined confirmed the absence of the chytrid fungus from the salamanders sampled (Figure 3). PCR using the violacein gene primers was successful in amplifying the gene from DNA collected from salamander samples

(Figure 4). Purified violacein PCR product sequencing results were inconclusive in determining the species of the amplicon.

**Discussion**

Salamander samples tested in this study were negative for the chytrid fungus (*Batrachochytrium dendrobatidis*). Though the violacein gene PCR sequencing results were inconclusive, it is most likely it was the violacein gene that was amplified. The lack of definitive sequencing results was most likely due to the presence of multiple violacein bacterial species. Ligating the PCR products into a bacterial vector would allow for determination of the violacein producing bacterial species and confirm the amplification of the violacein gene.

The violacein gene has been found on eastern red backed salamanders in other regions of its range, i.e. Virginia, where the species has been heavily researched (Becker et al. 2009; Harris et al. 2009). The presence of the gene on salamanders in New York City supports a species specific interaction found in previous studies (Brucker et al. 2008). The presence of the violacein gene may provide red backed salamanders protection if the chytrid fungus becomes established in New York State. The red backed salamander is the most common and well distributed vertebrate in north eastern forest (Townsend & Driscoll 2013; Gertzog et al. 2011) and they are essential for proper ecosystem functioning.

Future work should include a complete community analysis of violacein producing bacterial species, analysis of the entire skin microbial community, and skin microbial community analysis across an urban to rural gradient.

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**Tables & Figures**

| Primer Name | Sequence 5'-3' |
|---|---|
| Violacein Forward | TGTTCTACACCGACAGC |
| Violacein Reverse | GCTGCCTTCCATCCAGCCGCAATGCG |
| Chytrid Forward | TACAGTGTGCCATATGTCACGAG |
| Chytrid Reverse | GTTCATATCTGTCCAGTCAATTCGGAC |
| Universal Fungal Forward | ACCCGCTGAACTTAAGC |
| Universal Fungal Reverse | TCCTGAGGGAAACTTCG |

**Table 1.** Summary of primers used in the study

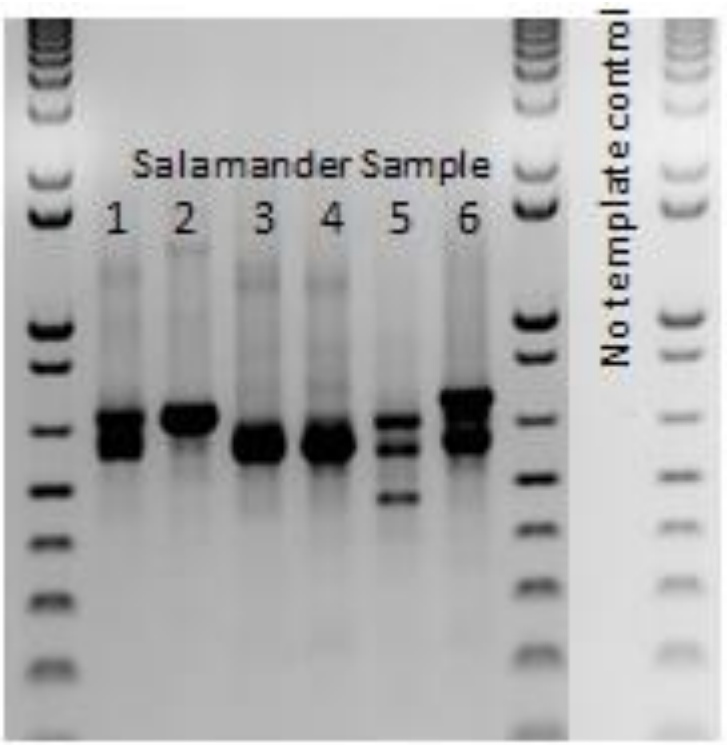


**Figure 1**. PCR results using universal fungal primers with salamander samples

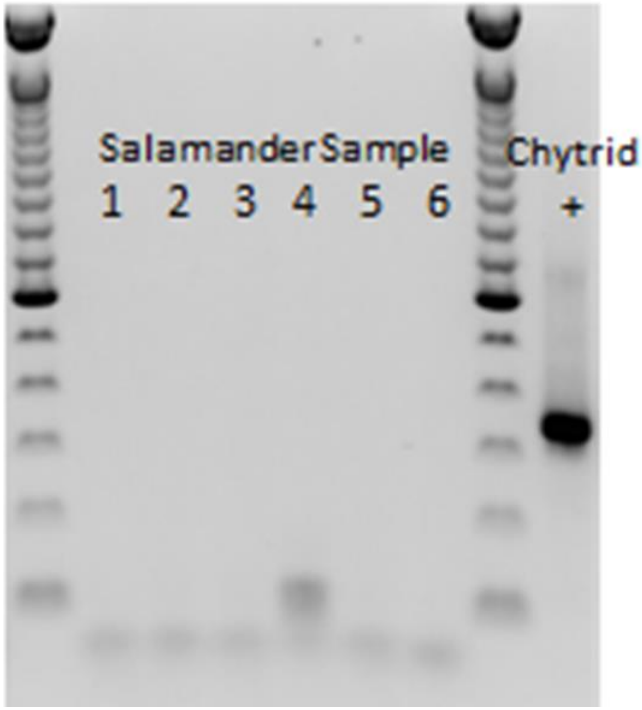


**Figure 2**. PCR results using chytrid specific primers with salamander samples and positive chytrid control samples

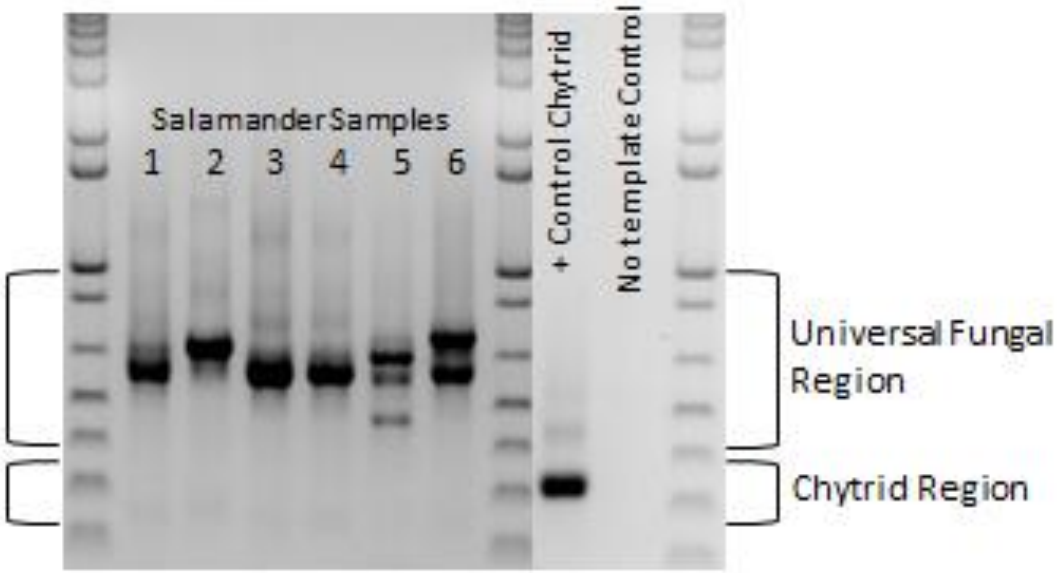


**Figure 3**. Results from PCR with universal fungal primers and chytrid primers

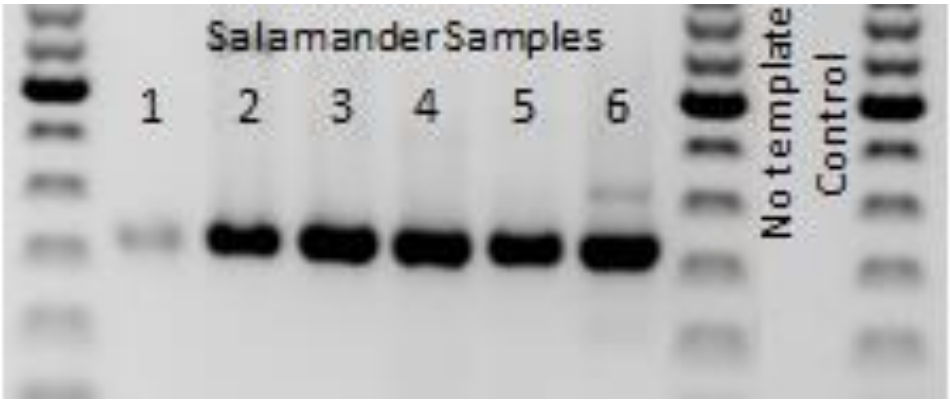


**Figure 4**. PCR results for detection of the violacein gene on salamander samples