DETECTION OF A SMALL STREAMSIDE SALAMANDER, THE NORTHERN TWO-LINED SALAMANDER (*EURYCEA BISLINEATA*), IN A STREAM WITH eDNA

by

Sarah M. Kuppert

A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Environmental Science and Policy

Committee:

_____	Dr. Thomas Wood, Thesis Director
_____	Dr. Patrick M. Gillevet, Committee Member
_____	Dr. Arndt Laemmerzahl, Committee Member
_____	Dr. Donald E. Moore, Committee Member
_____	Dr. Masoumeh Sikaroodi, Committee Member
_____	Dr. Donna M. Fox, Associate Dean, Office of Student Affairs & Special Programs, College of Science
_____	Dr. Peggy Agouris, Dean, College of Science

Date: _____ Summer Semester 2015
George Mason University
Fairfax, VA

Detection of a Small Streamside Salamander, the Northern Two-lined Salamander (*Eurycea bislineata*), in a Stream with eDNA

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

Sarah M. Kuppert
Bachelor of Arts
University of Kassel, 2012

Director: Thomas Wood, Professor
New Century College

Summer Semester 2015
George Mason University
Fairfax, VA

DEDICATION

This is dedicated to all the salamander populations that have declined due to human actions. I hope that my research will help develop alternatives to existing, invasive monitoring methods and hence give conservationists the opportunity to survey salamander populations without causing any harm to the animals.

This is dedicated to Eric, for being a positive influence in my life and a role model for perseverance. Thank you to the generous and patient Joe and Denise, who gave me a roof over my head and fantastic meals that kept me well-nourished during stressful times.

This is dedicated to my wonderful family and friends, who were always there for me in times of need, despite many of them being separated from me by an ocean. I could not have done it without your unconditional love and support, even though you often did not understand what I was talking about.

ACKNOWLEDGEMENTS

I would also like to thank Dr. Thomas Wood for his guidance, for helping me narrow my topic, and for providing the field site and materials.

I would like to thank the MBAC lab. and director Dr. Patrick Gillevet, for helping with the computational analysis, for the pointed questions that helped me navigate problems, and for funding for my research. Dr. Masoumeh Sikaroodi for her patience, advice and support.

I would also like to thank Dr. Moore, who always believed in my potential and helped with the transition from a different field of study by giving me the chance to intern with him. Dr. James Murphy, who was instrumental in my development as a herpetologist and Dr. Laemmerzahl, who was invaluably helpful.

I would like to thank Eric Hawkins, who helped with editing. The volunteers who helped me crawl through the stream for hours for my field survey: Matt Evans, Melissa Fuerst, Lorien Lemmon, Jesi Hessong, Angelo Vera, Emily, and Chelsie. Jos Kielgast for his advice and sharing his research experience with me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BLAST ..... Basic Local Alignment Search Tool
bp ..... base pairs
COI ..... Cytochrome Oxidase I
eDNA ..... environmental DNA
Gbp ..... billion base pairs
ha ..... hectares
HL ..... head length
HW ..... head width
OAL ..... overall length
pgs ..... picograms
SV ..... snout to vent length
TL ..... tail length

ABSTRACT

DETECTION OF A SMALL STREAMSIDE SALAMANDER, THE NORTHERN TWO-LINED SALAMANDER (*EURYCEA BISLINEATA*), IN A STREAM WITH eDNA

Sarah M. Kuppert, M.S.

George Mason University, 2015

Thesis Director: Dr. Thomas Wood

As the decline in the world's biodiversity threatens many species' survival, wildlife managers are in great need of information about species' status in the wild. This information is gathered through surveys that often have low cost-effectiveness, are time-intensive, and may harm the target species. For that reason, new surveying and monitoring methods should be explored. These new surveying and monitoring methods should improve the data gathering process and decrease or eliminate suboptimal aspects of traditional surveying methods.

A new genetic monitoring tool, environmental DNA (eDNA), has the potential to revolutionize aquatic surveys because, compared to the traditional methods, it is non-invasive, more cost-effective, and less time-intensive. However, protocols for different species have to be developed because detection probability may vary, depending on the

size and lifestyle of the species. In addition, molecular markers have to be developed that amplify the target species' DNA without amplifying closely related species.

In this study I was able to detect the DNA of Two-lined salamanders with the eDNA method in four water samples of controlled environments and two stream water sample. The protocol developed in this study should be further tested in different streams with known and unknown presence of the target species.

CHAPTER ONE: INTRODUCTION

1. Background

Our world is a patchwork of unique ecosystems that is comprised of biotic and abiotic factors that developed over the last 3.5 billion years to form a delicate equilibrium and intertwined networks of organisms that depend on each other for survival (Mojzsis et al., 1996). The biodiversity on our planet, "the number and variability of genes, species, and communities in space and time" (Sepkoski, 1997), plays an important role for the stability of ecosystems. However, the importance of biodiversity for humans may not be immediately obvious. While the most utilitarian reason for placing a high value on biodiversity is the linkage between ecosystems and human well-being, biodiversity should also be preserved simply because of its beauty and intrinsic value (Osbahr, 2001). Ecosystems provide services that are often taken for granted. As such, biodiversity plays an instrumental role in providing ecosystem services, such as water purification, flood regulation, nutrient cycling, and soil formation (World Health Organization, 2005). Even though the functions of a species are not always evident to humans, the smallest and most insignificant seeming species may play an important role in the ecosystem they are a part of and contribute to the stability and well-being of the plant and animal community as a whole (Tilman, 1996). Over the course of

billions of years, species evolved to adapt to environmental conditions by exploiting niches. Niches are the range of environmental conditions under which an organism can exist. These conditions differ among species, which makes them specialized areas of expertise and ensures limited competition from other species for resources. This is a strategy which ultimately increases the chances for survival of each species (Pianka, 1981). The interactions between species form an intricate web of dependencies, also known as food webs, whose links of species interactions ensure that species in any given ecosystem depend on each other for their continued existence. The loss of species is therefore highly problematic for the vigor of ecosystems.

The current rate of biodiversity loss is alarming, and it is believed that many species are even going extinct before we discover them (Lees & Pimm, 2015). The current rate of extinction far exceeds the natural background rate of extinctions, the latter of which is about 0.1 extinctions per million species per year (De Vos, Joppa, Gittleman, Stephens, & Pimm, 2014; IUCN, 2007; Pimm et al., 2014). The latest update of the IUCN Red List of Threatened Species shows that 22,413 species out of the 76,119 assessed species are threatened with extinction. Amphibians are among the most threatened taxa, with about 41% of all extant species threatened with extinction (IUCN, 2014).

This extraordinarily high rate of extinction is, however, not triggered by a catastrophic natural event, but is a key measure of humanity's global impact on other organisms. Although there are natural causes of species decline, the impact that humans have on flora and fauna is irrefutable. From early on in human history noticeable waves of extinction events were

caused by humans. The earliest wave happened as early as the existence of hunter and gatherer societies and led to the extirpation of several large vertebrate species (Alroy, 2001; Bulte, Horan, & Shogren, 2006). The most recent wave began in the 1800's with the Industrial Revolution, which was powered by the use of fossil fuels, and has been by far the largest extinction wave yet. With enormous amounts of cheap energy at their disposal, and the mechanization of agriculture, humans were able to produce more food and grow the human population rapidly (Zalasiewicz, Williams, Steffen, & Crutzen, 2010). In addition, the discovery in 1910 of the Haber process, which allowed the synthesis of ammonia, further helped boost food production and increase the human population (see Figure 1).

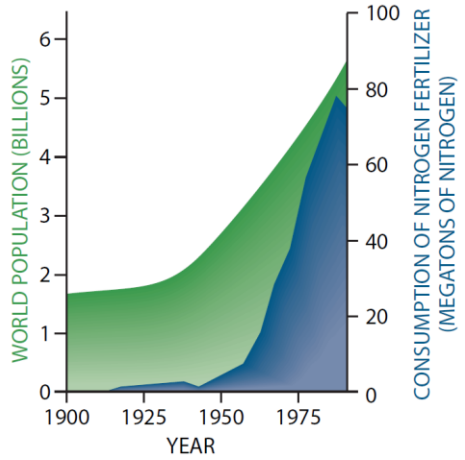


Figure 1: Human population growth and consumption of nitrogen fertilizer from 1900-2000 (Smil, 1997)

With cheap energy available and the ability to grow relatively more food, the human population grew rapidly from 1 billion in 1800 to 2 billion in 1930, 4 billion in 1975, and over 7 billion today (see Figure 2). If the current course is not altered, we will reach 8.3 to 10.6 billion by 2020 (United Nations, 2012; U.S. Census Bureau, 2006).

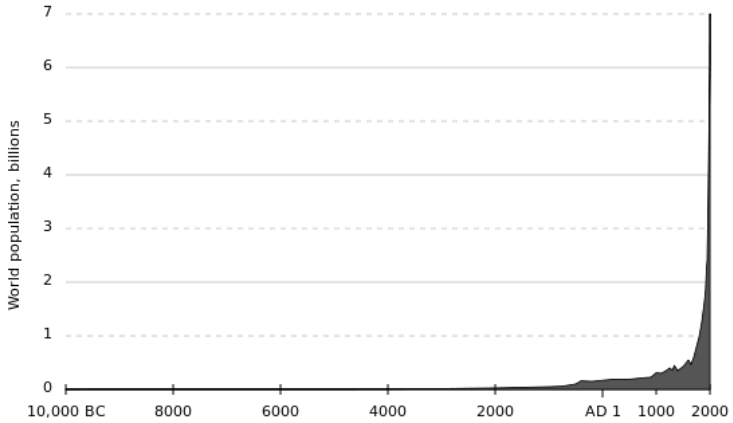


Figure 2: World human population (est.) 10,000 BC–2000 AD (U.S. Census Bureau, 2006).

As the human population increased, so did the rate of extinction. Reasons for human caused extinctions are manifold: habitat destruction and degradation, over-exploitation, pollution, disease, invasion of alien species, and global climate change (Hautier et al., 2015; IUCN, 2009; World Health Organization, 2005). All of those factors contribute to the high extinction risks of species. Salamanders are a particularly vulnerable group. According to the International Union for Conservation of Nature (IUCN), 49.8% of all salamander species assessed worldwide are threatened by extinction or have already gone extinct (IUCN, 2008).

2. Salamanders

Salamanders are part of the ancient class *Amphibia* that arose approximately 360 million years ago. They are part of the order *Caudata*, literally meaning "with tail." All members of this class are ectothermic vertebrates who possess a glandular skin and lack feathers, scales, and hairs. They have a long tail and four limbs. However, over the course of evolutionary history some species partially or fully lost their legs. Approximately 400 species of salamanders occur worldwide. A great diversity of salamander species is endemic to North America, including 127 species of 24 genera and all 9 extant families (Adler & Halliday, 2002; Bartlett & Bartlett, 2006; Petranka, 1998). The Appalachian Mountains in the eastern United States contain the largest salamander biodiversity in the world, which makes it a favorable area for salamander research (Petranka, Eldridge, & Haley, 1993).

Salamanders live in different habitats and their lifestyles can be divided into three major groups: totally aquatic, semi aquatic, and totally terrestrial. Most salamanders have a two-stage life cycle that starts with an aquatic larval stage and ends with a terrestrial adult stage. Some have a third stage where they become aquatic again. Other species are entirely aquatic or fully terrestrial for all stages of their lives. However, species of aquatic salamanders can differ in how much of their lives they actually spend in the water. Some salamanders prefer to spend more time at the stream side. Amphibians need moisture to survive, which is why salamanders are found in streams, ponds and vernal pools, and in moist soils or hidden under rocks or

logs. They are preferentially nocturnal, because at night they suffer the least water loss, are most protected from predators, and have more prey available (Bartlett & Bartlett, 2006; Vitt & Caldwell, 2008).

Salamanders are also remarkable from a genetic standpoint because they have extremely large genome sizes (see Figure 3). This is especially true of the plethodontid salamanders. Of the over 200 salamander genomes analyzed, genome sizes range from 14 to 120 Gbp, and are therefore between four and 35 times larger than the human genome (Palazzo & Gregory, 2014; Sun et al., 2012).

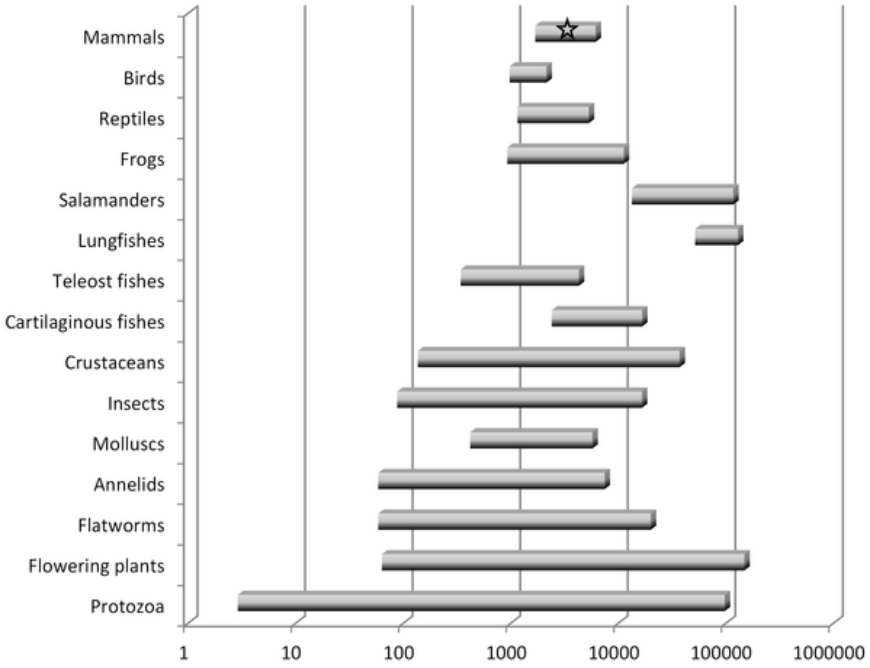


Figure 3: Summary of haploid nuclear DNA contents ("genome sizes") for various groups of eukaryotes (Palazzo & Gregory, 2014).

Salamanders play important roles in the ecosystems they inhabit. However, the effects of salamander population decline on ecological communities, have received little attention. Salamanders make up a large portion of the biomass in the Appalachian Mountains. They can have a significant effect on organic matter and they are the most abundant vertebrate in eastern hardwood forests (up to 18,000/hectare). Salamanders play a major role in leaf litter retention, the amount available for soil-building, and carbon capture at the litter-soil interface. This was demonstrated in a study by (Best & Welsh, Jr., 2014), which found that woodland salamanders (*Ensatina eschscholtzii*), potentially sequester an estimated 72.3 metric tons/year of carbon in forest soil rather than entering the atmosphere. Because leaf-eating insects release carbon into the air during the digestive process, the consumption of insects by salamanders ultimately hinders the release of carbon into the environment. Instead, leaves that are not eaten are becoming part of the soil forming process and carbon is stored in the ground as humic acids, rather than released into the air. Additionally, many of the invertebrates that salamander prey upon, are considered pests. Salamanders are, in turn, preyed upon by higher trophic-level predators, and therefore they fill an important ecological niche in terrestrial and aquatic communities (Blaustein et al., 2011). Furthermore, salamanders have been used for medical research because of their ability to regenerate limbs, which is a potential source for medical advancement in the area of tissue regeneration (Brockes & Kumar, 2005). Salamanders therefore provide important services and ecosystem services that would

be rather costly to replace if their role in the ecosystem were to cease due to population declines and extinctions. The functional extinction (which means the species no longer serves the ecological role it once did) of something as small as salamanders can therefore have a significant effect on the environment.

Declines of salamander populations are a global problem with a range of local causes. Salamander populations all around the world are affected by environmental problems, such as habitat loss, disease, pollution, acid rain, climate change, and ultraviolet radiation (Alford & Richards, 1999; Caruso & Lips, 2013; Cassie, 1999; Gibbon et al., 2000). Habitat loss and degradation are the most significant factors contributing to salamander population declines and extinctions (Blaustein et al., 2011). Most salamander species are relatively small and therefore have a limited ability to migrate, which is why a road can present a major barrier for salamanders to cross and ultimately leads to the separation of populations from one another. This is problematic because small population sizes are vulnerable to loss of genetic diversity through inbreeding and the consequent loss of alleles through genetic drift. The loss of genetic diversity leads to the decline of fitness of sexually reproducing organisms, because it increases the likelihood of inherited diseases and decreases reproductive success (Noël & Lapointe, 2010).

Logging is also a factor that may contribute to salamander population declines (Maigret et al., 2014; McDonald, 2001). Research by Verschuyl et al. (Verschuyl, Riffell, Miller, & Wigley, 2011) suggests that large-scale removal of biomass from Appalachian forests likely has an adverse effect on diversity and abundance of salamanders. Petranka, Eldridge, & Haley,

(1993) state that following timber harvest, declines of up to 80% for certain salamanders and species richness declines of up to 50% have been seen.

Pollution and acid rain are also major amphibian killers since amphibian skin is permeable and therefore especially vulnerable to pollutants in water. A study by (Burke, Bergeron, Todd, & Hopkins, 2010), for example, links mercury exposure to a decline in speed and responsiveness in hunting success of Northern two-lined salamanders (*Eurycea bislineata*). Ultimately, sub-lethal mercury concentrations may lead to a reduction of the ability to successfully execute tasks critical to their survival. Another pollutant that adversely affects salamanders is road deicing salt, which can be transported as runoff up to 170 meters from roads and end up in wetlands. It is known to impact the survival of salamander egg clutches (Karraker & Gibbs, 2011). Another threat to salamanders and other amphibians is acidity in the environment, as might be caused by acid rain. Acidic water, for example, interferes with the symbiosis between salamander eggs and green algae during early embryonic development (Bianchini, Tattersall, Sashaw, Porteus, & Wright, 2012). Similarly, low pH in soil and water is also known to decrease amphibian density and species richness (Wyman & Jancola, 1992).

2.1 Northern two-lined salamanders (*Eurycea bislineata bislineata*)

Northern two-lined salamanders (*Eurycea bisline*ata) belong to the *Plethodontidae,* a family of lungless salamanders. This is by far the largest family of salamanders, with over 380 species in the family and making up around 70% of the total salamander diversity (Min et al., 2005). *Bislineata* is Latin, meaning "two lined or striped" (Virginia Herpetological Society,

2015). *E. bislineata* is widely spread along the east coast of the United States, and is divided into four subspecies. The most widely spread subspecies, and the one that is found in Virginia, is the subspecies *E. bislineata bislineata* (see Figure 4 and Figure 5).

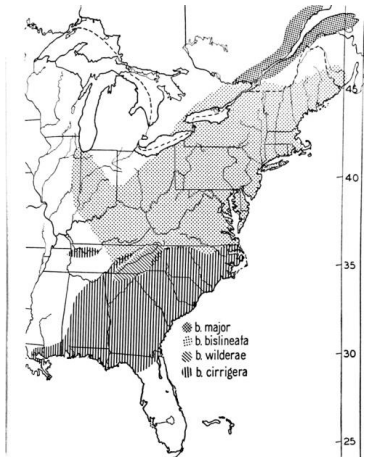


Figure 4: Distribution of the subspecies of *Eurycea bislineata* (Petranka, 1998).

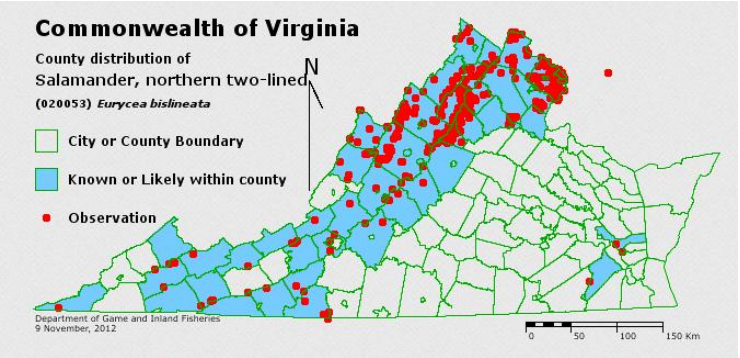


Figure 5: Distribution of the Northern two-lined salamander in the state of Virginia (Virginia Herpetological Society, 2015)

Northern two-lined salamanders are small and slender, with a greenish yellow or orange dorsum that is flanked on either side with a dark brown or black stripe. In between the stripes are dark brown or black spots or blotches. The venter is yellowish and their long tails are laterally compressed. They have 15 or 16 costal grooves and they are not sexually dimorphic in size, measuring between 6.3-12.1 cm overall length (Petranka, 1998; Virginia Herpetological Society, 2015). Individuals caught in this study measured between 4-9.9 cm OAL. Some individuals had partially missing tails, which significantly lowered their OAL. The average length of the surveyed individuals was 7.6 cm.

Hatchlings measure on average around 11-14 mm overall length (OAL) and have on average 14 costal grooves. Larval morphology is adapted to stream-living and they are brownish-bronze in color with six to nine pairs of dorsolateral spots (see

Figure 6). Hatchlings closely resemble *Desmognathus* hatchlings, but have a more squared snout and reddish gills which contain two series of long, slender rami (Eaton, 1956). The venter is light in color with numerous iridophores, which are light-reflecting cells that produce iridescent colors upon illumination by white light (Sutherland, Mäthger, Hanlon, Urbas, & Stone, 2008).

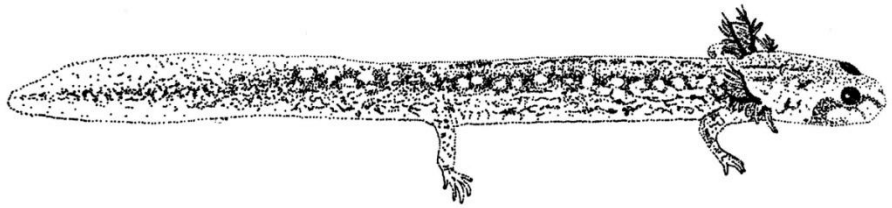


Figure 6: Stream adapted larval morphology of Eurycea (Petranka, 1998)

Adults are predominantly aquatic. They inhabit the margins of small rocky streams, seepages, and springs and hide beneath rocks, logs, and leaves (Bartlett & Bartlett, 2006). Their breeding season is likely between September and May, which is marked by the seasonal presence of secondary sexual characteristics in males during that time frame. Male *E.bislineata*, like several other *Plethodontid* salamanders, develop a mental hedonic gland (a glandular patch on the chin), seasonally enlarged premaxillary teeth, and nasal cirri, (see Figure 7), which are instrumental during the courtship ritual. The mental hedonic gland

releases secretions that act as stimulants for the females, the nasal cirri are thought to aid chemoreception. The protruding teeth are used to scrape the skin of the female during courtship and allow the secretions of the mental hedonic gland to enter the female's circulation (Sever, 1976). Breeding begins in late winter or early spring. In Virginia eggs are laid from late January to mid-April (Wood, 1953). Females typically guard their eggs, which they attach to the underside of submerged rocks. Each female can lay up to 30 eggs. The incubation period of the eggs ranges between 4-10 weeks, depending on water temperatures.

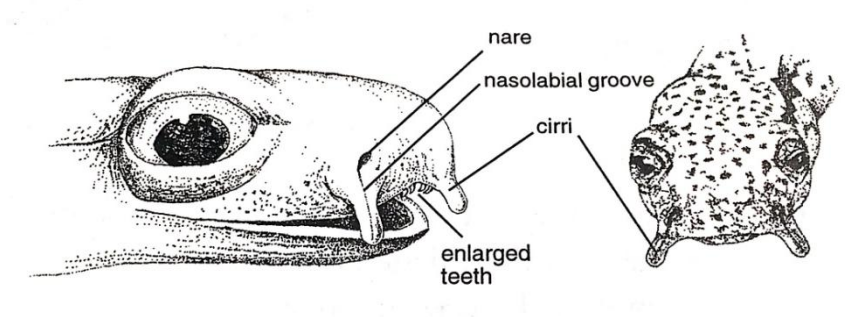


Figure 7: Examples of secondary sexual characteristics of *Eurycea bislineata* (Petranka, 1998)

Larvae inhabit beds of leaves in shallow to moderately deep streams and springs (Bartlett & Bartlett, 2006). They hatch in spring to early summer and may stay in the larval stage for 23-25 months before they metamorphose (Bruce, 1982). Larvae

are found in slow-moving pools within the streams. They are benthic feeders and are therefore mostly found prowling near the bottom (Petranka, 1998). *E. bislineata* is a fairly common species. However, it is sensitive to intensive land clearing, stream pollution, and siltation. It is mostly absent from urban areas and disturbed landscapes and soils with low pH (Petranka, 1998; Wyman & Jancola, 1992). Like most North American members of the *Plethodontidae*, *E. bislineata* have 28 chromosomes. Their genome size is relatively large, with 41.5 pg, or 40 billion bp (Olmo & Morescalchi, 1975).

3. Motivation

Due to the current biodiversity crisis, monitoring species is of utmost importance because it can help wildlife managers identify changes in the environment. Information on the current and past distribution of species is required to understand community dynamics, to limit the spread of invasive species, to protect rare species, or to mitigate consequences of range shifts. The exact knowledge of species distribution is therefore a key component of conservation biology (Hurlbert & Jetz, 2007). This is not only important for currently endangered species, but for other species as they may become endangered in the future. Monitoring and the creation of species inventories can help establish a baseline of species distribution and numbers that can help to see changes in populations more quickly (Dejean et al., 2011). More and more land managers and conservation practitioners are therefore turning to holistic, adaptive management and structured decision modeling (Allen, Fontaine, Pope,

& Garmestani, 2011). To get the data which is necessary for informed decision-making, extensive field work is required to obtain precise information.

However, species detection can be extremely challenging, time consuming and expensive, especially when the species are hard to detect at specific life stages, at very low population densities, or due to their secretive nature, small size, and habitat selection (Mackenzie, Nichols, Sutton, Kawanishi, & Bailey, 2005). Many aquatic and semi-aquatic salamander species, such as *E. bislineata*, are also difficult to identify in the larval stage, making it especially difficult to correctly classify them in the field. Additionally, not all parts of the habitat are always accessible because of difficult terrain or land ownership (Nickerson & Krysko, 2003). Furthermore, the impact of some survey methods, such as rock turning, may be counterproductive to conservation efforts, as they may disturb and possibly injure the target species and other species.

It is therefore a necessity for conservationists to develop surveying methods that are more efficient and informative. One of the most recent advances in surveying vertebrates is the eDNA approach, a genetic technique for the detection of aquatic species.

This thesis describes the development of a molecular protocol that allows the detection of Northern two-lined salamanders (*Eurycea bislineata*), a small streamside salamander, in its natural stream habitat. This study is based on a comparison of a rock-turning survey, an experiment with controlled environmental variables in holding containers, and directly

sampling in the stream. This thesis is intended to be a reference and resource for the detection of vertebrates, specifically streamside salamanders, in stream water in the Shenandoah watershed by means of molecular methods.

4. The eDNA approach

In recent years eDNA has been applied to detect the presence of amphibian species from environmental samples. DNA shed into the environment can be found in water, soil, sediment, or feces (Herder et al., 2014). In previous studies, eDNA has proven to be a sensitive and reliable tool for aquatic vertebrates with low population densities in water samples, including samples from fast flowing streams (Dejean et al., 2011; Ficetola, Miaud, Pompanon, & Taberlet, 2008; Goldberg, Pilliod, Arkle, & Waits, 2011). Detecting species relies on the presence of extracellular DNA present in the environment, stemming from cell lysis, excretions and secretions (Valentini, Pompanon, & Taberlet, 2009).

This method has several advantages over more traditional survey methods, such as rock turning, because it increases the detection probability of the target species, especially when the target species occur in low densities (see Figure 8). In several studies, eDNA surveys were also able to detect species whose presence could not be confirmed using traditional survey methods (Dejean et al., 2012; Jerde, Mahon, Chadderton, & Lodge, 2011; Pilliod, Goldberg, Laramie, & Waits, 2013).

Once a protocol has been established, it is easy to take water samples and therefore less time consuming and less costly to detect a species in the environment. eDNA provides for positive species identification, which eliminates the need for an

expert in identification working with the field team. Because larvae are notoriously hard to identify among closely related salamander species, molecular identification is preferable to identification based on morphological features. Water sampling does not suffer from land access, terrain or other access issues as do the traditional surveys methods because water samples may be obtained from easily accessible locations of streams and ponds. A study by (Deiner & Altermatt, 2014) found that DNA was transported over 9 km from its original source. Another advantage of eDNA surveys is that surveying does not disturb the animals, which makes it a preferable method when working with sensitive or endangered species. The fact that the animals do not have to be handled or disturbed also reduces permitting and other regulatory requirements.

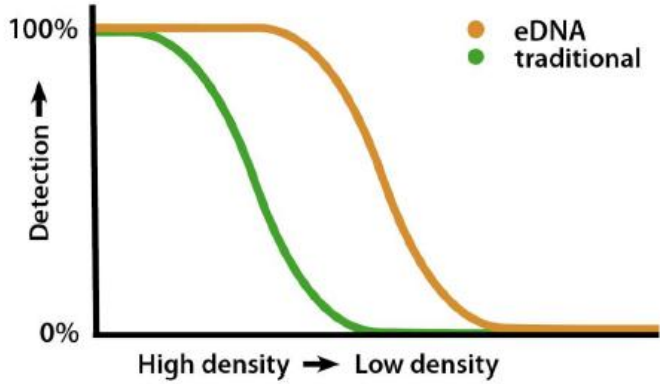


Figure 8: The relationship between detection probability and density for eDNA and traditional survey methods (Bellemain, 2013).

This tool is also advantageous because the DNA remains in the environment even after the animals are no longer physically present. In pond ecosystems the DNA can last from two weeks (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012) up to a month (Dejean et al., 2011), while the persistence of DNA in streams is limited to one hour (Pilliod, Goldberg, Arkle, & Waits, 2014). Once the DNA is released from the organisms, extracellular DNA is degraded by a multitude of environmental factors, such as UV radiation, bacteria, fungi, high temperatures, water, and pH level (Dejean et al., 2011; Herder et al., 2014). In streams, distance from the organism and the rate of water flow influences the detectability of the organisms. In addition, increased amounts of PCR inhibitors may occur in the environment during periods of high leaf deposition (Jane et al., 2015). Due to the variability of these factors, detection probability via eDNA can vary depending on the time of year and the location. Therefore, these factors have to be taken into account when planning an eDNA study. Generally, summer is a good time to sample streams because water flow is moderate and leaf deposition rates are low.

For those reasons, eDNA is ideal for non-invasive surveys and monitoring of secretive aquatic species. So far eDNA has been used to detect a large variety of animal classes present in aquatic environments, such as mammals (Foote et al., 2012; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012), freshwater and saltwater fish (Jerde et al., 2011; Takahara, Minamoto, & Doi, 2013; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012), invertebrates (Deiner & Altermatt, 2014), and reptiles (Piaggio et al., 2014).

In order to use eDNA as a tool for monitoring purposes, assays and species-specific primers have to be developed for each species. Detection probabilities of species in water samples may depend on the size of the species and how aquatic they are, e.g., how much time of their lives they actually spend in the water. So far, eDNA extraction from water samples has only been tested on large, mostly aquatic amphibian species, such as great crested newts (*Triturus cristatus*) (Biggs et al., 2015), American bullfrogs (*Lithobates catesbeianus*) (Dejean et al., 2011), Rocky Mountain tailed frogs (*Ascaphus montanus*), and Idaho giant salamanders (*Dicamptodon aterrimus*) (Goldberg et al., 2011). It was unclear whether the eDNA detection method would work for a small, streamside salamander species, like *E. bislineata*.

5. Hypothesis and outline

The hypothesis for this study was that I can detect a small, streamside salamander, the Northern two-lined salamander (Eurycea bislineata), in a watershed in Virginia with eDNA. To further develop eDNA as a tool for monitoring purposes, I developed a molecular assay for detecting Northern two-lined salamanders (*Eurycea bislineata*) using the eDNA approach. The overarching goal of the study was to detect the species that were observed during the stream surveys in water samples from the same stream with the eDNA approach. These findings lay the foundation for future monitoring efforts, and will help establish a basic understanding of the possibilities and limits of the eDNA method.

CHAPERT TWO: METHODS

The study was carried out in a first order stream at the *Environmental Studies at the Piedmont* and in Warrenton, Fauquier County, VA. The methods are divided into a traditional rock turning survey and an eDNA survey. The rock turning survey was conducted first to confirm the presence of the target species and closely related species. The goal of the study was to detect the species that were observed during the stream surveys in water samples from the same stream. The eDNA methods were divided into sampling of water from controlled environments and directly from the stream. Additionally, two sediment samples were taken and swabs of the skin of salamanders that were caught served as positive controls. The surveys were carried out under Virginia state permit # 051113.

1. Rock turning survey

The rock turning survey was conducted on two different occasions in the fall of 2014. I confirmed the presence of *E. bislineata* by using a traditional rock turning survey (also called stream side transect survey) that uses cover object searches, employing the National Capital Region Network's Amphibian Monitoring Protocol (Bailey, Campbell Grant, & Mattfeld, 2007). The surveys were conducted on two paired transects, which started at the source of the stream. The sets of transects

were 15 meters long, with a space of 15 meters in between. A 100-meter space was located between the first and the second set of transects.

To detect the animals, two to three surveyors concurrently conducted cover object visual encounter surveys in the stream channel and on both stream banks. Each transect was searched twice to help ensure that no salamanders were missed the first time. Another surveyor recorded where the animals were found and measured them. This stream survey was conducted twice over the period of a month to ensure a better coverage. For details and results see Table 3 and

Table 4 in the appendix.

2. Molecular methods

The molecular methods consisted of sampling and filtering the water, taking skin swabs, DNA extraction, primer choice and design, primer and protocol validation, fingerprinting, cloning, and sequencing of the product.

2.1 Sampling and filtering

The first step was sample collection. Samples were taken on three different occasions (for details see Table 1). The quantity of water filtered for a study depends on the environment that is sampled. Standing waters, such as ponds and lakes, require a much smaller sample sizes (15 mL) (Dejean et al., 2012), compared to stream samples, which should be larger because of the water flow rate (Herder et al., 2014). Generally, larger water samples, multiple sampling locations and a larger number of replicates increase the detection probability of the target species. However, there is a limit to how much water can be filtered depending on the amount of dissolved matter, which will ultimately clog the pores of the filter paper. During the first sampling, which followed the second rock turning survey, nine 500 mL water samples were collected in each sampling location along the transects (no larger water samples were available due to seasonal dryness). The water was filtered directly in the field, using a peristaltic flow-through pump with 250 mL, 0.45 µm single-use funnels (Fisher Scientific Company LLC) based on a protocol by Goldberg et al., 2011. After collection the filters were stored in 2 mL tubes filled with ≥95% ethanol. In addition to that, two 50 mL sediment samples and four skin swabs from two species of salamanders (*Eurycea bislineata* and *Plethodon cinereus*)

were collected. The animals were kept in a zip-lock bag for taking measurements and swabs were taken inside the bag afterwards by using a sterile cotton swab, which was stored in 2 mL tubes filled with ≥95% ethanol until extraction. At the lab, the filters, sediment, and swabs were stored at -20 °C until DNA extraction was done about a month later.

Since the amplification of the first samples could not be performed satisfactorily, the sampling approach was changed and another, intermediate step was added to allow for controlling some of the variables in the sampling design. To that end, 1-L plastic containers with lids were filled with 750 mL of water and a salamander was placed in each of them for a predetermined period of time (0 min, 10 min, 30 min, 1 h, and 2 h). On another sampling occasion four plastic containers were filled with 250 mL of water and a salamander was placed in each of them. An additional two 1-L water samples were taken directly from the stream. All salamanders were released immediately upon completion of measuring and swabbing, which followed the holding period. Skin swabs were taken as a positive control from the skin of a total of nine Northern two-lined salamanders (*Eurycea bislineata*) and one Red back salamander (*Plethodon cinereus*), which were collected over the course of all three sampling occasions. The samples were then immediately taken to the laboratory 40 minutes away, and filtered. Filters with 0.2 µm pore size were only used during the second sampling event because the water passed through it very slowly. For the third sampling event, filters with 0.45 µm pore size were used. Extraction was done immediately after the filtering, or the next day, to ensure minimal degradation

of the DNA. It should be noted that for the second and third sampling event, the filters and swabs were not stored in ethanol, but kept frozen until extraction.

2.2. Extraction

To extract DNA from the water and swab samples, the Fast DNA Extraction kit for Soil ® (MP Biomedicals, LLC) and the DNeasy® Blood & Tissue kit (QIAGEN) were used. Extractions were done using the manufacturer's protocols, using a third to half of the filter papers, 200 µl of sediment, and the whole tip of the swabs (cut directly above the cotton). To limit the degradation of the DNA for the second and third sampling event, the extractions were done immediately after filtering or the next day.

2.3 Primers and primer design

The unlabeled universal 12S rRNA primer set (UNI_12s), which amplifies the mitochondrial 12S rRNA gene, was initially used to determine if there was enough DNA present in the samples. This primer pair was used to find out if DNA extraction was successful. The Fam labeled 12S rRNA primer was then used for fingerprinting, which was done in order to find out if there was more than one species' DNA in the sample. Additionally, the labeled 18S primer set, which amplifies the eukaryotic nuclear18S rRNA gene was used once. This was done to find out about the amount and quality of the genomic DNA in the samples.

The species specific primers for Cytochrome Oxidase I gene were designed to amplify DNA fragments between 107-177 base pairs, called mini-barcodes. The COI gene was chosen because it is a conserved region, yet there is enough variation to be able to distinguish among different species. In addition, there are multiple copies of

mitochondrial DNA present in the cell, in contrast to nuclear DNA, where only one copy per cell is present (Robin & Wong, 1988). Due to the degradation of DNA in environmental samples, short fragments below 150 base pairs are more likely to be amplified from the sample (Meusnier et al., 2008). The three chosen species specific COI primers were then validated following three steps (see Figure 9).

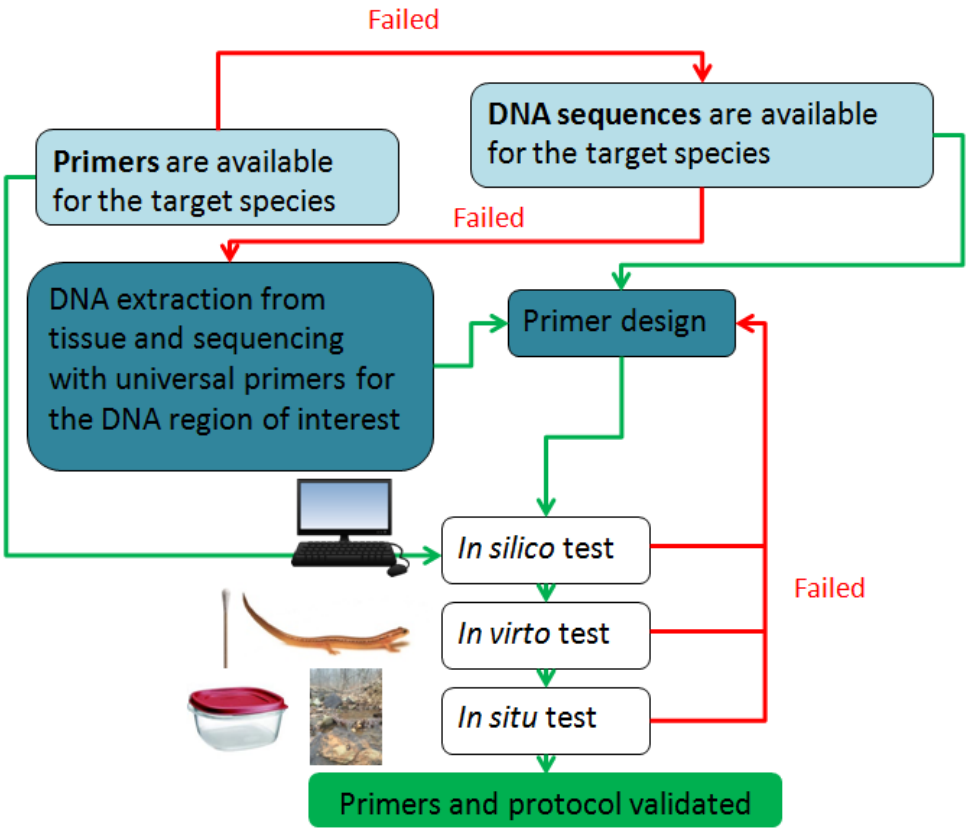


Figure 9: Procedure for testing primer (or probe) reliability, robustness and specificity (Herder et al., 2014).

The first step in the species specific primer design was done using bioinformatics (*In Silico*). To that end, a complete sequence of the mitochondrial genome of *Eurycea bislineata*, was downloaded from the National Center for Biotechnology information

(NCBI) nucleotide database (Mueller, Macey, Jaekel, Wake, & Boore, 2010) and loaded into the program Geneious, version R8 (Biomatters Limited), in order to determine possible sequences for primers with the primer tool. Then, the primers were compared to all known sequences stored in the NCBI database, using the tool Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). This was done in order to ensure that the primers would not amplify the DNA of other species present in the area. The new markers COI are located throughout the mitochondrial (mt) DNA loop. There are several copies of mtDNA present in a cell, whereas only one copy of nuclear DNA is present in each cell (Robin & Wong, 1988). This ultimately increases the detection probability because there are more copies of the genome per cell present. This ultimately increases the detection probability because there are more copies of the genome per cell present. We designed a total of three species specific primers for the Cytochrome Oxidase I gene. The primers amplify between 107-177 bp (see Table 1).

The second step was to test the primers and PCR protocol on swab samples of the target species and a closely related species, the Red back salamander (*Plethodon cinereus*) (*In Vitro*). An intermediary step, before testing the protocol in the stream was added for the second and third sampling, involved a procedure in which controlled treatment water samples were set up and exposed to salamanders for periods up to 24 hours. Finally, the primers were tested on field samples collected from a stream with known presence of the target species (*In Situ*) (Ficetola et al., 2008).

2.4 Amplification (PCR)

Overall, 37 PCR reactions were performed, with six different primer sets, three dilutions (1:1, 1:5, and 1:50), and four types of samples (skin swabs, sediment, controlled environment water samples, and stream samples) (for details see Table 9). The PCR reaction was performed with about 10ng of DNA in a mix that consisted of DEPC treated water, 1X Rx. buffer, 2.5 mM Mg++ mix,, 0.2 mM dNTPs ,0.5 µm of each forward and reverse primers, 0.01% BSA, 0.5 units of *Taq* Gold polymerase (Life Technologies), in a 20-µl total volume. Negative and positive controls were included for each PCR. Successful PCR amplifications were performed on a 2720 Thermal Cycler (Life Technologies) using the following protocols: an initial denaturation for 11 min at 95°C; then 40 cycles of 95°C for 30 seconds, 50-54ºC (depending on set of primers used), and 72ºC for one minute plus 5 seconds per cycle added and a final extension step at 72°C for 10 min. Amplification products were visualized by electrophoresis on a 1% agarose gel made with 1X TAE buffer and stained with ethidium bromide. Successful PCR products were used in fingerprinting, or cloning and sequencing via Sanger Sequencing.

2.5 Fingerprinting

Fingerprinting was run on a capillary sequencer (ABI 3130xl) with the LH-PCR products with the labeled 12S primers. The PCR product was diluted based on the product strength and added to ILS-600 and HiDi Formammaide mix (made in 1:20 ratio). The final plate was then denatured at 95ºC in a thermal cycler for 3 minutes and quickly cooled in the ethanol bath in the freezer. The fingerprint plate was then run on the ABI

capillary machine. After the fingerprinting analysis, data was analyzed with the Gene mapper v4.1 software.

2.6 Cloning

Cloning was done for four samples from PCR #31 using TOPO® TA Cloning® Kit (Life Technologies) according to the manufacturer's protocol.

2.7 Sequencing

Sequencing was done for samples from PCR # 11, 35, 36, and 37. After PCR was performed and the product exhibited bright bands in the expected size, the product was purified for sequencing using Agencourt AMPure XP (Beckman Coulter, Inc.) according to the manufacturer's protocol. The purified product was analyzed on a 1% agarose gel in 1xTAE and stained with ethidium bromide in order to visualize DNA bands.

If bands were visible on the gel, the product was sequenced via Sanger sequencing. The sequencing was done according to manufacturer's protocol. The sequences were then compared to all known sequences stored in the NCBI database, using the tool BLAST (Altschul et al., 1997).

CHAPTER THREE: RESULTS

1. Rock turning survey

During the rock turning survey, two species of salamanders were encountered, the Northern two-lined salamander (*Eurycea bislineata*) and the fully terrestrial Red back salamander (*Plethodon cinereus*). Only *E. bislineata* is a stream species, *P. cinereus* is a fully terrestrial species and was encountered only at the edge of the stream. Several other species of amphibians were also observed: green frog (*Lithobates clamitans*), pickerel frog (*Lithobates palustris*), American bullfrog (*Lithobates catesbeianus*), and spring peeper (*Pseudacris crucifer*).

The first survey took 3 hours and 51 minutes. The second survey took 4 hours and 34 minutes.. A total of 17 adult and 55 larval salamanders were caught during the first survey, and 33 adults and 36 larval salamanders during the second survey. The adults were measured in zip-lock bags. They were released where they were found at the end of each survey.

2. Fingerprinting

The fingerprinting on the 12S rRNA gene was done in order to confirm if the samples amplified a single species or multiple species. The results showed that there were multiple peaks, which means that the samples contained the DNA of a multitude of species and not just a single species. An overview of the results can be found in the Appendix in Figure 12.

3. Cloning

The cloning of the PCR product was done because of uncertainty of what species' DNA was amplified in the PCR. Cloning was only done to confirm that only the DNA of one species, Northern two-lined salamander, was amplified. The cloning, however, did not produce any white colonies that could be sequenced. The lack of white colonies means that either no DNA fragments or too small fragments got inserted into the clones.

4. Sanger sequencing

Sanger sequencing was performed on two occasions, for PCR # 11 and PCR # 35, 36, and 37. For PCR # 11, clean sequences could only be retrieved for samples that came from the swabs. Sequences of the target species were also successfully retrieved from the second sequencing experiment, for PCR # 35 and 37. The samples that were used for this were collected during the third sampling event and consisted of four 24 hour controls, two stream water samples and two swabs. All samples collected on that sampling occasion worked with two sets of primers. The primers that successfully amplified the salamander sequences were COI 78F, 254R (177 bp), and COI 329F, 435R (107bp). For an example of successful amplification visualized with gel electrophoresis on a 1% gel, see Figure 10.

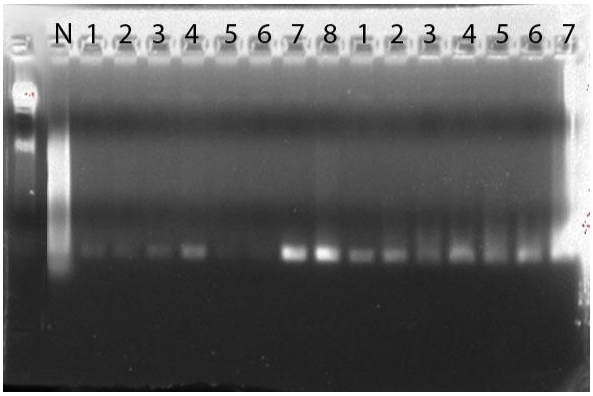


Figure 10: COI 329 F, 435 R primers were able to amplify salamander DNA found in controlled environment (1-4) stream (5,6) , and swab samples (7,8).

The sequences obtained are displayed in Table 10 in the appendix. The BLAST results revealed that all sequences except for one, Sample5_009 with the COI _ 254R Primer, had Northern two-lined salamander as the highest match. The sequence that was retrieved from Sample5_009 did not have such a match. It only showed a single match of 86% with *Methanococcus maripaludis*, a species of methanogen. An example of what the BLAST results looked like can be seen below in Figure 11.

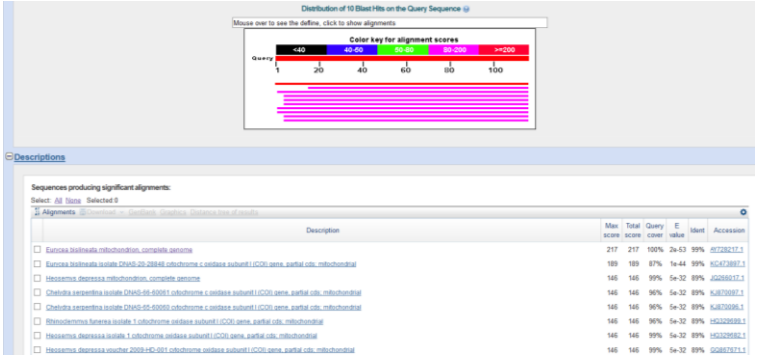


Figure 11: BLAST results for sample Sample1_COI_254R_001.

CHAPTER FOUR: DISCUSSION

Environmental changes can impact the abundance and distribution of biodiversity, therefore monitoring is fundamental for understanding presence or absence of species and then reducing negative impacts on wildlife. The sensitivity and reliability of monitoring techniques have important implications for management. The ability to create accurate species inventories non-invasively, quickly, at low cost, and with high accuracy is an absolute necessity for conservation managers. Importantly, it could allow them to intervene when biodiversity is in decline.

The results from this study confirm the applicability for eDNA surveys to detect the presence of a small streamside salamander, such as the Northern two-lined salamander in a small, fast flowing stream. Northern two-lined salamander DNA was amplified and sequenced successfully from four controlled environmental samples, two stream samples and two swab samples, which were all samples that were taken during the third sampling event. The samples from the first and second sampling event of stream samples, sediment samples, and the controlled environment samples did not result in successful amplifications. This was likely caused by storing the samples in ethanol and not extracting the DNA immediately after the first sampling event. For the second sampling event, the salamander in the controlled samples were likely not given enough time to shed cells into the water. Two of the primers that were developed were able to

amplify the DNA of the target species, and they were confirmed by sequencing and comparing the PCR products to known sequences in Genbank database. For further testing the broad applicability of the primers and protocol, other streams with known presence and absence of the target species and closely related stream salamander species should be performed. During this study, it became apparent that it is important to extract DNA as soon as possible after the samples are taken. The extraction of samples was also more successful when the filters were kept frozen and not stored in ethanol. The time it takes to transport to the lab and store samples is therefore an important consideration when designing an eDNA study.

Overall, it is necessary to develop protocols for other species to be able to use the eDNA approach for conservation purposes because it is rarely a single species' presence that needs to be detected. This can be done by developing additional primers for other species or by using a multispecific approach, called eDNA metabarcoding. eDNA metabarcoding has been done for several groups, such as detection of vertebrate DNA in soil (Andersen et al., 2012) and of marine fish DNA in sediment (Thomsen, Kielgast, Iversen, Møller, et al., 2012). However, it is extremely difficult to find short, universal primers that are versatile enough to cover a whole class of species, yet specific enough to identify individual species. Nevertheless, there is a way to avoid that problem, as (Thomsen, Kielgast, Iversen, Møller, et al., 2012) demonstrated, by using several sets of species-specific primers on a next-generation platform.

While presence/absence data is valuable information for wildlife managers, abundance is a fundamental biological factor that plays a major role for the conservation

of endangered species. It is also often difficult to accurately estimate abundance. While it is possible to estimate biomass with the eDNA approach, by using qPCR, there still remain a large number of uncertainties with that approach (Minamoto, Yamanaka, Takahara, Honjo, & Kawabata, 2012). The relationship between the densities of target species and amount of eDNA has been found to have a strong correlation in aquaria, but degradation of DNA under natural conditions makes it difficult to get accurate density estimates. Nevertheless, qPCR for eDNA surveys can be used as an indicator for species densities and to provide a comparison of species densities in one location between years (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012).

Furthermore, eDNA surveys cannot answer all the questions that wildlife managers have. The data gathered from eDNA surveys does not give any indication about demographics, age distribution, gender distribution, health, or amount of inbreeding. In addition, the life history of the target species has to be considered when estimating biomass. Ectothermic species may, for example, be less active when temperatures are low, due to their reliance on external heat for maintaining a high activity level. This means that they will have a lower metabolism when it is cold outside, and ultimately shed fewer cells into the environment. Therefore, there is a need to explore the applicability of biomass estimations across taxa and seasons.

Nevertheless, eDNA is a promising tool that can help revolutionize species monitoring and conservation. The eDNA approach also has the potential of being established as a citizen science monitoring program. Such a program was demonstrated in the United Kingdom with a national citizen science-based monitoring trial for the great

crested newt (*Triturus cristatus*) (Biggs et al., 2015). Because species identification expertise is not needed and because of the ease of taking samples, virtually anybody can take samples and send them to a laboratory for analysis.

APPENDIX

Table 1: Selected primer pairs.

Forward primer	Tm (ºC)	Reverse primer	Tm (ºC)	Type	Product length	Primer sequence
UNI-12sF	54.4	UNI-12sR	60	Universal	~ 500 bp	F: AACTGGGATTAGATACCCCACTAT R: GAGGGTGACGGGCGGTGTGT
Fam_12sF	54.4	UNI-12sR	60	Labled, universal	~ 500 bp	F: AACTGGGATTAGATACCCCACTAT R: GAGGGTGACGGGCGGTGTGT
Fam_18sF	50	18s Rat	67	Labled, universal	~ 400 bp	F: CTGGTTGATCCTGCCAGT R:TTTCTCAGGCTCCCTCTCCGGAATCGAACCCT
COI_329F	53.9	COI_435R	55.9	Species specific	107 bp	F: TACTTCTCCTCGCCTCGTCA R: TAGGTCTACAGAGGCTCCCG
COI_1299F	53.8	COI_1436R	51.8	Species specific	138 bp	F: CGCAGGTATACCACGACGTT R: TTTGAGGCAAAGGCYCCCA
COI_78F	55.9	COI_254R	53.8	Species specific	177 bp	F: CGGCATAGTGGGTACAGCTA R: AGTGGCAGCAGTCAGTTAC

Table 2: Sample overview from three different sampling events.

Sample #	Sample name	Type	Sample size	Preservation	Extraction	Corresponds with sample #
1.	1	Stream water	0.5 L	ethanol	DNeasy	
2.	2	Stream water	0.5 L	ethanol	DNeasy	
3.	3	Stream water	0.5 L	ethanol	DNeasy	
4.	4	Stream water	0.5 L	ethanol	DNeasy	
5.	5	Stream water	0.5 L	ethanol	DNeasy	
6.	6	Stream water	0.5 L	ethanol	DNeasy	
7.	7	Stream water	0.5 L	ethanol	DNeasy	
8.	8	Stream water	0.5 L	ethanol	DNeasy	
9.	9	Stream water	0.5 L	ethanol	DNeasy	
10.	10	Sediment	0.05 L	ice	Mp Bio (soil)	
11.	11	Sediment	0.05 L	ice	Mp Bio (soil)	
12.	12	Swab	-	ice	DNeasy	
13.	13	Swab	-	ice	DNeasy	
14.	14	Swab (*P. cinereus*)	-	ice	DNeasy	
15.	15	Swab	-	ice	DNeasy	
16.	1.2	Stream water	1 L	ice	DNeasy, Mp Bio	
17.	2.2	Aquarium- 10 min	0.4 L	ice	DNeasy, Mp Bio	15.
18.	3.2	Aquarium- 30 min	0.4 L	ice	DNeasy, Mp Bio	16.
19.	4.2	Aquarium- 1h	0.4 L	ice	DNeasy, Mp	17.

					Bio	
20.	5.2	Aquarium- 2 h	0.4 L	ice	DNeasy, Mp Bio	18.
21.	6.2	Swab	-	ice	DNeasy, Mp Bio	
22.	7.2	Swab	-	ice	DNeasy, Mp Bio	
23.	8.2	Swab	-	ice	DNeasy, Mp Bio	
24.	9.2	Swab	-	ice	DNeasy, Mp Bio	
25.	1.3	Aquarium- 24 h	0.25 L	ice	Mp Bio	
26.	2.3	Aquarium- 24 h	0.25 L	ice	Mp Bio	
27.	3.3	Aquarium- 24 h	0.25 L	ice	Mp Bio	
28.	4.3	Aquarium- 24 h	0.25 L	ice	Mp Bio	
29.	5.3	Stream water	1 L	ice	Mp Bio	
30.	6.3	Stream water	1 L	ice	Mp Bio	
31.	7.3	Swab	-	ice	Mp Bio	23.
32.	8.3	Swab	-	ice	Mp Bio	26.

Table 3: Survey details from the first rock turning survey for transect 1 and 2.

Sampling Sheet stream survey	**Survey**		0	**Time start**		11:10	**Time end**		01:14
Environmental Studies on the Piedmont	**Surveyer**		Sarah, Matt E., Mimi, Emily				**Time total**		01:04
TOP N: 38°47' 12.7" W: 77°48'40.8"	**Weather**		2.5	**Transects**		1 (upper),2 (lower)			
BOTTOM: N: 38°47'12.0" W: 77°48'39.1"	**Rainfall**		Yes	**Temp. Air**		25° C	**Temp. Water**		20° C
Species	**SV**	**TL**	**OAL**	**HW**	**HL**	**age**	**transect**	**remarks**	
Northern two-lined salamander (*E. bislineata*)	41	49	90	7	9	adult	1 (a)	11m	
Northern two-lined salamander (*E. bislineata*)	39	38	77	6	9	adult	1 (a)	11m	
Northern two-lined salamander (*E. bislineata*)	41	58	99	7	12	adult	1 (a)	11m	
Northern two-lined salamander (*E. bislineata*)	28	33	61	5	6	adult	1 (a)	11m	
Northern two-lined salamander (*E. bislineata*)	x	x	x	x	x	adult	1 (a)	4m, got away	
TOTAL: 5 adults									

Description: forested site dominated by hardwood (pawpaw, maple, poplar, oak). Ground vegetation consists of bushes, grasses and ferns. Stream has large flat rocks, pebbles, and clay and is on average two meters wide. Parts of it are dried up, but some small puddles remain.

Other species: green frog (*Lithobates clamitans*), crayfish (*Cambarus sp*.)

Table 4: Sampling details from the first rock turning survey for transect 3 and 4.

Sampling Sheet stream survey	**Survey**		1	**Time start**		03:43	**Time end**	06:30
Environmental Studies on the Piedmont	**Surveyer**		Sarah, Mimi, Matt		**Time total**		02:47	
TOP N: 38°47' 11.3" W: 77°48'35.9"	**Weather**		1	**Transects**		3 (upper), 4 (lower)		
BOTTOM: N: 38°47'10.4" W: 77°48'35.1"	**Rainfall**		Yes	**Temp. Air**		22.5° C	**Temp. Water**	20.5° C
Species	**SV**	**TL**	**OAL**	**HW**	**HL**	**age**	**transect**	**remarks**
Northern two-lined salamander (*E. bislineata*)	40	24	64	6	8	adult	4 (a)	35 m
Northern two-lined salamander (*E. bislineata*)	39	43	82	6	8	adult	4 (a)	39 m
Northern two-lined salamander (*E. bislineata*)	30	39	69	5	7	adult	4 (a)	35 m
Northern two-lined salamander (*E. bislineata*)	32	36	68	5	7	adult	4 (a)	35 m
Northern two-lined salamander (*E. bislineata*)	35	49	84	6	9	adult	3 (a)	2 m
Northern two-lined salamander (*E. bislineata*)	32	43	75	7	8	adult	3 (a)	3m
Northern two-lined salamander (*E. bislineata*)	39	47	86	6	7	adult	3 (a)	5 m
Northern two-lined salamander (*E. bislineata*)	35	29	64	6	8	adult	3 (a)	15 m, short tail
Northern two-lined salamander (*E. bislineata*)	28	31	59	6	9	adult	4 (b)	35 m
Northern two-lined salamander (*E. bislineata*)	26	30	56	5	7	adult	4 (b)	39 m
Northern two-lined salamander (*E. bislineata*)	41	34	75	5	8	adult	4 (b)	35 m, short tail
Northern two-lined salamander (*E. bislineata*)	39	58	97	6	8	adult	3 (b)	5 m
TOTAl: 12 adults, 55 larvae								

Description: forested site dominated by hardwood (pawpaw, maple, poplar, oak). Ground vegetation consists of bushes, grasses and ferns. Stream has large flat rocks, pebbles, and clay and is on average two meters wide. Parts of it are dried up, larger stretches of running water and pools are present.

Other species: green frog (*Lithobates clamitans*), crayfish (*Cambarus sp.*), pickerel frog (*Lithobates palustris*), white footed mouse (*Peromyscus leucopus*)

Table 5: Details from the second rock turning survey and first eDNA sampling for transect 1 and 2.

Sampling Sheet stream survey	**Survey**	1	**Time start**	10:48	**Time end**	12:50
Environmental Studies on the Piedmont	**Surveyer**	Sarah, Mimi, Jesi, Chelsie, Lorien			**Time total**	01:58
TOP N: 38°47' 12.7" W: 77°48'40.8"	**Weather**	0	**Transects**	1 (upper),2 (lower)		
BOTTOM: N: 38°47'12.0" W: 77°48'39.1"	**Rainfall**	Yes	**Temp. Air**	22° C	**Temp. Water**	17° C

Species	**SV**	**TL**	**OAL**	**HW**	**HL**	**age**	**transect**	**remarks**
Northern two-lined salamander (*E. bislineata*)	41	54	95	6	8	adult	1 (a)	11 m, Swab 1
Northern two-lined salamander (*E. bislineata*)	39	52	91	6	9	adult	1 (a)	7 m
Northern two-lined salamander (*E. bislineata*)	43	21	64	7	9	adult	1 (a)	6 m, Swab 3
Northern two-lined salamander (*E. bislineata*)	38	47	85	6	8	adult	1 (a)	3 m
Northern two-lined salamander (*E. bislineata*)	37	44	81	6	9	adult	1 (a)	0 m, Swab 2
Northern two-lined salamander (*E. bislineata*)	-	-	-	-	-	adult	1 (a)	3 escaped
Northern two-lined salamander (*E. bislineata*)	39	48	87	6	8	adult	2 (a)	34 m
Northern two-lined salamander (*E. bislineata*)	33	36	69	5	7	adult	2 (a)	32 m
Northern two-lined salamander (*E. bislineata*)	31	27	58	4	8	adult	1 (b)	10 m
Northern two-lined salamander (*E. bislineata*)	31	54	85	4	6	adult	1 (b)	9 m
Northern two-lined salamander (*E. bislineata*)	38	55	93	6	8	adult	1 (b)	5 m
Northern two-lined salamander (*E. bislineata*)	34	49	83	7	9	adult	2 (b)	34 m
Northern two-lined salamander (*E. bislineata*)	39	48	87	4	7	adult	2 (b)	33 m
Red back salamander (*P. cinereus*)	26	22	48	4	6	adult	2 (b)	31 m, Swab 4
TOTAL: 14 adults, 3 larvae								

Description: forested site dominated by hardwood (pawpaw, maple, poplar, oak). Ground vegetation consists of bushes, grasses and ferns. Stream has large flat rocks, pebbles, and clay and is on average two meters wide. Parts of it are dried up, larger stretches of running water and pools are present.

Other species: pickerel frog (*Lithobates palustris*), green frog (*Lithobates clamitans*), bullfrog (*Lithobates catesbeianus*), spring peeper (*Pseudacris crucifer*)

Table 6: Details from the second rock turning survey and first eDNA sampling for transect 3 and 4.

Sampling Sheet stream survey	**Survey/Sampling**	2/1	**Time**	2:55	**Time end**	04:31
Environmental Studies on the	**Surveyer**	Mimi, Jesi, Chelsie		**Time**	02:36	
TOP N: 38°47' 11.3" W:	**Weather**	0		**Transect**	3 (upper), 4 (lower)	
BOTTOM: N: 38°47'10.4" W:	**Rainfall**	No		**Temp.**	24° C	**Temp.** 18° C

Species	**SV**	**TL**	**OAL**		**H**		**HL**	**age**	**transec**	**remarks**
Northern two-lined salamander (*E.*		44	4	89	6	9	adult	3		11 m
Northern two-lined salamander (*E.*		34	4	83	5	7	adult	3		14 m
Northern two-lined salamander (*E.*		33	3	72	5	7	adult	3		6 m
Northern two-lined salamander (*E.*		38	4	86	5	7	adult	3		3 m
Northern two-lined salamander (*E.*		42	3	72	6	8	adult	4		43 m, short tail
Northern two-lined salamander (*E.*		35	2	59	5	7	adult	4		45 m, short tail
Northern two-lined salamander (*E.*		35	4	79	5	7	adult	4		40 m
Northern two-lined salamander (*E.*		28	3	61	4	6	adult	4		39 m
Northern two-lined salamander (*E.*		34	4	74	5	7	adult	4		39 m
Northern two-lined salamander (*E.*		36	4	81	6	7	adult	4		39 m
Northern two-lined salamander (*E.*		32	3	67	5	7	adult	4		38 m
Northern two-lined salamander (*E.*		36	4	77	5	8	adult	4		35 m
Northern two-lined salamander (*E.*		40	5	92	6	8	adult	4		32 m
Northern two-lined salamander (*E.*		31	3	70	5	6	adult	4		30 m
Northern two-lined salamander (*E.*		35	4	83	5	7	adult	3		14 m
Northern two-lined salamander (*E.*		42	4	84	5	8	adult	3		13 m
Northern two-lined salamander (*E.*		34	4	78	5	7	adult	3		4 m
Northern two-lined salamander (*E.*		38	1	56	5	7	adult	3		3 m, no tail
Northern two-lined salamander (*E.*		37	3	69	5	7	adult	4		38 m
Northern two-lined salamander (*E.*		41	4	84	6	7	adult	4		32 m
Northern two-lined salamander (*E.*		35	4	77	5	8	adult	4		33 m
TOTAL: 19 adults, 33 larvae										

Table 7: Details from second eDNA sampling.

Sampling Sheet eDNA	**Sampling**	2	**Time start**	11:26	**Time end**	04:52
Environmental Studies on the Piedmont	**Surveyer**	Sarah, Angelo	**Rainfall**	Yes		
N: 38°47' 09.8" W: 77°48'34.1"	**Weather**	1-2	**Temp. Air**	8.5° C	**Temp. Water**	10.6° C

Species	**SV**	**TL**	**OAL**	**HW**	**HL**	**age**	**held for (min)**	**water (l)**
Northern two-lined salamander (*E. bislineata*)	32	35	67	5	7	adult	10	0.75
Northern two-lined salamander (*E. bislineata*)	32	8	40	5	7	adult	30	0.75
Northern two-lined salamander (*E. bislineata*)	33	29	62	6	6	adult	60	0.75
Northern two-lined salamander (*E. bislineata*)	34	42	76	6	7	adult	120	0.75

Description: forested site dominated by hardwood (pawpaw, maple, poplar, oak). Ground vegetation consists of bushes, grasses and ferns. Stream has large flat rocks, pebbles, and clay and is on average two m wide. Heavy water flow.

Other species: pickerel frog (*Lithobates palustris*), crayfish (*Cambarus sp*.)

Table 8: Details from third eDNA sampling.

Sampling Sheet eDNA	**Sampling**	3	**Time start**	10:03	**Time end**	12:07
Environmental Studies on the Piedmont	**Surveyer**	Sarah, Angelo	**Rainfall**	Yes		
N: 38°47' 09.8" W: 77°48'34.1"	**Weather**	0	**Temp. Air**	15.5° C	**Temp. Water**	12.1° C

Species	**SV**	**TL**	**OAL**	**HW**	**HL**	**age**	**held for (h)**	**water (l)**
Northern two-lined salamander (*E. bislineata*)	30	40	70	5	7	adult	24	0.25
Northern two-lined salamander (*E. bislineata*)	32	32	64	5	7	adult	24	0.25
Northern two-lined salamander (*E. bislineata*)	30	38	68	5	7	adult	24	0.25
Northern two-lined salamander (*E. bislineata*)	35	34	69	5	7	adult	24	0.25

Table 9: Overview of PCR reactions performed

PCR ID	primers	Cycles & temperature	machine	Sampling	Extractions and other procedures
1	12s Uni	35-50	PMG 1	1	DNeasy extraction
2	12s Uni	40-50	PMG 1	1	
3	12s Uni	40-50	PMG 1	1	
4	COI 329 F	50-50	PMG 1	1	
5	12s Uni	50-50	PMG 1	1	
6	12s Uni	50-50	PMG 2	1	
					Fast DNA extraction
7	12s Uni	40	PMG 1	1	
8	12s Uni	40	PMG 2	1	
9	12s Uni	40	PMG 1	1	
10	COI 1299 F 12s Uni	40	PMG 1	1	
11	all COI	40	PMG 1	1	
12	COI 1299 F	40	PMG 1	1	
13	COI 329 F	40	PMG 1	1	
14	COI 78 F	40	PMG 2	1	
15	all COI	40	PMG 1	1	
16	COI 78 F	45	PMG 1	1	
					Sanger sequencing of PCR 11
17	COI 78 F	40	PMG 1	1	
18	COI 78 F	40	PMG 1	1	Fast DNA extraction
19	12s Uni	40	PMG 2	1	
20	COI 78 F	40	PMG 1	1	
21	18s Fam	40	PMG 2	1	

22	COI 78 F & 12s Uni	40	PMG 2	1	
23	12s Fam	40	ABI 421	1	
24	12s Fam	40	ABI 421	1	
					Fingerprinting of samples from PCR 23&24
25	12s Fam, 12s Uni	40	PMG 1	1	
26	COI 329 F	40	PMG 2	1	
27	COI 329 F	40	PMG 1	1	
28	12s Fam	40	PMG 1	2	
29	COI 329 F	40	PMG 2	2	DNeasy extraction
30	12s Fam	50	PMG 1	2	
31	COI 329 F	45	PMG 2	2	Fast DNA extraction
32	COI 329 F	50	PMG 2	2	
					Cloning of samples from PCR 32
33	COI 78 F	50	PMG 1	2	
34	COI 1299 F	50	PMG 2	2	
35	COI 78 F	50	PMG 3	3	
36	COI 1299 F	45	PMG 1	3	
37	COI 329 F	45	PMG 2	3	
					Sequencing of samples from PCR 35-37

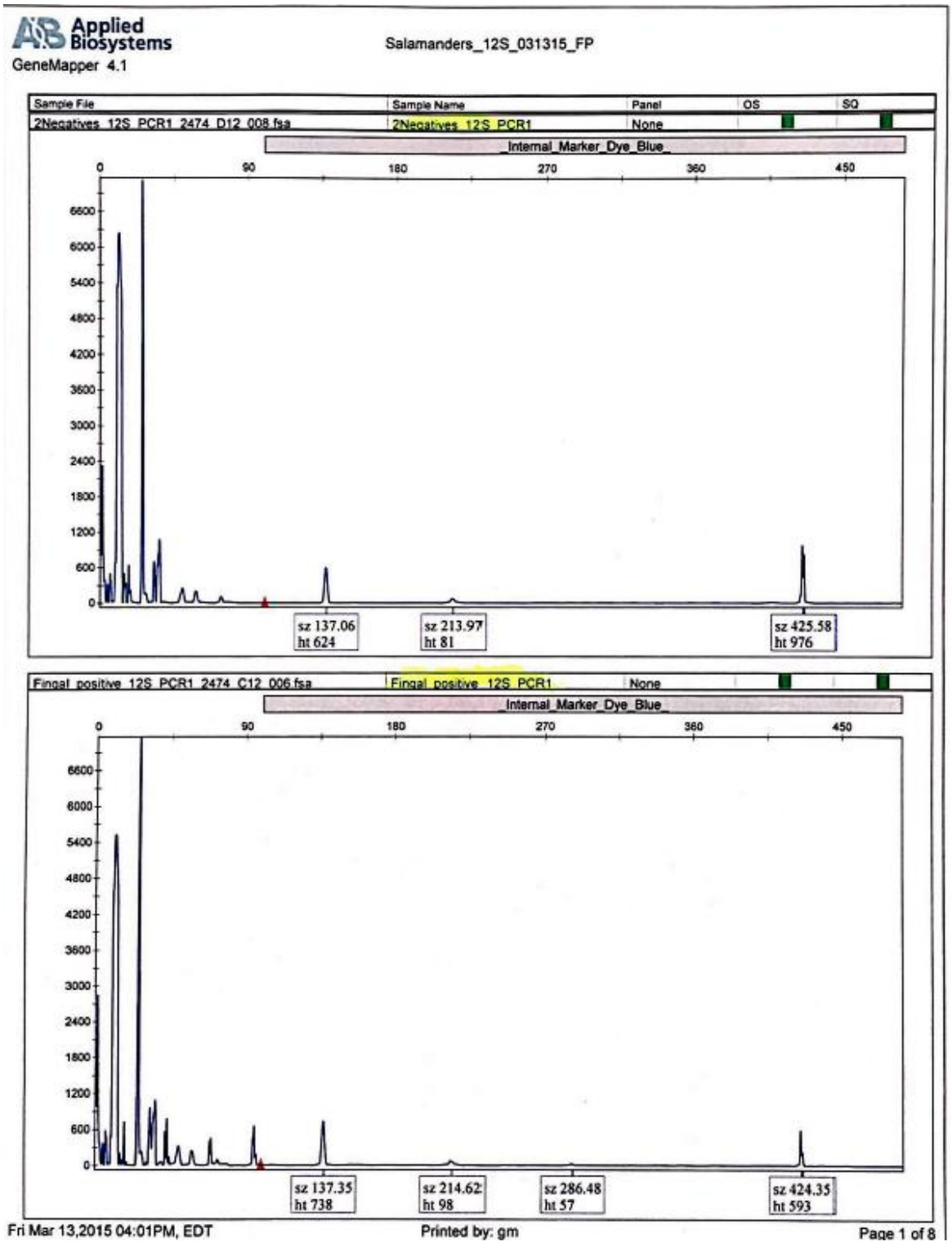
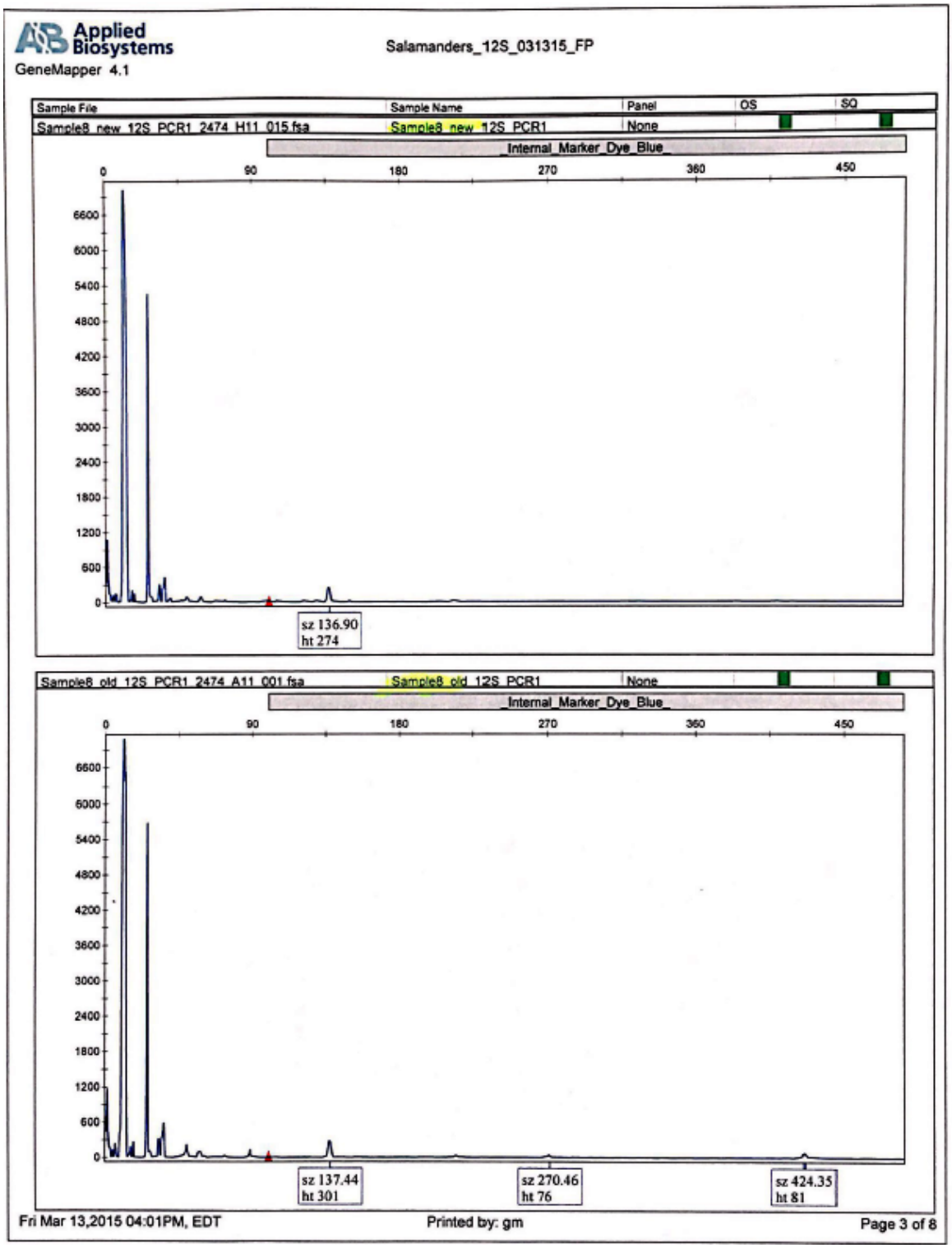
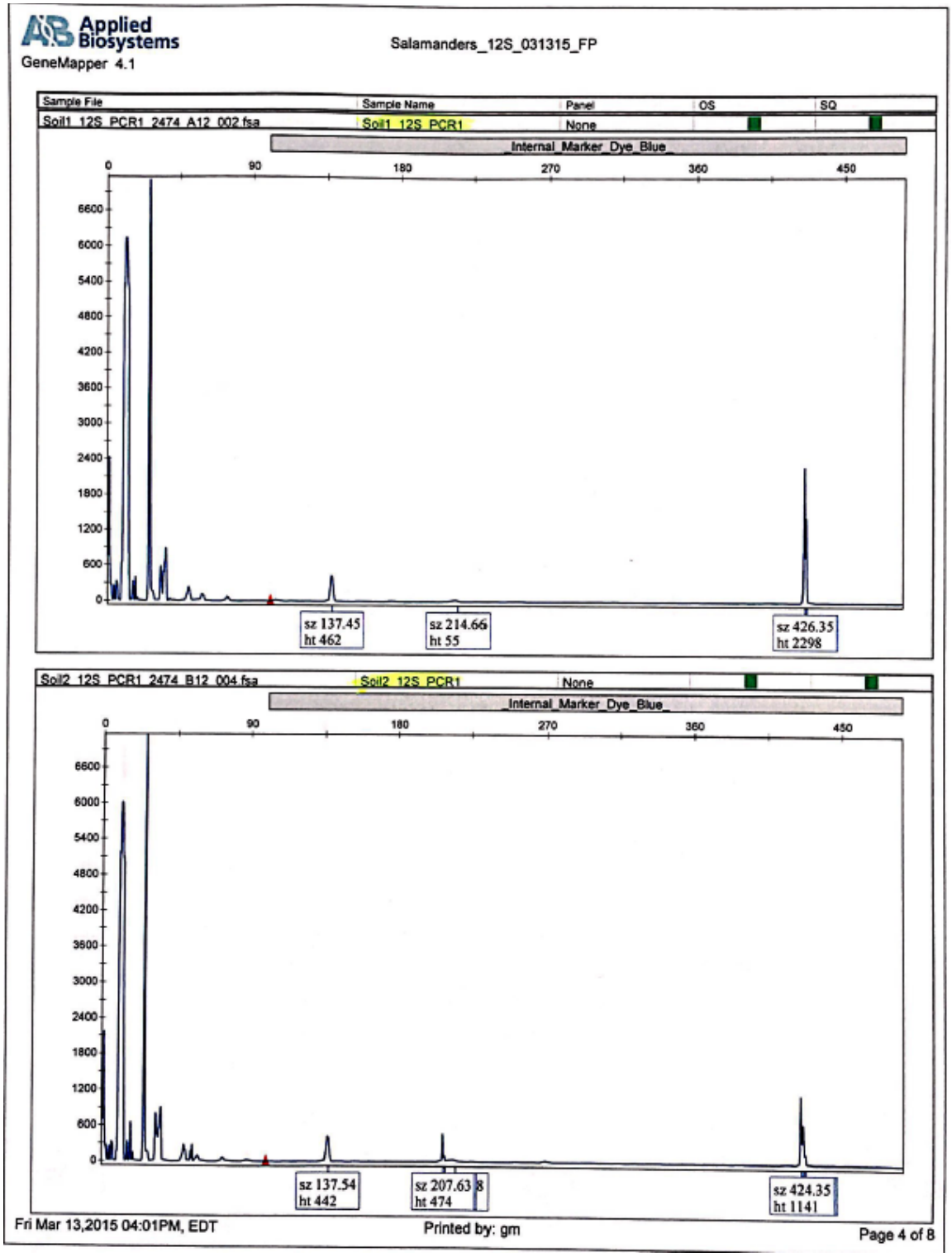
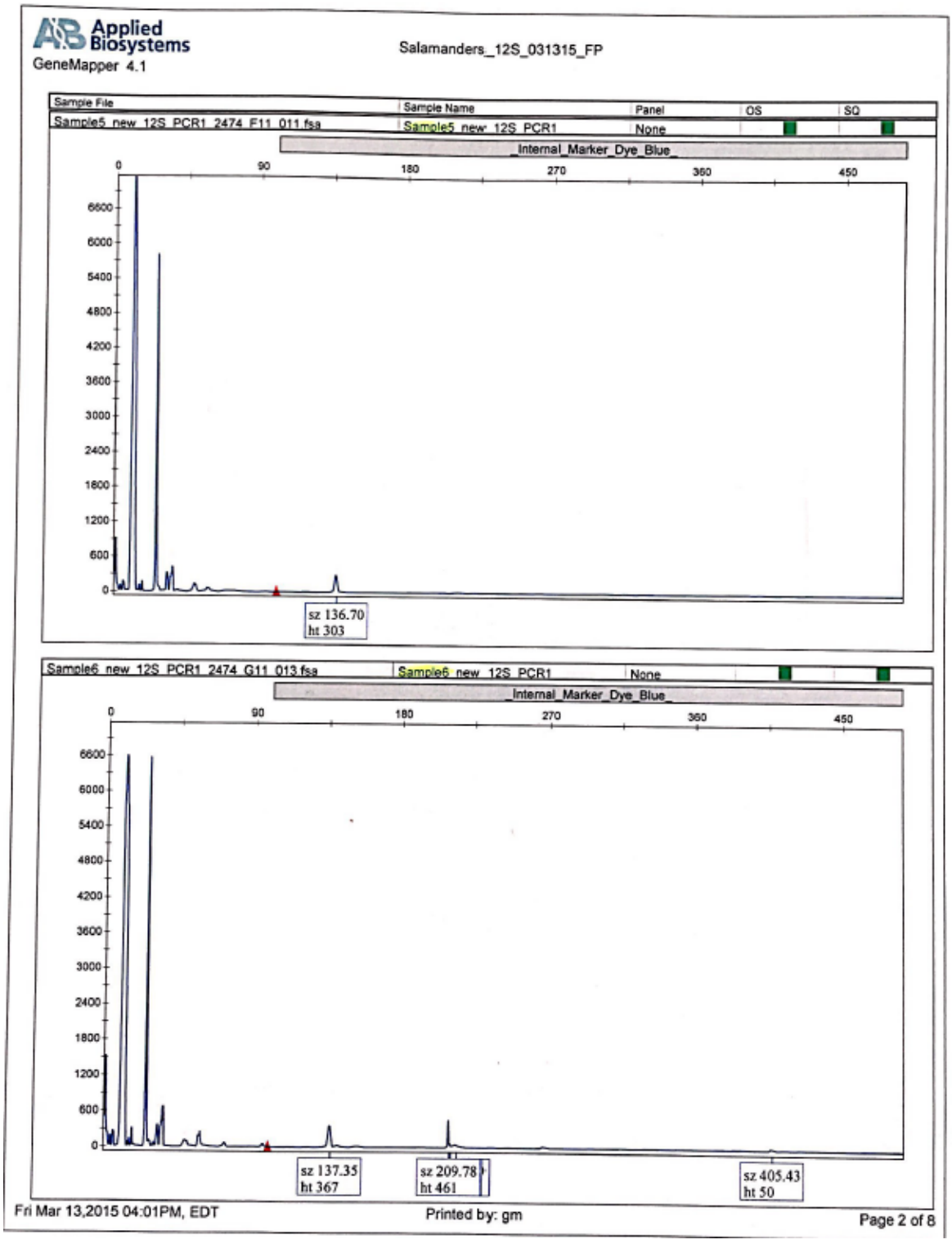
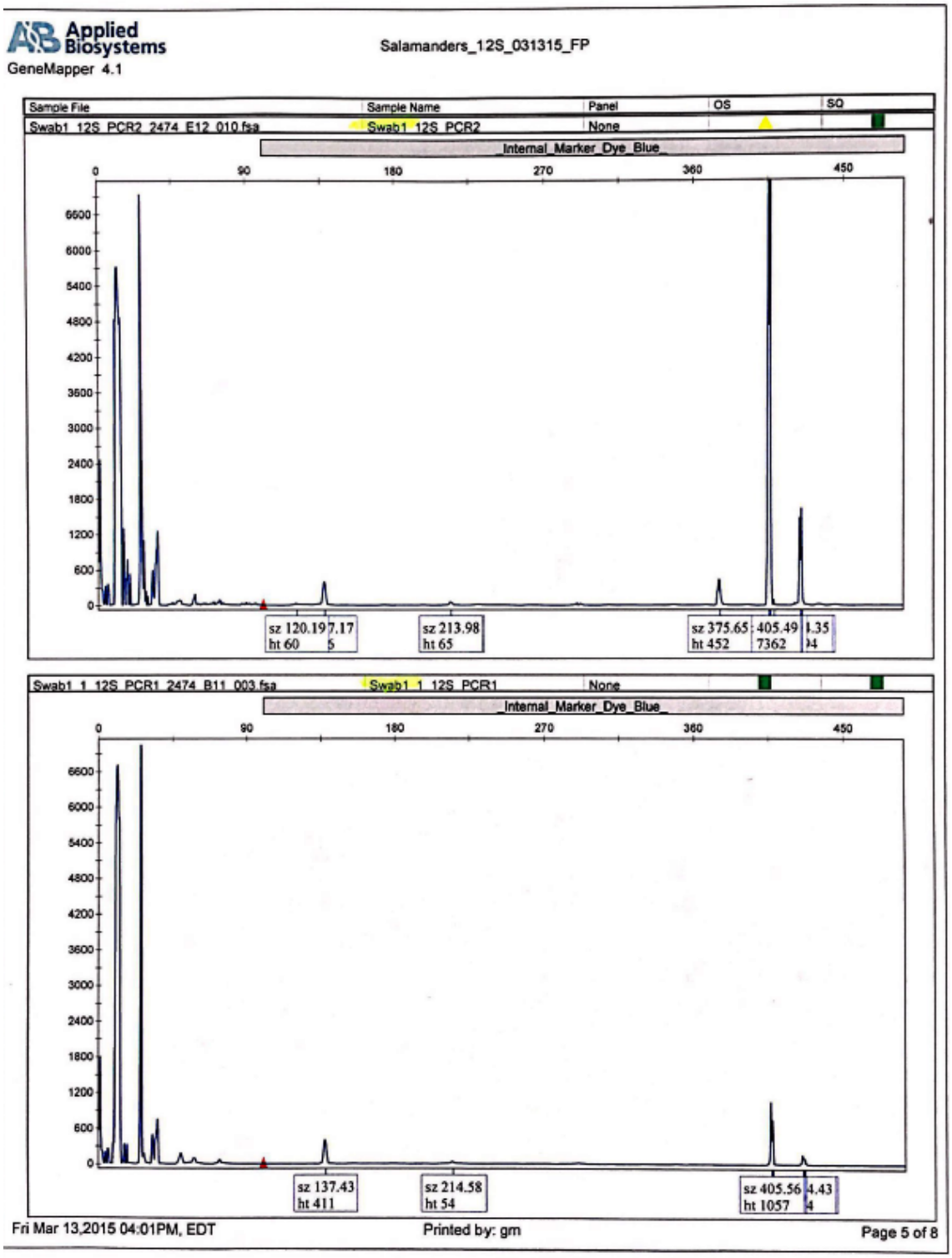


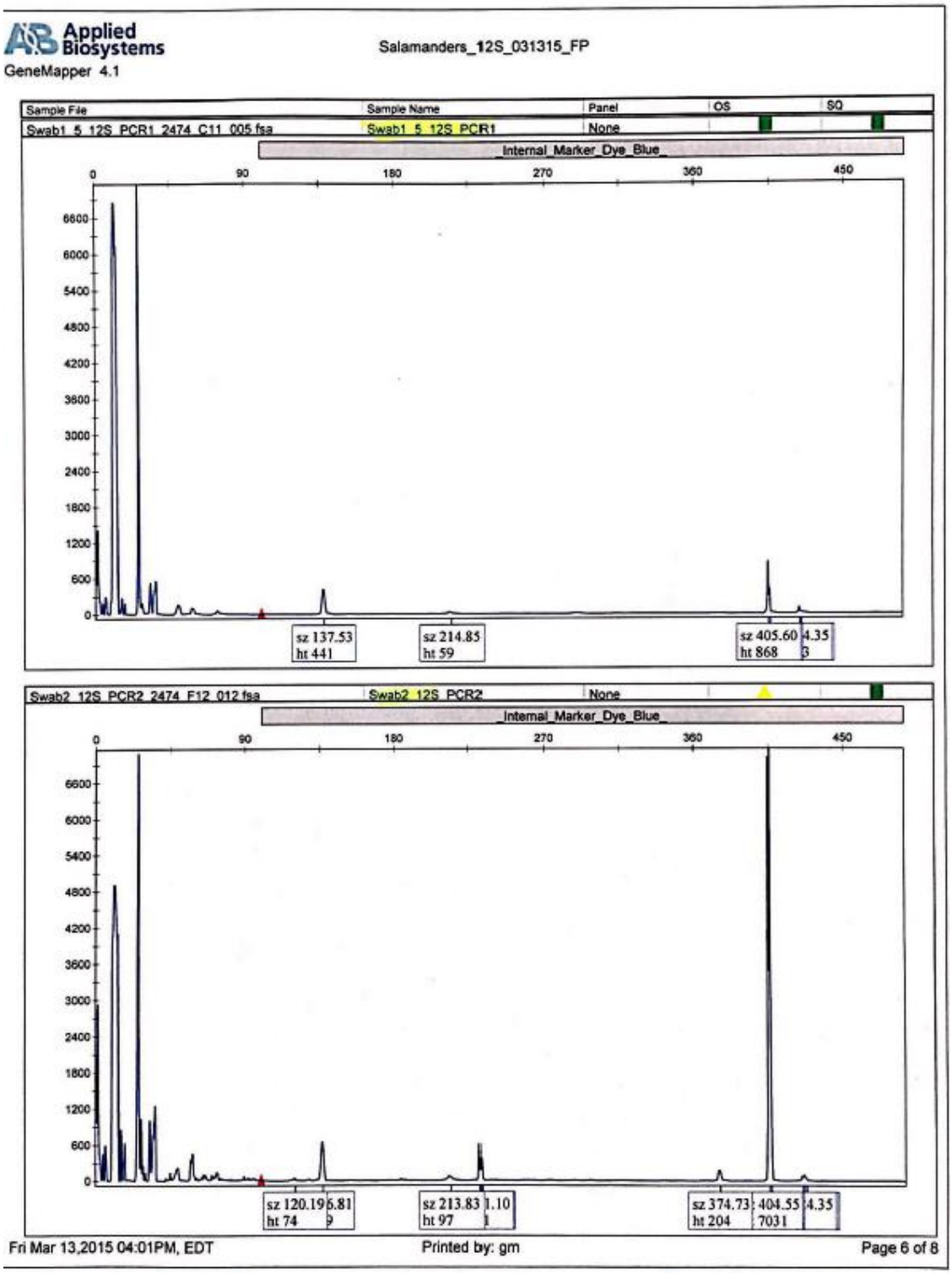
Figure 12: Results from Fingerprinting of samples from PCR # 23 and 24.

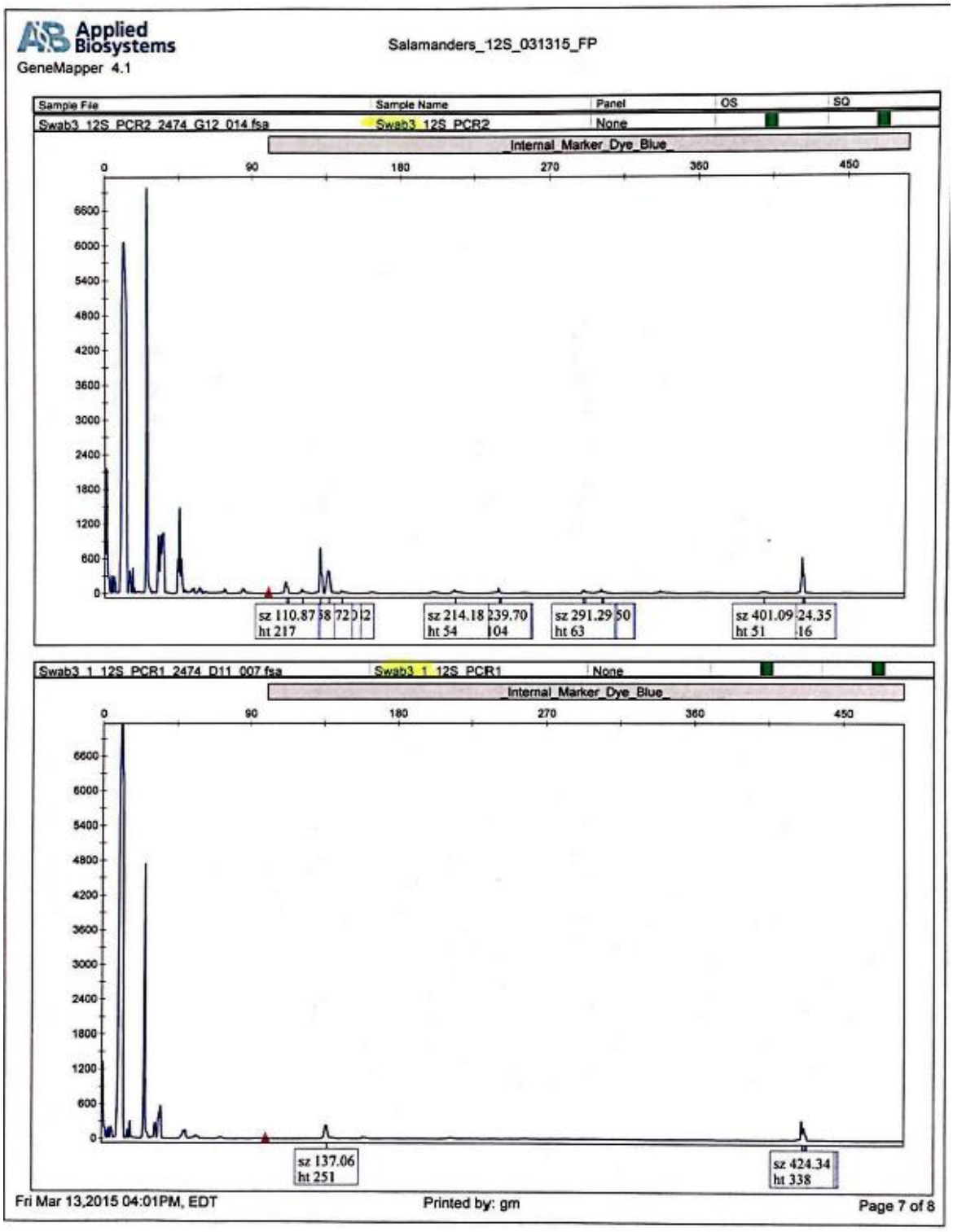

Applied Biosystems
GeneMapper 4.1
Salamanders_12S_031315_FP
Sample File
Sample Name
Panel
OS
SQ
Sample8_new_12S_PCR1_2474_H11_015.fsa
Sample8_new_12S_PCR1
None
_Internal_Marker_Dye_Blue_
0
90
180
270
360
450
6600
6000
5400
4800
4200
3600
3000
2400
1800
1200
600
0
sz 136.90
ht 274
Sample8_old_12S_PCR1_2474_A11_001.fsa
Sample8_old_12S_PCR1
None
_Internal_Marker_Dye_Blue_
sz 137.44
ht 301
sz 270.46
ht 76
sz 424.35
ht 81
Fri Mar 13,2015 04:01PM, EDT
Printed by: gm
Page 3 of 8


Applied Biosystems
GeneMapper 4.1
Salamanders_12S_031315_FP
Sample File
Sample Name
Panel
OS
SQ
Soil1_12S_PCR1_2474_A12_002.fsa
Soil1_12S_PCR1
None
Internal_Marker_Dye_Blue
sz 137.45
ht 462
sz 214.66
ht 55
sz 426.35
ht 2298
Soil2_12S_PCR1_2474_B12_004.fsa
Soil2_12S_PCR1
None
Internal_Marker_Dye_Blue
sz 137.54
ht 442
sz 207.63
ht 474
sz 424.35
ht 1141
Fri Mar 13,2015 04:01PM, EDT
Printed by: gm
Page 4 of 8


Applied Biosystems
GeneMapper 4.1
Salamanders_12S_031315_FP
Sample File
Sample Name
Panel
OS
SQ
Sample5_new_12S_PCR1_2474_F11_011.fsa
Sample5_new_12S_PCR1
None
_Internal_Marker_Dye_Blue_
0
90
180
270
360
450
6600
6000
5400
4800
4200
3600
3000
2400
1800
1200
600
0
sz 136.70
ht 303
Sample6_new_12S_PCR1_2474_G11_013.fsa
Sample6_new_12S_PCR1
None
_Internal_Marker_Dye_Blue_
sz 137.35
ht 367
sz 209.78
ht 461
sz 405.43
ht 50
Fri Mar 13,2015 04:01PM, EDT
Printed by: gm
Page 2 of 8


Applied Biosystems
GeneMapper 4.1
Salamanders_12S_031315_FP
Sample File
Sample Name
Panel
OS
SQ
Swab1_12S_PCR2_2474_E12_010.fsa
Swab1_12S_PCR2
None
Internal_Marker_Dye_Blue
sz 120.19 7.17
ht 60 5
sz 213.98
ht 65
sz 375.65 405.49 1.35
ht 452 7362 94
Swab1_1_12S_PCR1_2474_B11_003.fsa
Swab1_1_12S_PCR1
None
Internal_Marker_Dye_Blue
sz 137.43
ht 411
sz 214.58
ht 54
sz 405.56 4.43
ht 1057 4
Fri Mar 13,2015 04:01PM, EDT
Printed by: gm
Page 5 of 8


Applied Biosystems
GeneMapper 4.1
Salamanders_12S_031315_FP
Sample File
Sample Name
Panel
QS
SQ
Swab1_5_12S_PCR1_2474_C11_005.fsa
Swab1_5_12S_PCR1
None
Internal_Marker_Dye_Blue
sz 137.53 ht 441
sz 214.85 ht 59
sz 405.60 ht 868
4.35
Swab2_12S_PCR2_2474_F12_012.fsa
Swab2_12S_PCR2
None
Internal_Marker_Dye_Blue
sz 120.19 ht 74
6.81
sz 213.83 ht 97
1.10
sz 374.73 ht 204
404.55 7031
4.35
Fri Mar 13,2015 04:01PM, EDT
Printed by: gm
Page 6 of 8


Applied Biosystems
GeneMapper 4.1
Salamanders_12S_031315_FP
Sample File
Sample Name
Panel
OS
SQ
Swab3_12S_PCR2_2474_G12_014.fsa
Swab3_12S_PCR2
None
_Internal_Marker_Dye_Blue_
sz 110.87
ht 217
sz 214.18
ht 54
sz 291.29
ht 63
sz 401.09
ht 51
Swab3_1_12S_PCR1_2474_D11_007.fsa
Swab3_1_12S_PCR1
None
_Internal_Marker_Dye_Blue_
sz 137.06
ht 251
sz 424.34
ht 338
Fri Mar 13,2015 04:01PM, EDT
Printed by: gm
Page 7 of 8

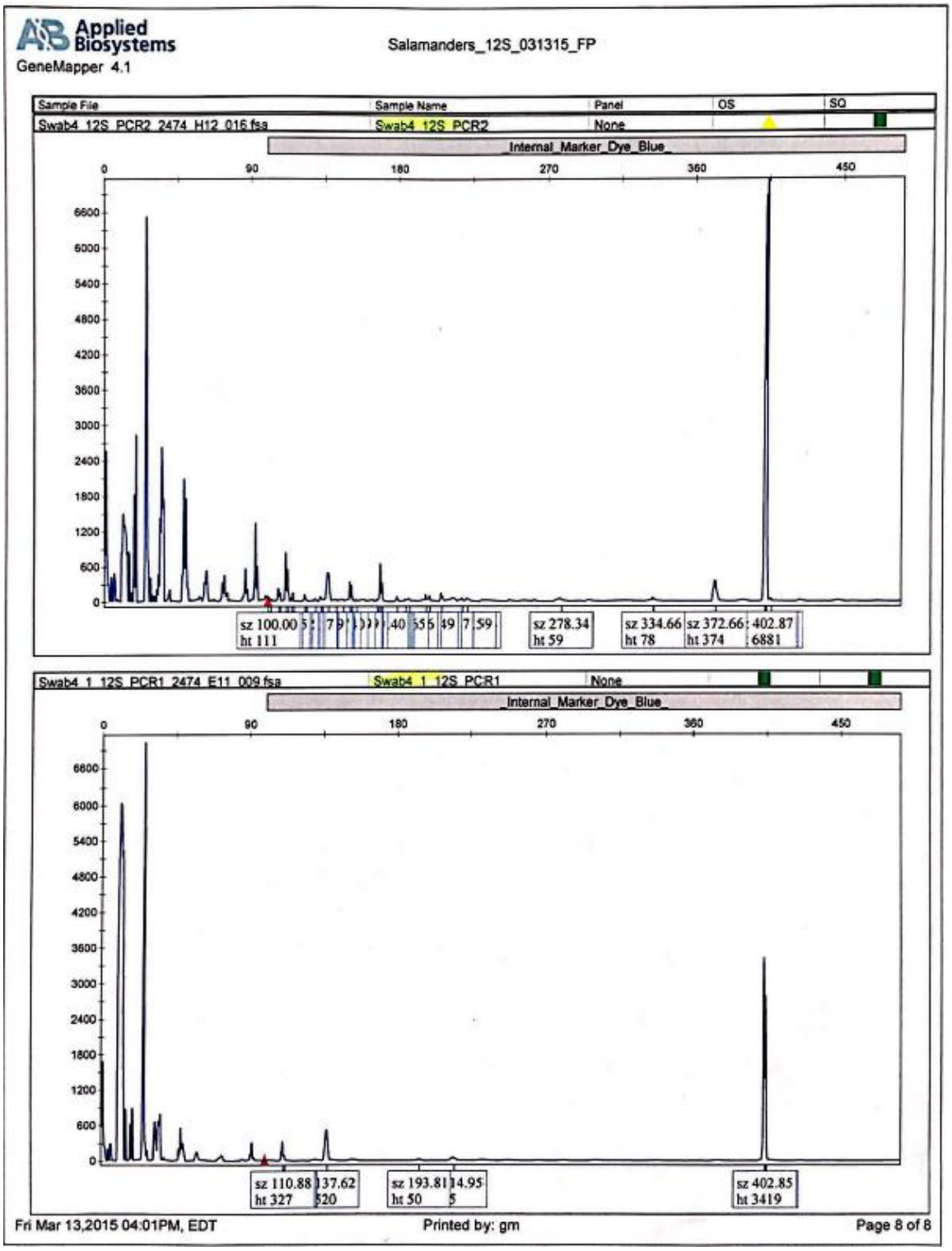

Applied Biosystems
GeneMapper 4.1
Salamanders_12S_031315_FP
Sample File
Sample Name
Panel
OS
SQ
Swab4_12S_PCR2_2474_H12_016.fsa
Swab4_12S_PCR2
None
_Internal_Marker_Dye_Blue_
0
90
180
270
360
450
6600
6000
5400
4800
4200
3600
3000
2400
1800
1200
600
0
sz 100.00
ht 111
sz 278.34
ht 59
sz 334.66
ht 78
sz 372.66
ht 374
sz 402.87
ht 6881
Swab4_1_12S_PCR1_2474_E11_009.fsa
Swab4_1_12S_PCR1
None
_Internal_Marker_Dye_Blue_
sz 110.88
ht 327
sz 193.81
ht 50
sz 402.85
ht 3419
Fri Mar 13,2015 04:01PM, EDT
Printed by: gm
Page 8 of 8

Table 10: Sequences that retrieved matches with *E. bislineata* from Sanger sequencing of PCR # 35 and 37.

Sample name	Reverse primer	Sample type	Sequence retrieved from Sanger sequencing
Sample1_ 001	COI_254R 177 bp	24 h control	CGGCATAGTGGGTACAGCTCTAAGTTTATTAATTCGCGCAGAATTAAGCCAACCAGGTACCCT ATTAGGAGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCCTTTGTTA
Sample1_ 002	COI_435R 107 bp	24 h control	TTACTTCTCCTCGCCTCGTCAGGGGTCGAAGCGGGAGCCGGGACCGGGTGAACTGTTATCCCC
Sample2_ 003	COI_254R 177 bp	24 h control	TCGGCATAGTGGGTACAGCTCGAAGGTTATTAATTTGTGCAGAATTCAACCAACCAGGTACC GTATTAGGAGATGATCAAAGACTGTAATGGTATTGTTTCTGCAACAGCACTATGCGA
Sample2_ 004	COI_435R 107 bp	24 h control	TTACTTCTCCTCGCCTCGTCAGGGGTCGAAGCGGGAGCCGGGACCGGGTGAACTGTTATCCC
Sample3_ 005	COI_254R 177 bp	24 h control	CGGCATAGTGGGTACAGCTCTAAGTTTATTAATTCGCGCAGAATTAAGCCAACCAGGTACCCT ATTAGGAGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCCTTTGTTA
Sample4_ 007	COI_254R 177 bp	24 h control	CGGCATAGTGGGTACAGCTCTAAGTTTATTAATTCGCGCAGAATTAAGCCAACCAGGTACCCT ATTAGGAGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCCTTTGTTA
Sample4_ 008	COI_435R 107 bp	24 h control	TTACTTCTCCTCGCCTCGTCAGGGGTCGAAGCGGGAGCCGGGACCGGGTGAACTGTTATCCCC
Sample5_010	COI_435R 107 bp	Stream water	TTACTTCTCCTCGCCTCGTCAGGGGTCGAAGCGGGAGCCGGGACCGGGTGAACTGTTATCCC
Sample6_ 011	COI_254R 177 bp	Stream water	CGGCATAGTGGGTACAGCTCTAAGTTTATTAATTCGCGCAGAATTAAGCCAACCAGGTACCCT ATTAGGAGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCCTTTGTTA
Sample6_012	COI_435R 107 bp	Stream water	TTACTTCTCCTCGCCTCGTCAGGGGTCGAAGCGGGAGCCGGGACCGGGTGAACTGTTATCCCC
Sample7_ 013	COI_254R 177 bp	Swab	CGGCATAGTGGGTACAGCTCTAAGTTTATTAATTCGCGCAGAATTAAGCCAACCAGGTACCCT ATTAGGAGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCCTTTGTTA

Sample7_ 014	COI_435R 107 bp	Swab	TTACTTCTCCTCGCCTCGTCAGGGGTCGAAGCGGGAGCCGGGACCGGGTGAACTGTTATCCC
Sample8_ 016	COI_435R 107 bp	Swab	TTACTTCTCCTCGCCTCGTCAGGGGTCGAAGCGGGAGCCGGGACCGGGTGAACTGTTATCCC

REFERENCES

Adler, K., & Halliday, T. (2002). *Firefly Encyclopedia of Reptiles and Amphibians*. Firefly Books.

Alford, R. A., & Richards, S. J. (1999). GLOBAL AMPHIBIAN DECLINES: A Problem in Applied Ecology. *Annual Review of Ecology and Systematics*, *30*(1), 133–165. http://doi.org/10.1146/annurev.ecolsys.30.1.133

Allen, C. R., Fontaine, J. J., Pope, K. L., & Garmestani, A. S. (2011). Adaptive management for a turbulent future. *Journal of Environmental Management*, *92*(5), 1339–1345. http://doi.org/10.1016/j.jenvman.2010.11.019

Alroy, J. (2001). A Multispecies Overkill Simulation of the End-Pleistocene Megafaunal Mass Extinction. *Science*, *292*(5523), 1893–1896. http://doi.org/10.1126/science.1059342

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389–3402.

Andersen, K., Bird, K. L., Rasmussen, M., Haile, J., Breuning-Madsen, H., Kjaer, K. H., … Willerslev, E. (2012). Meta-barcoding of "dirt" DNA from soil reflects vertebrate biodiversity: META-BARCODING OF "DIRT" DNA FROM SOIL. *Molecular Ecology*, *21*(8), 1966–1979. http://doi.org/10.1111/j.1365-294X.2011.05261.x

Bailey, L. L., Campbell Grant, E. H., & Mattfeld, S. D. (2007, January 10). National Capital Region Network Amphibian Monitoring Protocol. Retrieved from irmafiles.nps.gov/reference/holding/462214

Bartlett, R. D., & Bartlett, P. (2006). *Guide and Reference to the Amphibians of Eastern and Central North America* (1st edition). Gainesville: University Press of Florida.

Bellemain, E. (2013, April). *eDNA barcoding and metabarcoding*. Presented at the Symposium: metabarcoding of environmental samples, Royal Belgian Institute of Natural Sciences, Brussels, Belgium. Retrieved from http://bebol.myspecies.info/node/80

Best, M. L., & Welsh, Jr., H. H. (2014). The trophic role of a forest salamander: impacts on invertebrates, leaf litter retention, and the humification process. *Ecosphere*, *5*(2), art16. http://doi.org/10.1890/ES13-00302.1

Bianchini, K., Tattersall, G. J., Sashaw, J., Porteus, Cosima S., & Wright, P. A. (2012). Acid Water Interferes with Salamander–Green Algae Symbiosis during Early Embryonic Development. *Physiological and Biochemical Zoology*, *85*(5), 470–480. http://doi.org/10.1086/667407

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R. A., … Dunn, F. (2015). Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus). *Biological Conservation*, *183*, 19–28. http://doi.org/10.1016/j.biocon.2014.11.029

Blaustein, A. R., Han, B. A., Relyea, R. A., Johnson, P. T. J., Buck, J. C., Gervasi, S. S., & Kats, L. B. (2011). The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Annals of the New York Academy of Sciences*, *1223*(1), 108–119. http://doi.org/10.1111/j.1749-6632.2010.05909.x

Brockes, J. P., & Kumar, A. (2005). Appendage Regeneration in Adult Vertebrates and Implications for Regenerative Medicine. *Science*, *310*(5756), 1919–1923. http://doi.org/10.1126/science.1115200

Bruce, R. C. (1982). Egg-Laying, Larval Periods and Metamorphosis of Eurycea bislineata and E. junaluska at Santeetlah Creek, North Carolina. *Copeia*, *1982*(4), 755–762. http://doi.org/10.2307/1444083

Bulte, E., Horan, R. D., & Shogren, J. F. (2006). Megafauna extinction: A paleoeconomic theory of human overkill in the pleistocene. *Journal of Economic Behavior & Organization*, *59*(3), 297–323. http://doi.org/10.1016/j.jebo.2005.04.010

Burke, J. N., Bergeron, C. M., Todd, B. D., & Hopkins, W. A. (2010). Effects of mercury on behavior and performance of northern two-lined salamanders (Eurycea bislineata). *Environmental Pollution*, *158*(12), 3546–3551. http://doi.org/10.1016/j.envpol.2010.08.017

Caruso, N. M., & Lips, K. R. (2013). Truly enigmatic declines in terrestrial salamander populations in Great Smoky Mountains National Park. *Diversity & Distributions*, *19*(1), 38–48. http://doi.org/10.1111/j.1472-4642.2012.00938.x

Cassie, B. (1999). *National Audubon Society First Field Guide: Amphibians*. Scholastic.

Deiner, K., & Altermatt, F. (2014). Transport Distance of Invertebrate Environmental DNA in a Natural River. *PLoS ONE*, *9*(2). http://doi.org/10.1371/journal.pone.0088786

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., & Miaud, C. (2011). Persistence of Environmental DNA in Freshwater Ecosystems. *PLoS ONE*, *6*(8), e23398. http://doi.org/10.1371/journal.pone.0023398

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., & Miaud, C. (2012). Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus. *Journal of Applied Ecology*, *49*(4), 953–959. http://doi.org/10.1111/j.1365-2664.2012.02171.x

De Vos, J. M., Joppa, L. N., Gittleman, J. L., Stephens, P. R., & Pimm, S. L. (2014). Estimating the Normal Background Rate of Species Extinction. *Conservation Biology*, n/a–n/a. http://doi.org/10.1111/cobi.12380

Eaton, T. H., Jr. (1956). Larvae of Some Appalachian Plethodontid Salamanders. *Herpetologica*, *12*(4), 303–311.

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*, 423–425. http://doi.org/10.1098/rsbl.2008.0118

Foote, A. D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., … Gilbert, M. T. P. (2012). Investigating the Potential Use of Environmental DNA (eDNA) for Genetic Monitoring of Marine Mammals. *PLoS ONE*, *7*(8), e41781. http://doi.org/10.1371/journal.pone.0041781

Gibbon, J. W., Scott, D. E., Ryan, T. J., Buhlmann, K. A., Tuberville, T. D., Metts, B. S., … Winnie, C. T. (2000). The Global Decline of Reptiles, Déjà Vu Amphibians. *BioScience*, *50*(8), 653–666. http://doi.org/10.1641/0006-3568(2000)050[0653:TGDORD]2.0.CO;2

Goldberg, C. S., Pilliod, D. S., Arkle, R. S., & Waits, L. P. (2011). Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders. *PLoS ONE*, *6*(7), e22746. http://doi.org/10.1371/journal.pone.0022746

Hautier, Y., Tilman, D., Isbell, F., Seabloom, E. W., Borer, E. T., & Reich, P. B. (2015). Anthropogenic environmental changes affect ecosystem stability via biodiversity. *Science*, *348*(6232), 336–340. http://doi.org/10.1126/science.aaa1788

Herder, J., Valentini, A., Bellemain, E., Dejean, T., van Delft, Jeroen, Thomsen, Phillip Francies, & Taberlet, Pierre. (2014). Environmental DNA- a review of the possible applications for the detection of (invasive) species. RAVON.

Hurlbert, A. H., & Jetz, W. (2007). Species Richness, Hotspots, and the Scale Dependence of Range Maps in Ecology and Conservation. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(33), 13384–13389.

IUCN (Ed.). (2007, May). Species Extinction - The Facts. Retrieved from http://cmsdata.iucn.org/downloads/species_extinction_05_2007.pdf

IUCN. (2008). IUCN Red List Status. Retrieved May 13, 2015, from http://www.iucnredlist.org/initiatives/amphibians/analysis/red-list-status

IUCN. (2009, November 3). Extinction crisis continues apace. Retrieved from http://www.iucn.org/?4143/Extinction-crisis-continues-apace

IUCN (Ed.). (2014). The IUCN Red List of Threatened Species. Version 2014.3. Retrieved from http://www.iucnredlist.org

Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., … Whiteley, A. R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, *15*(1), 216–227. http://doi.org/10.1111/1755-0998.12285

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. *Conservation Letters*, *4*(2), 150–157. http://doi.org/10.1111/j.1755-263X.2010.00158.x

Karraker, N. E., & Gibbs, J. P. (2011). Road deicing salt irreversibly disrupts osmoregulation of salamander egg clutches. *Environmental Pollution*, *159*(3), 833–835. http://doi.org/10.1016/j.envpol.2010.11.019

Lees, A. C., & Pimm, S. L. (2015). Species, extinct before we know them? *Current Biology*, *25*(5), R177–R180. http://doi.org/10.1016/j.cub.2014.12.017

Mackenzie, D. I., Nichols, J. D., Sutton, N., Kawanishi, K., & Bailey, L. L. (2005). Improving Inferences in Population Studies of Rare Species That Are Detected Imperfectly. *Ecology*, *86*(5), 1101–1113.

Maigret, T. A., Cox, J. J., Schneider, D. R., Barton, C. D., Price, S. J., & Larkin, J. L. (2014). Effects of timber harvest within streamside management zones on

salamander populations in ephemeral streams of southeastern Kentucky. *Forest Ecology and Management*, *324*, 46–51. http://doi.org/10.1016/j.foreco.2014.03.043

McDonald, H. N. (2001). *The impact of logging on aquatic salamander communities* (M.S.). Ann Arbor, United States. Retrieved from http://search.proquest.com/docview/304693235/abstract/1793D044A31649AAPQ/1?accountid=14541

Meusnier, I., Singer, G. A., Landry, J.-F., Hickey, D. A., Hebert, P. D., & Hajibabaei, M. (2008). A universal DNA mini-barcode for biodiversity analysis. *BMC Genomics*, *9*(1), 214. http://doi.org/10.1186/1471-2164-9-214

Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M. N., & Kawabata, Z. (2012). Surveillance of fish species composition using environmental DNA. *Limnology*, *13*(2), 193–197. http://doi.org/10.1007/s10201-011-0362-4

Min, M. S., Yang, S. Y., Bonett, R. M., Vieites, D. R., Brandon, R. A., & Wake, D. B. (2005). Discovery of the first Asian plethodontid salamander. *Nature*, *435*(7038), 87–90. http://doi.org/10.1038/nature03474

Mojzsis, S. J., Arrhenius, G., McKeegan, K. D., Harrison, T. M., Nutman, A. P., & Friend, C. R. (1996). Evidence for life on Earth before 3,800 million years ago. *Nature*, *384*(6604), 55–59.

Mueller, R. L., Macey, J. R., Jaekel, M., Wake, D. B., & Boore, J. L. (2010, February 1). Eurycea bislineata mitochondrion, complete genome. National Center for Biotechnology Information. Retrieved from http://www.ncbi.nlm.nih.gov/nuccore/NC_006329.1

Nickerson, M. A., & Krysko, K. L. (2003). Surveying for hellbender salamanders, Cryptobranchus alleganiensis (Daudin): A review and critique. *Applied Herpetology*, *1*(1), 37–44.

Noël, S., & Lapointe, F.-J. (2010). Urban conservation genetics: Study of a terrestrial salamander in the city. *Biological Conservation*, *143*(11), 2823–2831. http://doi.org/10.1016/j.biocon.2010.07.033

Olmo, O., & Morescalchi, A. (1975). Evolution of the genome and cell sizes in salamanders. *Experientia*, *31*(7), 804–806. http://doi.org/10.1007/BF01938475

Osbahr, K. (2001). Biodiversity. In C. E. Bell, *Encyclopedia of the World's Zoos* (pp. 124–125). Taylor & Francis.

Palazzo, A. F., & Gregory, T. R. (2014). The Case for Junk DNA. *PLoS Genetics*, *10*(5). http://doi.org/10.1371/journal.pgen.1004351

Petranka, J. W. (1998). *Salamanders of the United States and Canada*. Washington and London: Smithsonian Institution Press.

Petranka, J. W., Eldridge, M. E., & Haley, K. E. (1993). Effects of Timber Harvesting on Southern Appalachian Salamanders. *Conservation Biology*, *7*(2), 363–370.

Pianka, E. R. (1981). Competition and niche theory. *Ariel*, (128), 205–172.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources*, *14*(1), 109–116. http://doi.org/10.1111/1755-0998.12159

Pilliod, D. S., Goldberg, C. S., Laramie, M. B., & Waits, L. P. (2013). *Application of environmental DNA for inventory and monitoring of aquatic species*. US Department of the Interior, US Geological Survey. Retrieved from http://pubs.usgs.gov/fs/2012/3146/

Pimm, S. L., Jenkins, C. N., Abell, R., Brooks, T. M., Gittleman, J. L., Joppa, L. N., … Sexton, J. O. (2014). The biodiversity of species and their rates of extinction, distribution, and protection. *Science*, *344*(6187), 1246752.

Robin, E. D., & Wong, R. (1988). Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *Journal of Cellular Physiology*, *136*(3), 507–513. http://doi.org/10.1002/jcp.1041360316

Sepkoski, J. J., Jr. (1997). Biodiversity: Past, Present, and Future. *Journal of Paleontology*, *71*(4), 533–539.

Sever, D. M. (1976). Morphology of the Mental Hedonic Gland Clusters of Plethodontid Salamanders (Amphibia, Urodela, Plethodontidae). *Journal of Herpetology*, *10*(3), 227–239. http://doi.org/10.2307/1562984

Smil, V. (1997). Global population and the nitrogen cycle. *Scientific American*, *277*(1), 76–81.

Sun, C., Shepard, D. B., Chong, R. A., Arriaza, J. L., Hall, K., Castoe, T. A., … Mueller, R. L. (2012). LTR Retrotransposons Contribute to Genomic Gigantism in Plethodontid Salamanders. *Genome Biology and Evolution*, *4*(2), 168–183. http://doi.org/10.1093/gbe/evr139

Sutherland, R. L., Mäthger, L. M., Hanlon, R. T., Urbas, A. M., & Stone, M. O. (2008). Cephalopod coloration model. I. Squid chromatophores and iridophores. *JOSA A*, *25*(3), 588–599.

Takahara, T., Minamoto, T., & Doi, H. (2013). Using Environmental DNA to Estimate the Distribution of an Invasive Fish Species in Ponds. *PLoS ONE*, *8*(2), e56584. http://doi.org/10.1371/journal.pone.0056584

Thomsen, P. F., Kielgast, J., Iversen, L. L., Møller, P. R., Rasmussen, M., & Willerslev, E. (2012). Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *PLoS ONE*, *7*(8), e41732. http://doi.org/10.1371/journal.pone.0041732

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., … Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA: Species Monitoring by Environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. http://doi.org/10.1111/j.1365-294X.2011.05418.x

Tilman, D. (1996). Biodiversity: Population Versus Ecosystem Stability. *Ecology*, *77*(2), 350. http://doi.org/10.2307/2265614

United Nations. (2012). World Population Prospects: The 2012 Revision, Highlights and Advance Tables. Retrieved May 18, 2015, from http://www.un.org/en/development/desa/population/theme/trends/index.shtml

U.S. Census Bureau. (2006, December 31). Historical Estimates of World Population. Retrieved May 18, 2015, from https://web.archive.org/web/20061231163421/http://www.census.gov/ipc/www/worldhis.html

Valentini, A., Pompanon, F., & Taberlet, P. (2009). DNA barcoding for ecologists. *Trends in Ecology & Evolution*, *24*(2), 110–117. http://doi.org/10.1016/j.tree.2008.09.011

Verschuyl, J., Riffell, S., Miller, D., & Wigley, T. B. (2011). Biodiversity response to intensive biomass production from forest thinning in North American forests – A meta-analysis. *Forest Ecology and Management*, *261*(2), 221–232. http://doi.org/10.1016/j.foreco.2010.10.010

Virginia Herpetological Society. (2015). Northern Two-lined Salamander. Retrieved May 31, 2015, from http://www.virginiaherpetologicalsociety.com/amphibians/salamanders/northern-two-lined-salamander/northern_two-lined_salamander.php

Vitt, L. J., & Caldwell, J. P. (2008). *Herpetology: An Introductory Biology of Amphibians and Reptiles*. Academic Press.

Wood, J. T. (1953). *The Nesting of the Two-lined Salamander, Eurycea bislineata, on the Virginia Coastal Plain, 1953, Natural History Miscellanea, Number 122 : 7 pages with 1 figure.* The Chicago Academy of Sciences.

World Health Organization. (2005). Millennium Ecosystem Assessment- Ecosystems and Human Well-Being. Island Press, Washington, DC.

Wyman, R. L., & Jancola, J. (1992). Degree and Scale of Terrestrial Acidification and Amphibian Community Structure. *Journal of Herpetology*, *26*(4), 392–401. http://doi.org/10.2307/1565115

Zalasiewicz, J., Williams, M., Steffen, W., & Crutzen, P. (2010). The New World of the Anthropocene. *Environmental Science & Technology*, *44*(7), 2228–2231. http://doi.org/10.1021/es903118j

BIOGRAPHY

Sarah M. Kuppert graduated from Thomas Strittmatter High School, St. Georgen, Germany, in 2006. She received her Bachelor of Arts in *English and American Culture and Business Studies* (English, Business, and Economics) from Kassel University in 2012. For her Bachelor's Thesis she studied reptile and amphibian communication for seven months at the Smithsonian National Zoo in Washington, D.C. She then went on to pursue a Master's degree in Environmental Science and Policy, with a focus on conservation, at George Mason University.