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Detection of the Mole Salamander (*Ambystoma talpoideum)* in Aquatic Habitats using Environmental DNA

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Abstract

Environmental DNA (eDNA) is a noninvasive method used to determine species presence or abundance, measuring and monitoring populations. eDNA can detect chloroplast, nuclear or mitochondrial DNA that has been shed from an organism in water, air, or soil. eDNA requires both field and laboratory procedures for detecting species. In Western North Carolina, three freshwater ponds were indirectly monitored to detect the presence of mole salamanders, *Ambystoma talpoideum*. Mole salamanders breed in freshwater ponds during winter months, releasing cellular material. The cellular material left behind in ponds was genetically analyzed by Polymerase Chain Reaction (PCR) to amplify the *COX1* gene. We were able to detect mole salamanders, along with other algae and fish species in vernal pools and permanent ponds. Future studies will focus on using eDNA to quantify mole salamander populations.

1. Introduction

Biodiversity is rapidly declining as humans over exploit natural resources. Loss of biodiversity from anthropogenic factors is the result of loss of habitats, habitat fragmentation, invasive species and climate change. Species are being lost at an increasing rate, with little to no documentation. Monitoring species is more important than ever, and one relatively new method of monitoring populations involves collecting DNA from the environment. Environmental DNA (eDNA) can be used to study many different species in various habitats, including invasive or imperiled taxq.

eDNA can detect cellular material in biological cast offs like saliva, urine, skin cells, or feces. Environmental DNA samples contain nuclear, chloroplast, or mitochondrial DNA released from organisms into the environment. Samples such as soil, air, or water can be analyzed through single species detection, metabarcoding, or metagenomics for measuring and monitoring populations[1]. This noninvasive method makes it feasible to detect the presence of organisms without having to spend time searching for the organisms, accessing difficult areas, or paying traditional expensive fees[2]. Environmental DNA can be used to detect species more accurately than conventional survey techniques. eDNA can also reveal cryptic and endangered species that are difficult to find because of small population size. For instance, a turtle species (*Rafetus swinhoei*) was believed to have a population size of only three males in Vietnam, until scientists discovered a female in 2020 using eDNA sampling methods[3].

The mole salamander, *Ambystoma talpoideum,* is a species of special concern in North Carolina. They come from the *Ambystoma* genus (tiger, blue-spotted, Jefferson, small-mouthed, and the paedomorphic axolotl) from Mexico. Mole salamanders occupy pond habitats, and like most amphibians, they utilize an aquatic larval stage and terrestrial adult stage. Some populations of mole salamander have individuals that never leave their larval stage and spend their entire lives in the water. Ambystomatids are heavy-tailed salamanders with short legs for burrowing, and usually >160 mm in total length[5]. In the larval stage, *Ambystoma talpoideum* are often found in stagnant bodies of water that may dry up seasonally. The aquatic larvae have gills that are lost when they metamorphose into their terrestrial adult stage. There are two types of *Ambystoma talpoideum*: a metamorphic and paedomorphic type. The metamorphic type are terrestrial adults that lack larval traits. The paedomorphic type keep their gills, remain in aquatic habitat, and

reproduce in the aquatic stage. The paedomorphic type is selected for in stagnant ponds at low densities, while the metamorphic type is selected in ponds with seasonal drying, found at high densities[7]. As adults, they typically live underground, and only come above ground to reproduce. Mole salamanders breed during the winter months, and a prior study done by Takahashi et at. (2018) revealed that there is an increase in salamander eDNA concentrations detectable in aquatic systems during the breeding season[8].

Environmental DNA could be used to confirm the presence and monitor aquatic salamander populations by analyzing water samples. This study used eDNA to indicate if *Ambystoma talpoideums* is present in selected aquatic sites in western North Carolina. Two of the sites are in south Asheville, one is a vernal pool that dries in the summer called Sandy Bottom Vernal Pools (SBVP), and the second one is a permanent pond called Sandy Bottom Pond (SBP). The third site is a retention pond on the University of North Carolina Asheville campus (CP).

2. Materials and Methods

2.1 Sample Sites

The Campus Retention Pond (CP) is located on the University of North Carolina Asheville campus, and is a stormwater retention pond. Paedomorphic mole salamanders have been found at this site in the past. The Sandy Bottom Pond (SBP) is a permanent pond that has not been assessed for *Ambystoma talpoideum*. The third site, Sandy Bottom Vernal Pool (SBVP), typically floods in the winter and spring, but dries out in the summer. This site is a known breeding site for metamorphic *Ambystoma talpoideum*, and metamorphic adults were in the breeding pools at the time of this study. Water samples were collected and filtered from each sample site in March of 2021.

2.2 Field and Filtration Protocol

Four 1L jars were sterilized with 50% bleach solution for five minutes. Each jar was rinsed 10x to remove any remaining bleach and labeled CP (Campus Retention Pond), SBP (Sandy Bottom Pond), SBVP (Sandy Bottom Vernal Pool). All samples were taken back to the lab for filtration.

Samples were vacuum filtered with an electrical pump connected to a 1 L Erlenmeyer flask with 0.45 µm cellulose nitrate filters. Filters were removed using forceps sterilized in bleach, folded, and placed in 100 mL tubes with enough ethanol (95%) to cover the filter paper. Samples were then placed in a freezer for later use in DNA extraction assay. To develop a reference sample, a mole salamander was captured from Sandy Bottom and placed into 250 mL of distilled water for 45 min before releasing it. Theoretically, the water should be saturated with species DNA.

In February of 2021 samples were collected from CP and SPB and filtered water using sample kits from Jonah Ventures DNA™. The filtered water samples were mailed to Jonah Ventures DNA™ lab where sample kits were analyzed to learn what fish species live in those two bodies of water. Jonah Ventures DNA provided state of the art eDNA sequencing that identified fish species from our samples.

2.3 DNA Extraction

The Witzel et al. protocol was used to develop our DNA extraction protocol[2]. Our filter papers were removed from ethanol and allowed to air dry. The filter paper was cut into thin strips (to aid in the extraction of DNA) and placed in 50 mL tubes with 20 mL of CTAB extraction buffer in each tube. The samples were incubated for 20 h at 55ºC and shaken occasionally. Sample amounts were doubled by adding an 20 mL of phenol:chloroform to each tube and inverted it for 30 minutes. The remnants of the filter paper were carefully removed using sterilized forceps. Samples were transferred into clean labeled centrifuged tubes and centrifuge for 5 min. An equal amount of isopropanol was added to each tube. Samples were centrifuged again for 15 min at 8000 rpm to extract the aqueous phase from the organic solution. The isopropanol poured right off and pelleted DNA was rinsed with 70% ethanol. Samples were air dried in a fume hood, and 100 µL of TE buffer was added to each sample to dissolve pellets.

Five primers were purchased from Genbank, an annotated collection of National Institutes of Health (NIH) genetic sequence databases of all publicly available DNA sequences. A specific species search for the *Ambystoma talpoideum* Cytochrome C oxidase (*COX1*) gene provided multiple primers, and five primers were selected to test our samples.

Table 1. Primers selected for *COX1* gene in *Ambystoma talpoideum* from NCBI GenBank.

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity
Forward primer	CAACTCGAACTCACGGGACA	Plus	20	10102	10121	59.97	55.00	4.00
Reverse primer	CTGCTGAGCCGTATCCTGAG	Minus	20	13809	13790	59.97	60.00	5.00
Product length	3708							

Primer pair 2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity
Forward primer	AAACTCTTGGTGCAACCCCA	Plus	20	11705	11724	60.03	50.00	4.00
Reverse primer	AGGCCTAGTAGTGAGCCGAA	Minus	20	14214	14195	60.03	55.00	6.00
Product length	2510							

Primer pair 3

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity
Forward primer	AGAAGCCCCGGCGAAAATTA	Plus	20	5160	5179	60.03	50.00	4.00
Reverse primer	TGTCCCGTGAGTTCGAGTTG	Minus	20	10121	10102	59.97	55.00	4.00
Product length	4962							

Primer pair 4

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity
Forward primer	ACCAACACGACAACGCCTAT	Plus	20	10414	10433	59.97	50.00	2.00
Reverse primer	TGGGGTTGCACCAAGAGTTT	Minus	20	11724	11705	60.03	50.00	4.00
Product length	1311							

Primer pair 5

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity
Forward primer	TTCCCACACCAACAAACCGA	Plus	20	8001	8020	60.03	50.00	2.00
Reverse primer	TTGGGGTTGCACCAAGAGTT	Minus	20	11725	11706	60.03	50.00	5.00
Product length	3725							

Polymerase Chain Reaction (PCR) was used to test our primers and amplify the selected *COX1* gene. The PCR protocol included 1 µL of both forward and reverse primers, 6µL of PCR H_2O, and 10 µL of GoTaq™ master mix was added into a clean PCR tube and centrifuged for 3 minutes. An aliquot of 2 µL of DNA sample was added to the mixture and placed into the thermocycler.

The thermocycler amplified DNA at the *COX1* locus using the following program. DNA was initially denatured at 94 ºC for 2 min, then the thermocycler repeated the following for 35 cycles: 94 ºC for 40 s, 59 ºC for 40 s, and 72 ºC for 60 s. After a 10 min extension at 72 ºC, PCR products were held at 4 ºC until analysis.

The DNA concentration of all samples was tested using a Nanodrop N100 Spectrophotometer™ to evaluate DNA concentration and purity in our reference sample that contained the water used to soak *A. talpoideum*, and all our filtered water samples. All samples showed a significant amount of DNA and relatively low contamination. Polymerase chain reaction (PCR) was used to amplify the *COX1* gene. Gel electrophoresis was used to separate DNA fragments according to their size. The primers were tested first using this technique on our reference samples. Of the five primers tested on the reference sample, Primer #5 worked the best. Overall, protocol was successful for creating a reference for *Ambystoma talpoideum*, without harming the specimen. The same technique was used on all of the samples from the three field sites using Primer #5 in our PCR.

3. Results

Overall, we successfully detected *Ambystoma talpoideum* in the Campus Pond and Sandy Bottom vernal pool. Of the five primers tested on the reference sample, Primer #5 had the most consistent and clear amplicons. Gel electrophoresis revealed that Primer #5, visible in the bottom of Figure 1, showed clear bands in the 1,000 kb range. Primers #3 and #4 are visible in the top portion of the gel, but neither had bands in the correct size range for the selected gene.

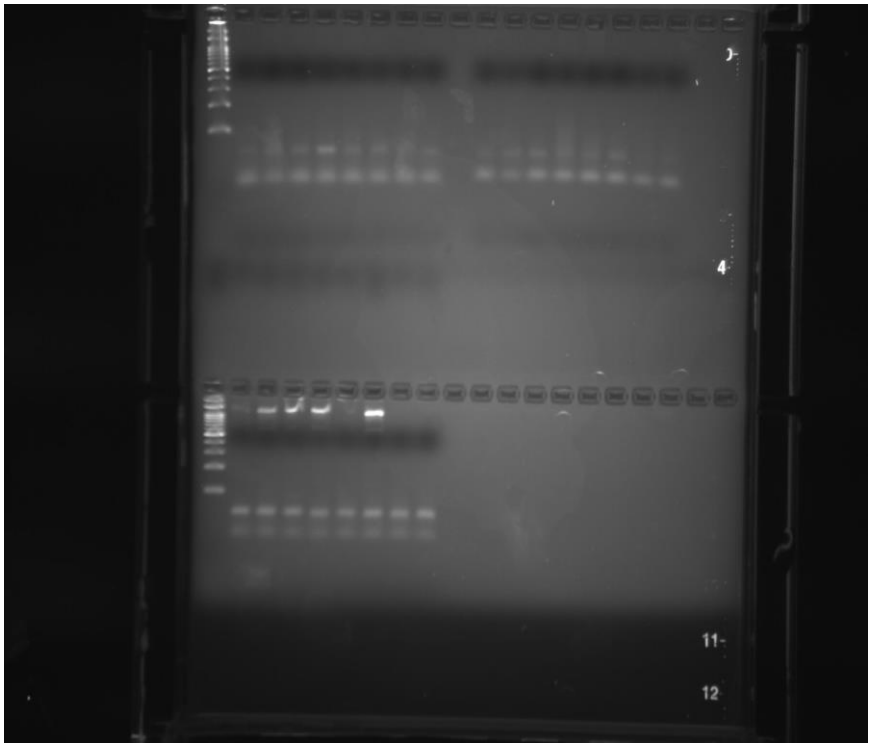

Figure 1. Gel electrophoresis from three of the five primers we tested. Primer #5 showed clear DNA amplification for the selected gene.

Water samples were collected and filtered using eDNA kits purchased from Jonah Ventures™. The samples were sent back to Jonah Ventures for an eDNA analysis of which fish species occupied the two permanent ponds (CP and SBP). The data revealed some similarities between the fish at these sites but also some notable differences. Table 1 shows the species detected at the sites. The SBP site contained some genetic sequences from large predatory fish species like chain pickerel and warmouth, which were absent from the CP site.

Table 1. Fish species identified by Jonah Ventures DNA from eDNA water samples collected at the UNC Asheville Campus Retaining Pond (CRP) and the permanent pond at Sandy Bottom (SBP). Note the species identified match the DNA sequences in the samples, but other fish species may contain the same sequence; therefore species identified may not be entirely accurate.

Common Name	Species	CRP	SBP
Black Bullhead	*Ameiurus melas*	Present	Present
Brook Stickleback	*Culaea inconstans*	Absent	Present
Chain Pickerel	*Esox niger*	Absent	Present
Common Carp	*Cyprinus carpio*	Present	Present
Creek Chub	*Semotilus atromaculatus*	Absent	Present
Rainbow Trout	*Oncorhynchus mykiss*	Present	Absent
Redbreast Sunfish	*Lepomis auritus*	Present	Absent
Warmouth	*Chaenobryttus gulosus*	Absent	Present

We detected *A. talpoideum* DNA in two samples sites, SBVP and CP. Of these two sites, CP has a paedomorphic population and SBVP is used as a breeding site for metamorphic mole salamanders. PCR and Gel electrophoresis revealed *A. talpoideum* for three of the eight samples from the campus pond site and four of the eight samples from the Sandy Bottom vernal pool site. None of the samples from SBP yielded mole salamander eDNA.

4. Discussion

Soaking the mole salamander to acquire a reference sample for eDNA was highly effective. Unlike traditional methods where species are killed for blood and tissue, no organisms were harmed to collect DNA for this study. Over the years eDNA studies, including the Witzel. et al. protocol, used blood and tissue samples. This technique could be a useful way for collecting reference samples in the future without the need for a dead specimen or procedures that could harm a living specimen.

Overall, our protocols were successful for detecting *Ambystoma talpoideum* in studied sites, and Jonah DNA detected a variety fish species. Both, the campus pond and the Sandy Bottom Vernal Pool sites now have a known mole salamander populations. Three out of the eight samples from the CP site had the correct band sizes, from SBVP four out of eight, and no samples from SBP fit the predicted band size. Water samples were taken in March during the breeding season, and metamorphic adult *Ambystoma talpoideum* were observed in the vernal pool site at Sandy Bottom at the time of our sampling. A study conducted by Takahashi[8] found a significant increase in DNA concentration for the Eastern Hellbender during the breeding season. Sampling during the breeding season may provide the best chance of detecting mole salamander populations.

The three sites differ in terms of physical conditions, but also in biotic factors. The Sandy Bottom Vernal Pool site often dries out in the summer, preventing the establishment of large fish species that might prey on or compete with *A. talpoideum*. The Campus Pond and Sandy Bottom Pond both have established fish populations. However, the Jonah eDNA kits were able to detect different fish species at both Sandy SB and CP, including some large predatory species. These two sites are quite different in terms of fish species, while containing some of the same species. Sandy bottom pond is a permanent body of water and has the potential to have paedomorphic and metamorphic populations. This site is also located right next to a larger body of water, the French Broad River, and Jonah DNA results indicated that there may be large predatory fish like warmouth and chain pickerel that may have made it into that pond from the river. These predatory fish may prey on or outcompete the mole salamanders, and could explain the absence of *A. talpoideum* in the SBP site. The Jonah DNA data also detected the presence of possible rainbow trout in the campus pond, but this is likely erroneous. The campus pond has shallow stagnant water and is not the kind of habitat typically associated with rainbow trout. The company provided a disclaimer that noted that multiple species could contain a sequence, and that misidentifications are possible.

The use of eDNA for detecting the presence of species can complement or replace traditional methods. DNA assay can be helpful for detecting the presence of a single species, invasive species, and rare or threatened species. New populations can be detected using this method and known populations can be monitored. Our study was successful at potentially detecting the presence of mole salamanders. The next step would be to send in our PCR products for genetic sequencing to make sure that our amplified DNA came from mole salamanders, and not one of the closely related species of *Ambystoma* that also occur in Western North Carolina.

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6. References

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