Multiple methods of detection of semiaquatic salamanders in small lotic systems: A comparison of eDNA and leaf litter bags

ASBURY UNIVERSITY

Rebecca R. Piche, Eliza M. Crawford, and Ben F. Brammell

Department of Natural Sciences, Asbury University, Wilmore, KY 40390

Introduction

In the past decade environmental DNA (eDNA) has become firmly established as an effective method for detecting macroinvertebrate presence and promises to greatly increase the ease, efficacy, and scope of ecological studies.[1-4] Numerous studies have utilized eDNA to quantify salamander populations [5-7]. We utilized species specific eDNA primers to detect salamander species in three Kentucky streams.

Figure 1: *Eurycea cirrigera,* the southern two-line salamander (left), and *Pseudotriton ruber,* the northern dusky salamander. Photos by Todd Pierson.

Of the 35 different salamander species in Kentucky,[8] we focused on *Eurycea lucifuga, Eurycea cirrigera, Desmognathus fuscus, and Pseudotriton ruber.*

Table 1: Salamander species included in eDNA analysis. Species selection was based on anticipated abundance in Kentucky streams and availability of molecular tools.

Scientific Name	Common Name
Eurycea lucifuga	cave
Eurycea cirrigera	southern two-line
Desmognathus fuscus	northern dusky
Pseudotriton ruber	northern red

Figure 2: Sampling location in the outflow of Lake Edmiston in Maywoods, KY.

Study Area

Both leaf litter bag sampling and eDNA water collection are being conducted in two streams located in Maywoods Natural Area (EKU) as well as an additional stream (Stony Run) located in Northern Madison County. Fall Lick Creek is located on the northeastern edge of Maywoods and the outflow stream of Lake Edmiston exits the Lake and joins Fall Lick Creek near the entrance to Maywoods. (Figure 3).

Methods

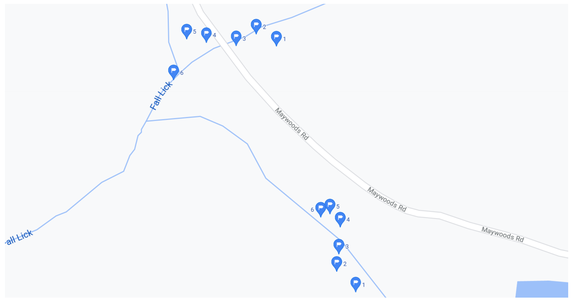

Figure 3: Sampling site locations at Maywoods Natural Area. Six leaf litter bags were deployed at each sampling site (outflow of Lake Edmiston and Fall Lick Creek).

Leaf litter bags

¾" plastic mesh was cut into 0.45 × 0.45 m squares and the corners of each square were tied together to form a bag-like configuration. These bags were placed along selected Maywoods streams at 3 m intervals. Bags were marked with orange flagging tape and held in place using stream rocks. The contents of each bag were dumped into a container and the number and species of salamanders recorded. Bags were checked for salamanders at two week intervals.

Figure 4: Leaf litter bag deployed in the outflow of Lake Edmiston in Maywoods Natural Area.

Water Sample Collection

1-liter water samples were collected 10 m below the lowest leaf bag at each site on each sampling day. Briefly, water was collected in triple rinsed containers, held on ice, and filtered in the lab within 24-48 hours using vacuum filtration and 4.7 cm fine particle filters (VWR).

Environmental DNA extraction

Briefly, filters were dried and cut into 12-15 sections before transfer to 360 μl ATL buffer (Qiagen), mixed with 40 μl proteinase K, and digested overnight (57ºC) before extraction according to DNeasy kit (Qiagen) protocol.

Molecular tools

Quantitative PCR assays for all four species of salamanders to be quantified using eDNA have been developed and validated in silico, in vitro, and in situ in our laboratory.[9,10]

Methods

eDNA quantification

Extracted DNA was quantified using a Step One Plus Real-Time PCR system. Each 20 μl reaction contained: TaqMan EMM (10 μL), nuclease free water (7 μL), eDNA extract (2 μL), and primer/probe mix (1 μL). Thermocycler conditions were as previously described[11] with the exception of varied annealing temperatures. Standard curves were generated using synthetic DNA (gBlock, IDT) to both enable data reporting in copy number and assess lowest observed limits of detection and quantification.[12]

Figure 5: *Eurycea lucifuga*, the cave salamander (left), and *Desmognathus fuscus*, the northern dusky salamander (right). Photos by Todd Pierson.

Results

Leaf Litter Bags

Leaf litter bags were emptied into clean bins and sorted to find salamander larvae. Pictures of each larvae were taken. Number of salamander larvae found at each site were entered into a master table for quantification.

Table 2: Master table of salamanders found in leaf litter bags. Salamanders are counted and identified at two week intervals. Number of salamander larvae found are listed with correlating sample locations and exact dates.

Site	Coll. Date	Total # of larvae
Edmiston Outflow	8/15/21	7
	8/29/21	5
	9/12/21	7
	9/26/21	8
	10/10/21	9
	10/25/21	9
Stony Run	8/15/21	1
	8/29/21	0
	9/12/21	4
	9/26/21	4
	10/10/21	1
	10/25/21	0
Fall Lick	8/15/21	0
	8/29/21	1
	9/12/21	3
	9/26/21	2
	10/10/21	0
	10/25/21	1

eDNA Results

Eurycea cirrigera PCR assay has been used to quantify eDNA in extracted samples. Two trials have been run since the first extraction, each in triplicate. Positive and negative controls were run in duplicate with each trial.

Table 3: Quantitative PCR results from *Eurycea cirrigera* PCR assay. After eDNA was extracted, samples were run through Step One Plus Real-Time PCR system. All samples were run with southern two-line PCR assay. CT (cycle threshold) values are inversely proportional to DNA levels within the samples.

Site	Collect. date	CT Mean
F.L.	8/31/21	36.1
S.R.	8/31/21	37.1
L.C.	8/31/21	37.4

Conclusions

Preliminary data indicates detection of *Eurycea cirrigera* (southern two lined salamander) in leaf litter bags in two of three sites but in all three sites using eDNA. The final results should both provide interesting insight into the relationship between traditional and novel methods of amphibian detection and useful data concerning the species present in Maywoods Environmental and Educational Laboratory.

Bibliography

1. Xia, Z. *et al.* Screening marker sensitivity : Optimizing eDNA- based rare species detection. *Diversity and Distributions* 1–8 (2021) doi:10.1111/ddi.13262.

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

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

4. Amphibians. KY Fish & Wildlife [accessed 2021 Sept 6]. https://fw.ky.gov/Wildlife/Pages/Amphibians.aspx

5. Thomsen, P. F. *et al.* Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology* (2012) doi:10.1111/j.1365-294X.2011.05418.x.

6. Spear, S. F., Groves, J. D., Williams, L. A. & Waits, L. P. Using environmental DNA methods to improve detectability in a hellbender (Cryptobranchus alleganiensis) monitoring program. *Biological Conservation* (2015) doi:10.1016/j.biocon.2014.11.016.

7. Santas, A. J., Persaud, T., Wolfe, B. A. & Bauman, J. M. Noninvasive Method for a Statewide Survey of Eastern Hellbenders Cryptobranchus alleganiensis Using Environmental DNA . *International Journal of Zoology* (2013) doi:10.1155/2013/174056.

8. Goldberg, C. S., Pilliod, D. S., Arkle, R. S. & Waits, L. P. Molecular detection of vertebrates in stream water: A demonstration using rocky mountain tailed frogs and Idaho giant salamanders. *PLoS ONE* (2011) doi:10.1371/journal.pone.0022746.

9. Bell, F. *et al.* Use of eDNA to detect multiple salamander species in eastern Kentucky streams. *104th Annual Kentucky Academy of Science Meeting* (2018).

10. Sams, R., McGuire, C., Nesselroade, A., Stull, M. & Brammell, B. Seasonal fluctuations in salamander eDNA in central KY streams. *Kentucky Water Resources Annual Symposium* (2017).

11. Guivas, R. A. & Brammell, B. F. Use of Environmental DNA to Determine Fantail Darter (Etheostoma flabellare) Density in a Laboratory Setting: Effects of Biomass and Filtration Method. *International Journal of Zoology* **2020**, (2020).

12. Klymus, K. E. *et al.* Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA* (2020) doi:10.1002/edn3.29.

Acknowledgements

Research was funded by the EKU Division of Natural Areas Grant, the Asbury University, Department of Science and Health provided additional support. The authors would like to sincerely thank grounds manager Homer Strong for invaluable assistance in providing information regarding sample sites. We would also like to thank Tina Piche and Andrew Morlote for their assistance in the field.