Detecting fossorial salamanders using eDNA: Development and validation of quantitative and end-point PCR assays for the detection of five species of Ambystomid salamanders

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Introduction

Small ephemeral ponds serve as important breeding grounds for many amphibian species including a number of salamander species. In Kentucky at least five salamander species of the genus *Ambystoma* and eastern newts (*Notophthalmus viridescens)* utilize ponds during one or more portions of their life cycle. *Ambystoma* species spend most of their life in burrows, primarily emerging to breed in ponds where their larvae will develop for a period of months before metamorphosis. *Ambystoma* larvae can be challenging to identify, and although relatively easy to capture, determining the presence or absence of a particular species in an environment containing multiple species may be difficult.

Figure 1. *Ambystoma barbouri* (left), the streamside salamander and *Ambystoma tigrinum* (right), the tiger salamander. Photos by Todd Pierson.

Environmental DNA (eDNA) refers to DNA shed into the environment by sloughed cells, feces, gametes, or other particles (Ficetola et al. 2008, Lodge et al. 2012). In aquatic systems this DNA disperses through the environment and can be recovered through water samples and the organisms that released it identified, offering great promise in improving species detection and conservation (Jerde et al. 2011, Thomsen et al. 2012). eDNA detection of macroscopic organisms is still a relatively new technique and many unanswered questions exist regarding detection limits, nucleic acid degradation rates, and other issues.

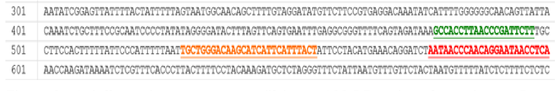

Figure 2. *A. jeffersonianum* assay amplifying a 123 BP region of cytochrome B.

We developed primers to identify five *Ambystoma* species found in central KY using eDNA. Water samples were collected from a number of sites in central and eastern Kentucky known to serve as breeding grounds for four of these five *Ambystoma* species. We demonstrated, using quantitative RT PCR, the utility of this technique in detecting the presence of salamander species in these habitats. These assays provide an effective means of determining species present in particular habitats rapidly and definitively and therefore offer to increase the ease of range delineation and spawning habitat studies.

Figure 3. Ambystoma breeding pond located in Jessamine County, KY.

Methods

Sequencing

Initial primers designed based on published sequences proved inadequate in distinguishing central Kentucky *Ambystoma* species. Tissue samples were obtained (KYDFWR Permit # - SC1811153) from locally collected specimens and portions of cytochrome b amplified and sequenced from the following species (Table 1). Sanger sequencing was completed by ACGT in triplicate and all sequences aligned and edited prior to submission.

Table 1. Specimens sequenced for oligo development.

Species	Collection site	Accession #	Amplicon length
A. jeffersonianum	Jessamine Co., KY	KT780869.1	726
A. jeffersonianum	Powell Co., KY	MZ962318	749
A. tigrinum	Green Co., KY	MZ962317	770
A. tigrinum	Hart Co., KY	OL456143	769
A. barbouri	Jessamine or Madison Co., KY	OK040169	337
A. barbouri	Madison Co., KY	OL456142	935
A. maculatum	Jessamine Co., KY	MZ485477	411
A. opacum	Powell Co., KY	KT780868.1	720
N. viridescens	Powell Co., KY	MZ962319	272

Assay Design

Primer and probe sequences were designed using sequencing results and in some cases (*A. barbouri*) published sequences (Bi and Bogart 2010). Primers were designed using IDT's primer design software checked for specificity with sympatric species using BLAST and MEGAX.

Table 2. Amplicon length produced by the quantitative PCR assay for each *Ambystoma* species.

Species	Amplicon length (BP)
Ambystoma jeffersonianum	123
Ambystoma maculatum	159
Ambystoma opacum	131
Ambystoma tigrinum	111
Ambystoma barbouri	134

Tissue Extract Testing

Primers were tested *in vitro* for effectiveness and specificity using tissue extracted DNA from the four other *Ambystoma* species and ten other sympatric salamander species. DNA was extracted via a DNeasy kit (Quiagen).

Figure 4. *Ambystoma opacum* (left), the marbled salamander and *Ambystoma maculatum* (right) the spotted salamander. Photos by Todd Pierson.

Water Sample Collection and Extraction

One liter water samples were collected from ponds and small streams in central and eastern KY. Samples were collected in high density polyethylene containers. Water was filtered in the lab using a 47 mm filter apparatus (VWR) and 4.7 cm fine particle filters (VWR). eDNA was extracted using a modified version of the procedure described by Goldberg et al. (2011).

qPCR

All qPCR reactions were run on a Step One Plus (Life Technologies) instrument using Life Technologies Environmental Master Mix 2.0 and IDT primer/probe assays.

Methods

Field Sampling

Larvae were collected using dip nets, identified to species, and released aside from a small number from which tail clips were taken for tissue DNA.

Figure 5. *A. barbouri* (left) and *A. opacum* (right) larvae. Photos by Todd Pierson.

Results

Species Specificity Testing

Primers amplified target species but not sympatric species in PCR reactions run with tissue extracted DNA from all *Ambystoma* species and other sympatric species (Figure 6). The end point PCR reactions below represent 40 cycles, annealing temp. = 60ºC.

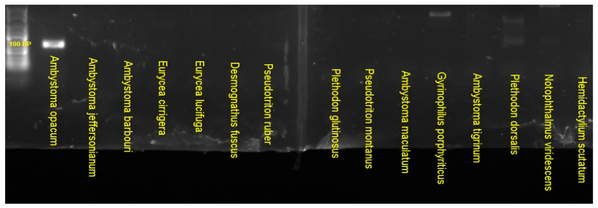

Figure 6. Species specificity testing for primers used to identify *A. jeffersonianum* (Jefferson) (A), *A. opacum* (marbled) (B), and *A. barbouri* (streamside) (C).

eDNA detection

Initial eDNA results reveal detection of target species from filtered water consistent with field observations (Table 3). In two sites (#3 and #8) species were detected via qPCR that were not detected in field collection surveys. These species are known to occur in the area where these samples were collected. In the case of #3 *A. opacum* larvae were not observed on 6/28/21 but were abundant in this habitat earlier in the spring.

Parallel endpoint PCR analysis is being completed to compare the effectiveness of this simpler and more cost-effective method. Although incomplete, the samples processed thus far all show positives if qPCR detection was positive. Additionally, Site #5 was positive (1 of 3 reps) for *A. jeff.,* eggs for which were observed at this site earlier in the 2021.

Table 3. Field collection, qPCR eDNA detection, and endpoint PCR eDNA detection of of four Ambystomid species in study sites in eastern and central Kentucky. For field collection X = collected and the absence of an X means no observation, for both qPCR and endpoint PCR X/3 represents the number of positive replicates out of 3, 0 = no amplification of any replicate, - means has not yet been analyzed.

	Site		Coll. date	Field collection				qPCR				Endpoint PCR			
				B. jeff.	*B. mac.*	*B. bar.*	*B. opac.*	*A. jeff.*	*A. mac.*	*A. bar.*	*A. opac.*	*A. jeff.*	*A. mac.*	*A. bar.*	*A. opac.*
1	Pond,	Powell Co.	7/1	X	X			3/3	1/3	0	0	2/2	-	-	-
2	Pond,	Powell Co.	7/1	X	X			3/3	3/3	0	0	3/3	3/3	-	-
3	Pond,	Rowan Co.	6/28		X			1/3	3/3	0	1/3	-	-	-	1/2
4	Pond,	Rowan Co.	6/28					0	0	0	0	-	-	-	-
5	Pond,	Rowan Co.	6/28		X			0	3/3	0	0	1/3	3/3	-	-
6	Pond,	Rowan Co.	6/28		X			0	3/3	0	0	0	3/3	0	0
7	E. stream,	Madison Co.	6/27			X		0	0	1/3	0	-	-	-	-
8	E. stream,	Madison Co.	6/28			X		1/3	0	3/3	3/3	-	-	-	-
9	Stream,	Madison Co.	6/27					-	0	0	-	-	-	-	-

Results

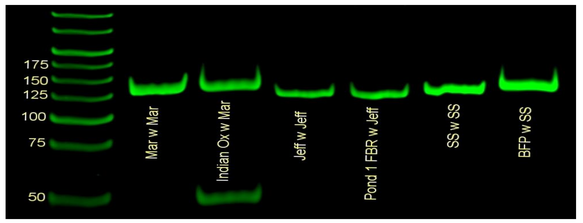

Figure 7. Polyacrylamide electrophoresis showing expected sizes of salamander cytochrome b amplicons from both tissue and water samples. From left to right *A. opacum* (marbled) tissue (LN 1) and water (LN2), *A. jeffersonianum* (Jefferson) tissue (LN 3) and water (LN 4), *A. barbouri* tissue (LN 5) and water (LN 6).

Amplicon size confirmation

The polyacrylamide gel run with both tissue and water samples showed that amplicons from both tissue and water samples of *A. opacum* (131 BP), *A. Jeffersonianum* (123 BP), and *A. barbouri* (134 BP) migrate according to expected size.

Table 4. In silico comparison of *A. barbouri* oligos in the present study with sequences obtained in this study (Madison Co., KY) and previously published *A. barbouri* cytb sequences (Bi and Bogart 2010). Numbers represent number of mismatches between cytochrome b sequence and oligo.

Region		Central KY				W. Central KY			Ohio				TN	WK
County		Madison Co, KY	Jessamine Co., KY	Fayette Co., KY	Franklin Co., KY	Mercer Co., KY	Anderson Co, KY	Oldham Co, KY	Hamilton Co., OH	Warren Co, OH	Montgomery Co.,	Butler Co., OH	Rutherford Co., TN	Livingston Co., KY
Accession number		OL456142	GU078501	GU078484	GU078482	GU078496	GU078478	GU078490	GU078470	GU078512	GU078474	GU078511	GU078495	GU078504
F	GTAAACTATGGCTGACTCATAC	0	1	0	1	1	1	1	0	0	0	0	2	0
R	CCGATGTTTCATGTTTCTTTG	0	0	0	0	1	1	1	0	0	0	0	1	0
P	TATTGGCCGAGGGTTATACTACGG	0	0	0	0	4	5	4	0	0	0	0	2	0

Conclusions

- *Ambystoma* assays detect five *Ambystoma* species but not congeneric species or ten other sympatric species in in silico and in vitro tests.
- Initial field tests demonstrate eDNA detection consistent with field observations for four of these *Ambystoma* species.
- Our *A. barbouri* assay (designed for central KY specimen) appears to work well for central KY specimens but distinctly less well for specimens collected slightly to the west (Table 3, W. Central KY) or the disjunct TN population (Table 3, TN).

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