Development and in-vitro validation of PCR assays for use in eDNA detection of *Ambystoma texanum* and *Ambystoma talpoideum*

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Introduction

Salamanders of the genus *Ambystoma* are fossorial and rarely observed above ground outside of their reproductive seasons. Several members of this genus are of conservation concern and the conservation status of others uncertain, at least in portions of their range, as a result of limited data. Multiple species of the genus *Ambystoma* and *Notophthalmus viridescens* (eastern newt) often coexist in ephemeral ponds, creating identification challenges. We developed molecular tools to enable the identification of species present in such habitats effectively with relative ease.

Figure 1. *Ambystoma texanum* (left), the smallmouth salamander and *Ambystoma talpoideum* (right), the mole salamander. Photos by John P. Clare (left) and Todd Pierson (right).

Environmental DNA is an emerging method that utilizes DNA shed into the environment by sloughed cells, feces, gametes, or other particles to detect the presence of organisms that are otherwise difficult and/or time-consuming to locate (Ficetola et al. 2008, Lodge et al. 2012). Since the first publication utilizing this method to identify macroinvertebrates in 2008 the number of studies utilizing eDNA has increased in an exponential manner. eDNA shows great promise in enabling the collection of ecological data in an efficient and effective manner (Jerde et al. 2011, Thomsen et al. 2012).

Figure 2. *A. texanum* (top) assay amplifying a 149 BP region of cytochrome B and *A. talpoideum* (bottom) assay amplifying a 131 BP region of cytochrome B.

We developed species-specific qPCR assays for *Ambystoma texanum* and *Ambystoma talpoideum* and tested them in silico, in vitro, and in situ. Tissue tests confirm specificity against sympatric Ambystomids and other native species of salamanders. Primers were validated with in-vitro water tests from water exposed over a period of time to the target species. These assays demonstrate great promise in providing an easy and efficient method of locating these species, contributing significantly to a wide variety of salamander studies.

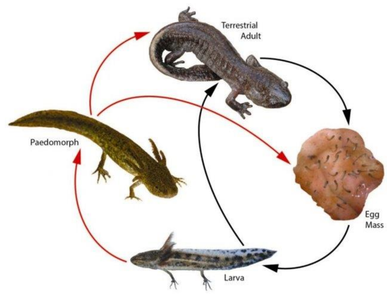

Figure 3. Complex life cycle of *A. talpoideum* (Wilbur 1980).

Methods

Sequencing

Initial primers were designed based on published sequences and tested against locally acquired specimens. Tissue samples were obtained (KYDFWR Permit # - SC1811153) from locally collected specimens and portions of cytochrome b amplified and sequenced from the following species. We are in the process of completing sequencing of cytb from both species.

Assay Design

Primer sequences were designed using sequencing results and 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 1. Mismatch table for *Ambystoma talpoideum* against sympatric species.

Sympatric species	FP mismatches	RP mismatches	P mismatches	% sim.	Seq. accession #
Ambystoma talpoideum	0	0	0	100	EF036640.1
Ambystoma talpoideum	0	0	0	100	EF036639.1
Ambystoma texanum	3	4	6	84.2	EF036656.1
Ambystoma barbouri	3	5	6	84.7	GU078513.1
Ambystoma opacum	3	7	3	84.1	KT780868.1
Ambystoma jeffersonianum	5	7	3	81.7	MZ962318
Ambystoma maculatum	4	8	7	83.1	EF036637.1
Ambystoma tigrinum	4	6	4	85.4	EF036667.1
Notophthalmus viridescens	9	9	4	78.8	AY691731

Table 2. Mismatch table for *Ambystoma texanum* against sympatric species.

Sympatric species	FP mismatches	RP mismatches	P mismatches	% sim.	Seq. accession #
Ambystoma texanum	0	0	0	100	GU078506.1
Ambystoma texanum	0	0	2	98.1	EF036656.1
Ambystoma texanum	0	0	2	97.7	EF036650.1
Ambystoma barbouri	1	1	3	92.6	GU078513.1
Ambystoma opacum	3	2	4	86.1	KT780868.1
Ambystoma jeffersonianum	2	2	1	87.1	MZ962318
Ambystoma maculatum	5	3	3	83.8	EF036637.1
Ambystoma tigrinum	3	4	2	88.4	EF036667.1
Notophthalmus viridescens	2	4	4	81.9	AY691731

Table 3. Amplicon length produced by the quantitative PCR assay for each *Ambystoma* species.

Species	Amplicon length (BP)
Ambystoma texanum	149
Ambystoma talpoideum	131

Tissue Extract Testing

Primers were tested *in vitro* for effectiveness and specificity using tissue extracted DNA from six other *Ambystoma* species and ten other sympatric salamander species. DNA was extracted via a Qiagen DNeasy kit.

Methods

Figure 4. *Ambystoma barbouri* (left), the streamside salamander and *Ambystoma texanum* (right) the smallmouth salamander. Photos by Todd Pierson.

Water Sample Collection and Extraction

Water samples were collected from an in-lab water test. Specimens were set in 500 mL of water for 96 hours. Water was then diluted 20:1 with deionized water (Davy et al. 2015). Water was then 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).

End- point PCR

All PCR reactions were run on a Step One Plus (Life Technologies) instrument GoTaq Green MasterMix and IDT primer/probe assays.

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.

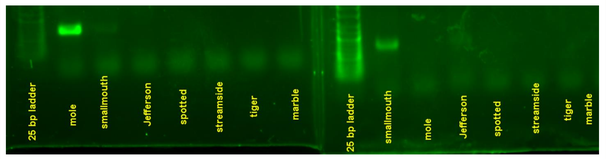

Figure 5. Species specificity testing for primers used to identify *A. talpoideum* (mole) (A) and *A. texanum* (smallmouth) (B).

eDNA detection

Initial testing of eDNA assays reveals that the primers do bind to the target species when run with tissue DNA, and in-lab water tests will soon determine if the assays are capable of picking up small amounts of DNA from water samples known to contain the target DNA. The goal of the in-lab water tests is to detect trace amounts of DNA in even smaller quanitieis than predicted in ephemeral ponds and slow-moving streams.

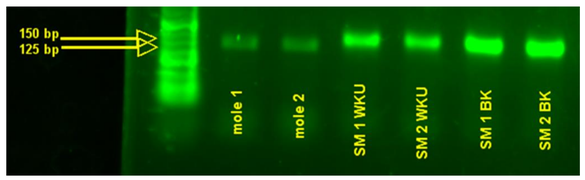

Figure 6. Polyacrylamide electrophoresis showing expected sizes of salamander cytochrome b amplicons from tissue samples. From left to right *A. talpoideum* (mole) tissue (LN 1 and 2), *A. texanum* (smallmouth) tissue (LN 3, 4, 5, & 6).

Conclusions

- Both *Ambystoma* assays detect target species, but not congeneric species or ten other sympatric species tested *in silico* and *in vitro.*
- Laboratory water tests are the next step for confirming specificity of primers at low levels of DNA.
- Future field water tests will be run when these species breed in the spring.
- *Ambystoma talpoideum* cytb has proven difficult to amplify, more testing needs to be done to confirm what primers are suitable. Previous studies suggest MVZ15 and MVZ16 but these were unsuccessful both in silico and in vitro.
- *Ambystoma texanum* cytb has been sequenced and will be submitted to GenBank.

Figure . *A. texanum* larvae (left) by Jeromi Hefner and *A. talpoideum* larvae (right) by Jason Gibson.

Bibliography

Bi, Ke and J. P. Bogart. 2010. "Time and time again: unisexual salamanders (genus *Ambystoma*) are the oldest unisexual vertebrates." *BMC evolutionary biology* 10:238.

C. M. Davy, A. G. Kidd, and C. C. Wilson. 2015. "Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles," *PLoS One*, 10(7), e0130965.

Ficetola, G. F., C. Miaud, F. Pompanon, and P. Taberlet. 2008. Species detection using environmental DNA from water samples. *Biology Letters*, *4*(4), 423-425.

Goldberg, C. S., D. Pilliod, S. Arkle, and R. Waits. 2011. Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PloS one*, *6*(7), e22746.

Jerde, C. L., A. Mahon, W. Chadderton, and D. Lodge, D. M. 2011. Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*(2), 150-157.

Lodge, D. M., C. Turner, C. Jerde, M. Barnes, L. Chadderton, S. Egan, and M. Pfrender. 2012. Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. *Molecular ecology*, *21*(11), 2555 -2558.

Thomsen, P., J. Kielgast, L. Iversen, C. Wiuf, M. Rasmussen, M. Gilbert, and E. Willerslev. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565-2573.

Wilbur, H.M. Complex life cycles. Ann. Rev. Ecol. Syst. 1980, 11, 67±93.

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