DEVELOPMENT AND VALIDATION OF PCR PRIMERS FOR DETECTING *AMBYSTOMA TEXANUM* (SMALLMOUTH SALAMANDER) FROM EDNA SAMPLES

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Abstract.—The smallmouth salamander (*Ambystoma texanum)* is a widely distributed member of the family Ambystomatidae found throughout much of the central United States. We developed primers targeting a 147 base pair fragment of the mitochondrial cytochrome b region of the smallmouth salamander and tested these primers *in silico*, *in vitro*, and *in vivo* in laboratory-based studies. We believe these validated tools will be widely useful in the detection of *A. texanum* in breeding ponds, particularly in habitats where multiple species of *Ambystoma* occur.

Key Words.—conservation, eDNA, salamander, Ambystomatidae, *Ambystoma texanum*

Environmental DNA (eDNA) has rapidly become a firmly established method for detecting organisms of research and conservation interest and promises to greatly increase the ease, efficacy, and scope of ecological studies (Dysthe et al. 2018; Mauvisseau et al. 2019; Sawaya et al. 2019). Since its inception in 2008 eDNA has been used to detect rare and endangered species of fish (Laramie et al. 2015; Paine et al. 2021), amphibians (Spear et al. 2015; Pierson et al. 2016; Witzel et al. 2020), crustaceans (Ikeda et al. 2016; Rusch et al. 2020), and mussels (Rusch et al. 2020; Coghlan et al. 2021; Schmidt et al. 2021), in some cases detecting cryptic species where traditional methods were unsuccessful (Sigsgaard et al. 2015; Gargan et al. 2017). Additionally, eDNA has been widely used in the detection of invasive species including Asian carp (Jerde et al. 2011) and many other species of fish (Adrian-Kalchhauser and Burkhardt-Holm 2016; Robson et al. 2016), molluscs (Klymus et al. 2017; Xia et al. 2018), crustaceans (Carim et al. 2016; Dougherty et al. 2016), amphibians (Dejean et al. 2012; Secondi et al. 2016), and even reptiles (Piaggio et al. 2014). The number of eDNA studies has increased at an exponential pace. Single species eDNA assay studies increased from one in 2008 to 56 in 2019 (Xia et al. 2021), eDNA studies focusing on fish ecology increased from one in 2011 to nearly 90 in 2021 (Xing et al. 2022), and fifty eDNA review articles were published in 2020 and 2021, comprising nearly 50% of the 105 of eDNA review articles published since the inception of the technique (Hinz et al. 2022). Clearly eDNA promises to expand the questions scientists are able to address in ecological studies.

Recent works have highlighted the need for carefully tested assays for use in species-specific marker studies (Klymus et al. 2020; Xia et al. 2021). Such studies rely on the development of primers that recognize the target, but not sympatric non-target species. A well-defined, species-specific primer pair will present a minimum of two mismatched DNA base pairs with sympatric species sequences (Wilcox et al. 2013), ensuring that the primer does not bind to non-target species. Recent studies have emphasized the need for thorough vetting of eDNA primers using as many local sequences as available given the potential for

false positives (amplification of a sympatric species' DNA) and false negatives (failure to amplify target species DNA) at any given location (Wilcox et al. 2013; Kaganer 2021; Bell et al. 2022). In vitro testing against sympatric species ensures specificity and provides assurance that false positives should not occur. Only 30.4% of specific marker studies published between 2008 and 2019 use previously developed primers (Xia et al. 2021), likely a result of lack of availability of these tools and an indication of the time and effort required to develop and thoroughly vet these molecular tools. Carefully validated species-specific markers that have been rigorously screened to prevent both false positives and negatives hold significant value that extends well beyond their time and location of origin.

As global amphibian communities continue to decline at alarming rates (McCallum 2007; Collins 2010; Fisher and Garner 2020), the need for methods enabling rapid identification of the presence/absence of specific organisms will continue to increase. Collins (2010) and McCallum (2007) suggest that all amphibians are in need of monitoring regardless of current conservation status. Molecular assays that enable the rapid detection of fossorial species such as smallmouth salamanders would provide great benefit to conservationists and ecologists studying these organisms.

The smallmouth salamander is a cryptic, fossorial salamander found throughout much of the central United States (Kraus and Petranka 1989; Petranka 1988). *A. texanum* was initially classified as having two forms: a widely distributed ephemeral pond breeding form and a stream breeding form with a more restricted range (Garcia et al. 2003). The stream breeding form was eventually recognized as the streamside salamander *(Ambystoma barbouri)* (Kraus and Petranka 1989), hypothesized to have diverged from *A. texanum* during the late Pleistocene (Kraus and Petranka 1989; Petranka and Sih 1987). Differentiation of adults of these species using external characteristics is difficult (Miller and Miller 2019). Although the larvae of *A. barbouri* are reported to be darker than *A. texanum* (Garcia et al. 2003), both species display cryptic, background color change responses (Garcia and Sih 2003), making them also difficult to differentiate as larvae. Although *A. texanum* and *A. barbouri* are mostly allopatric, several zones of parapatry do exist (Miller and Miller 2019). The molecular tools provided by this work will facilitate quantification of the presence of *A. texanum*, including most habitats where *A. texanum* and *A. barbouri* occur sympatrically. These assays have been designed based on locally obtained sequences and validated in silico and in vitro as recommended (Klymus et al. 2017; Langlois et al. 2020; Xia et al. 2021) providing ready-made tools to facilitate future conservation efforts.

METHODS AND MATERIALS

Tissue collection of target and non-target species. – We collected tissue from adult streamside salamanders and Eastern newts *(Notophthalmus viridescens)* (KYDFW Permit# SC2111188). Tissue for the smallmouth and other sympatric species was generously donated by collaborators. All information concerning origin of species used in laboratory testing is found in Table 2, Supplemental Data. We extracted tissue DNA using a DNeasy blood and tissue kit (Qiagen) according to the provided protocol. Tissue was lysed overnight at 56 °C in proteinase K and eluted twice (400 µl total) to increase DNA yield.

Sequencing of target species. – We amplified portions of cytochrome b (cyt b) from both *A. texanum* and sympatric species using published primers (Roe et al. 1985) (Table 2, Supplementary Data). Sequencing was completed in duplicate or triplicate, and sequences were edited prior to Gen Bank submission, all sequencing was completed by ACGT (ACTG inc.com).

Assay development and testing. – We aligned published cyt b sequences with nineteen potential sympatric Kentucky salamander species using MegaX and Clustal W. Accession numbers of cyt b sequences used in alignments are found in Table 2. We designed F and R primer pairs using PrimerQuest software (IDT) and aligned these with sympatric or potentially sympatric species to verify specificity (Table 2). All primers have at least 2 mismatches in the F or R primer. We also designed a probe (Table 1, Supplemental Data) and tested it *in silico* but not *in vitro*.

We evaluated F and R primers via a temperature gradient approach to determine optimal annealing temperature (53.1 – 61.6 ºC) (Figure 1, Supplemental Data). For in-vitro testing we ran end-point PCR on tissue extracts with six sympatric or potentially sympatric *Ambystoma* species and *Notophthalmus viridescens* collected in various localities in Kentucky (Table 5). Tissue extracted DNA concentration was quantified using a Qubit™2.0 (Life Technologies, Carlsbad, CA, USA) and DNA from all species diluted in nuclease free water to a concentration of 1.0 µg/ml. 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega, Madison, Wisconson, U.S.A.), 9.5 µl nuclease free water, 2 µl tissue extracted DNA and 2.0 µl of F and R primers (reaction concentration = 1.6 µm). Cycling conditions consisted of an initial denaturation stage of 95.0 ºC for 2 minutes followed by 40 cycles of 95.0 ºC for 45 s, 57.0 ºC for 60 s, and 72.0 ºC for 60 s.

Laboratory water tests. – We tested the validity of our primers through water exposure studies in the laboratory using two smallmouth salamanders: one larva and one adult (both collected in Logan County, KY). 500 mL of deionized water was added to a plastic Tupperware container containing each salamander. The larval salamander's enclosure was left on a flat surface, while the adult's was tilted to allow for resting partially out of the water. The bottom of this tilted container had a paper towel to facilitate traction, but the water still reached the bottom all around the base of the container. These containers were left for 96 hours based on previous studies (Maruyama et al. 2014; Takahara et al. 2012) in a room maintained at 68 °C. Following removal of salamanders exposed water was diluted 20:1 in DI water to approximate in situ concentrations (Davy et al. 2015). Water samples were processed through a 47mm diameter glass microfiber filter (VWR, 0.42mm thickness and 0.7 µm pore size) in a manner similar to previous studies (Eichmiller et al. 2014; Guivas and Brammell 2020; Jerde et al. 2011).

DNA Extraction. – eDNA extraction was performed using a DNeasy blood and tissue kit (Qiagen – Valencia, CA, USA) in the manner described by Guivas and Brammell (2020). Briefly, entire filters were cut into 30–40 pieces and incubated at 56°C overnight in 720 µl ATL buffer and 80 µl Proteinase K. Final elutions were performed twice into a total of 400 µl of AE buffer, and the extracted DNA was stored at −20 °C until analysis.

Amplicon Sequencing. – Amplicons obtained from the larval water test were sequenced in duplicate with both the forward and reverse primer to generate a consensus sequence including the entire amplicon. Sequencing was conducted by ACGT (ACGTinc.com – Wheeling, IL, USA).

RESULTS

In silico testing. – Smallmouth salamander forward and reverse primers have a minimum of two mismatches with all species and four or more mismatches with all species except streamside salamanders (Table 2). When the probe is considered, six or more mismatches exist with all 18 potential sympatric species (Table 2); therefore, amplification of sympatric species is highly unlikely. Additionally, when compared to published smallmouth salamander sequences from other portions of the range, the number of mismatches in only the forward and

TABLE 1. Primers developed to detect smallmouth salamander (*A. texanum*), designed based on sequence from Kentucky (Butler Co.) collected *A. texanum* specimen (Acc# OM236537). Bold, blue bases indicate location mismatches with streamside salamander (*A. barbouri*) (Table 2).

Amplicon length (BP)	Oligo	Sequence (5'-3')
147	F	TCAATGAATTTGAGGCGGATTT
	R	CCTGT**A**GG**G**TTATTAGATCCTGTT
	P	ACTCGATTCTTTGCCTTCCACTTCT

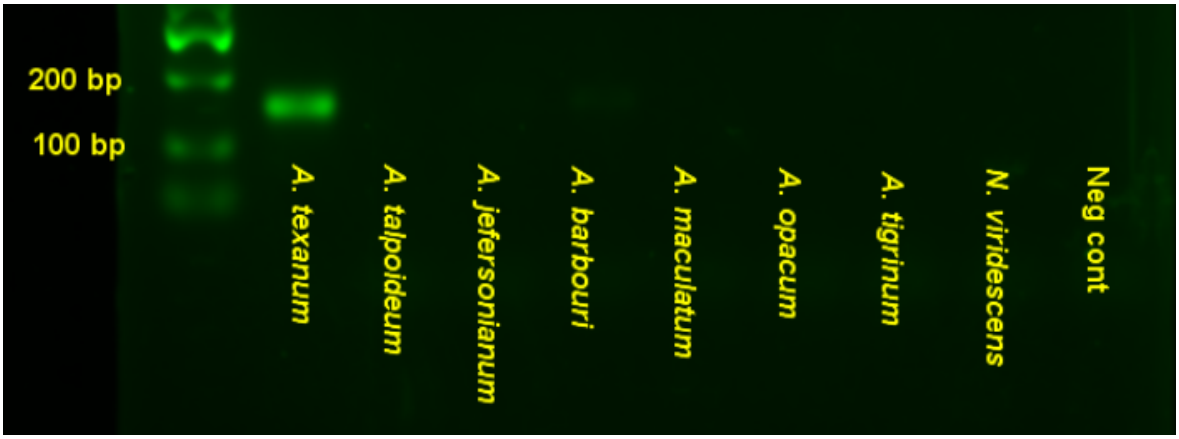

FIG. 1. Tissue extracted DNA specificity tests for smallmouth salamander (*A. texanum*) primers using tissue extracts of six sympatric or potentially sympatric species of *Ambystoma* species and eastern newt (*Notophthalmus viridescens*). 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 9 µl nuclease free water, 2 µl tissue extracted DNA and 1.5 µl of F and R primers. Cycling conditions consisted of an initial denaturation stage of 95.0ºC for 2 minutes followed by 40 cycles of 95.0ºC for 60 s, 57.0ºC for 60 s, 72.0ºC for 60 s. Ladder displayed is Hyper 25 BP ladder (Bioline).

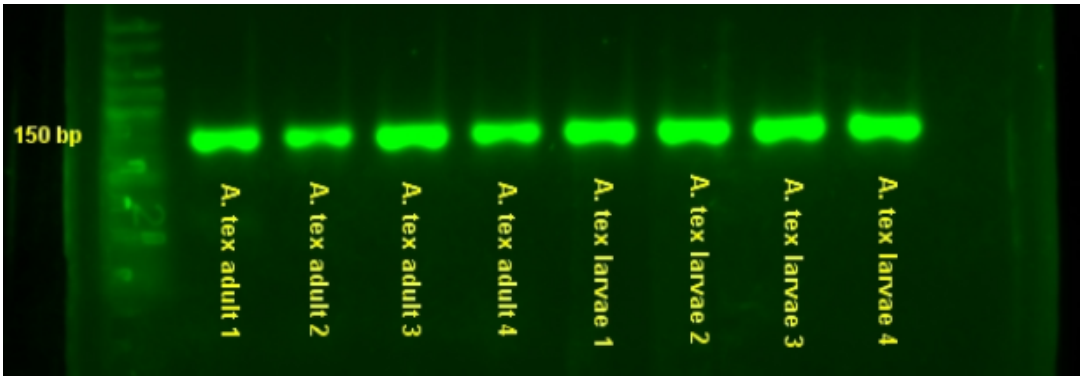

FIG. 2. Filtered water extracted DNA reactions run with smallmouth salamander (*A. texanum*) primers. Lanes 2 – 5 are replicates of the adult water test and Lanes 6 – 9 are replicates of the larval water test. 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 6.75 µl nuclease free water, 3.75 µl filtered water extracted DNA and 2.0 µl of F and R primers. Cycling conditions consisted of an initial denaturation stage of 95.0ºC for 2 minutes followed by 55 cycles of 95.0ºC for 30 s, annealing temperature of 56.5ºC for 30 s, 72.0ºC extension for 30 s with a final extension of 72.0ºC for 5 minutes. Ladder displayed is Hyper 25 BP ladder (Bioline).

reverse primer is zero except for a published sequence from Texas (EF036664.1) although mismatches (up to two) are found in the probe for some of these sequences (Table 3). A comparison of these primers with streamside salamander sequences from various portions of its range indicate a minimum of four mismatches when the probe is considered, with the interesting exception of one *A. barbouri* sequence from Rutherford Co., TN, which had only one mismatch among all oligos (Table 4). However, 5/13 sequences do have a single mismatch with only the forward and reverse primer (Table 4).

In vitro testing. – Endpoint PCR reactions followed by gel electrophoresis successfully amplified cytochrome b from smallmouth salamander but not sympatric salamander species (Figure 1) following 40 cycles.. Additionally, the amplicon produced with the smallmouth salamander DNA migrated according to the expected size (147 BP, Figure 1).

Laboratory water tests. – Diluted water extracted DNA from both the larvae and adult exposure trials produced strong bands that migrated according to size (Figure 2). Amplicons from the larval tank test were sequenced and produced an amplicon 100% similar to the complete smallmouth salamander cyt b sequenced in this study (Table 3, Supplemental Data).

TABLE 2. Mismatches in smallmouth salamander (*A. texanum*) oligos when compared to cytb sequences of other salamander species. FP = forward primer, RP = reverse primer, P = probe, % sim. = percent similarity of the smallmouth salamander cytb sequence obtained in this project (Acc# OM236537) to the sequence indicated by the accession # in the table, Symp. = the species does or may occur sympatrically with smallmouth salamanders, *In vitro* = the primers were screened in laboratory tissue tests with this species.

Sympatric species	FP mismatches	RP mismatches	P mismatches	% sim.	Seq. accession #	Symp.	In vitro
Ambystoma texanum	0	0	0	-	OM236537	-	-
Ambystoma talpoideum	4	3	3	84.2	NC_039182.1	Y	Y
Ambystoma barbouri	0	2	4	93.4	OL456142	Y	Y
Ambystoma opacum	3	2	4	85.3	KT780868.1	Y	Y
Ambystoma jeffersonianum	2	2	2	86.8	MZ962318	Y	Y
Ambystoma maculatum	5	3	3	84.1	EF036637.1	Y	Y
Ambystoma tigrinum	3	4	3	88.2	OL456143	Y	Y
Notophthalmus viridescens	2	4	5	80.6	AY691731	Y	Y
Eurycea cirrigera	3	2	2	78.1	NC_035494.1	Y	N
Eurycea lucifuga	1	3	6	79.0	KT873718.1	Y	N
Eurycea longicauda	3	3	6	77.4	AY528403.1	Y	N
Eurycea bislineata	1	4	3	78.4	AY528402	Y	N
Desmognathus monticola	5	3	3	79.6	MW319719.1	N	N
Desmognathus ochrophaeus	2	3	3	79.8	MW319718.1	N	N
Desmognathus conanti	3	5	4	79.2	KY659024.1	Y	N
Pseudotriton ruber	4	3	3	78.7	AY728220	Y	N
Pseudotriton montanus	3	4	4	77.7	KR054760.1	Y	N
Gyrinophilus porphyriticus	3	2	6	77.9	AY728230	Y	N
Hemidactylium scutatum	5	5	8	77.2	AY728231	Y	N

DISCUSSION

As anticipated, of the six sympatric Ambystomatid species tested, streamside salamanders possessed a cytochrome b sequence most similar to smallmouth salamanders. The streamside salamander cyt b sequence used for in vitro testing in this study was 93.3% similar to the smallmouth salamander sequence (Table 2). The sufficiency of two mismatches (Table 2) to distinguish these species is similar to that reported by Wilcox et al. (2013) who noted greatly reduced amplification of non-target DNA with one mismatch and nearly no amplification when two mismatches total were present in both F and R primers. Interestingly, the significance of the proximity of a mismatch to the 3' end of the primer has been noted in enhancing specificity (Stadhouders et al. 2010; Whiley and Sloots 2005; Wright et al. 2014). The mismatches between our smallmouth salamander R primer and streamside salamander sequence (OL456142) occur at the 6th and 9th base (Table 1) from the 5' end of the primer (24 BP total) but were still sufficient to produce specificity (Fig. 1). Furthermore, we note that the addition of the probe (Table 3, Supplemental Data) tested in this study *in silico* (four mismatches, Table 2) but not *in vitro*, would provide an additional level of security in preventing the possibility non-specific binding with streamside salamander DNA.

This work underscores the importance of local sequences in assay validation, consistent with recent studies (Czechowski et al. 2021; Goldberg et al. 2016). Variation in mitochondrial genes, and cytochrome b in particular, is consistently observed throughout the range of salamander species (Kuchta et al. 2016; Page et al. 2020; Sweet and Jockusch 2021), creating the potential for both Type I and II errors in eDNA surveys. We utilized locally collected specimens for our in vitro testing and sequenced five of the seven species utilized (Table 2, Supplemental Data) as well as our target species, smallmouth salamander. As expected, our primers work well for published smallmouth salamander sequences from the central portion of their range but not with a published smallmouth salamander sequence from Texas (Table 3), presumably because they are designed based on a specimen collected from Kentucky (Table 1). When our smallmouth salamander primers are compared with published streamside salamander sequences, all sequences compared have a minimum of four mismatches when the probe is included in the comparison with one previously noted exception (Table 4), indicating they should be sufficient to distinguish smallmouth from streamside in most areas of sympatry.

Although tools for monitoring all amphibian species have value, one of the primary advantages of tools enabling eDNA detection of smallmouth salamanders is the deployment of these tools in the detection of this fossorial species in ephemeral breeding ponds where they may occur with other Ambystomatid species and field identification is difficult. The smallmouth salamander range extends throughout much of the central United States (Kraus and Petranka 1989; Petranka 1988) (Figure 3) where it occurs sympatrically with as many as four other Ambystomatid species (Niemiller and Reynolds 2011). As previously noted, the streamside salamander is particularly difficult to distinguish from the smallmouth salamander and these two salamanders occur sympatrically in several portions of their range (Figure 3). The use of these primers presented here should enable the conduction of eDNA studies distinguishing these two species, particularly when used in conjunction with recently published tools for the eDNA detection of streamside salamanders (Witzel et al. 2020).

TABLE 3. Comparison of mismatches between smallmouth salamander oligos (including probe not tested in vitro) and published smallmouth salamander sequences (Bi and Bogart, 2010; Robertson et al., 2006).

	Collection locality	Butler Co, KY	Clarke Co., OH	Erie Co., OH	Montgomery Co., OH	Washington Co., OH	Jay Co., IN	Jennings Co., IN	Wabash Co., IN	Mclennon Co., TX	Essex Co., ON	Essex Co., ON	Essex Co., ON
	Gen Bank Accession Number	OM236537	GU078506.1	EF036641.1	GU078471	EF036656.1	EF036657.1	EF036660.1	EF036659.1	EF036664.1	EF036643.1	EF036648.1	EF036644.1
F	TCAATGAATTTGAGGCGGATTT	0	0	0	0	0	0	0	0	1	0	0	0
R	CCTGTAGGGTTATTAGATCCTGTT	0	0	0	0	0	*	0	0	3	0	0	0
P	ACTCGATTCTTTGCCTTCCACTTCT	0	1	1	1	1	2	1	1	1	1	1	1

*Primer falls outside published sequence.

TABLE 4. Comparison of mismatches between smallmouth salamander oligos (including probe which was not tested in vitro) and published streamside salamander sequences (Bi and Bogart, 2010; Robertson et al., 2006).

	Collection locality	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., OH	Butler Co., OH	Rutherford Co., TN	Livingston Co., KY
	Gen Bank Accession Number	OL456142	GU078501	GU078484	GU078482	GU078496	GU078478	GU078490	GU078470	GU078512	GU078474	GU078511	GU078495	GU078504
F	TCAATGAATTTGAGGCGGATTT	0	0	1	0	0	0	1	0	1	0	0	0	1
R	CCTGTAGGGTTATTAGATCCTGTT	2	2	2	1	1	2	0	2	1	2	2	1	*
P	ACTCGATTCTTTGCCTTCCACTTCT	4	4	4	4	3	3	3	3	4	4	3	0	4

*Primer falls outside published sequence.

TABLE 5. Salamander specimens used in in vitro specificity testing. All localities are in the state of Kentucky, the county in which the individual was collected appears in table.

Species	Collection locality	DNA conc. (µg/ml)
A. tigrinum	Warren	680
A. maculatum	Rowan	133
A. barbouri	Madison	449
A. opacum	Hart	261
A. jeffersonianum	Powell	154
A. talpoideum	Logan	354
A. texanum	Butler	261
N. viridescens	Powell	189

Recent works have emphasized the need for thorough specificity testing validation (Goldberg et al. 2016; Klymus et al. 2020; Loeza-Quintana et al. 2020). The assays presented here and tested, both *in silico*, *in vitro*, and in laboratory water exposure tests should serve as valuable tools enabling the detection of this widespread salamander species.

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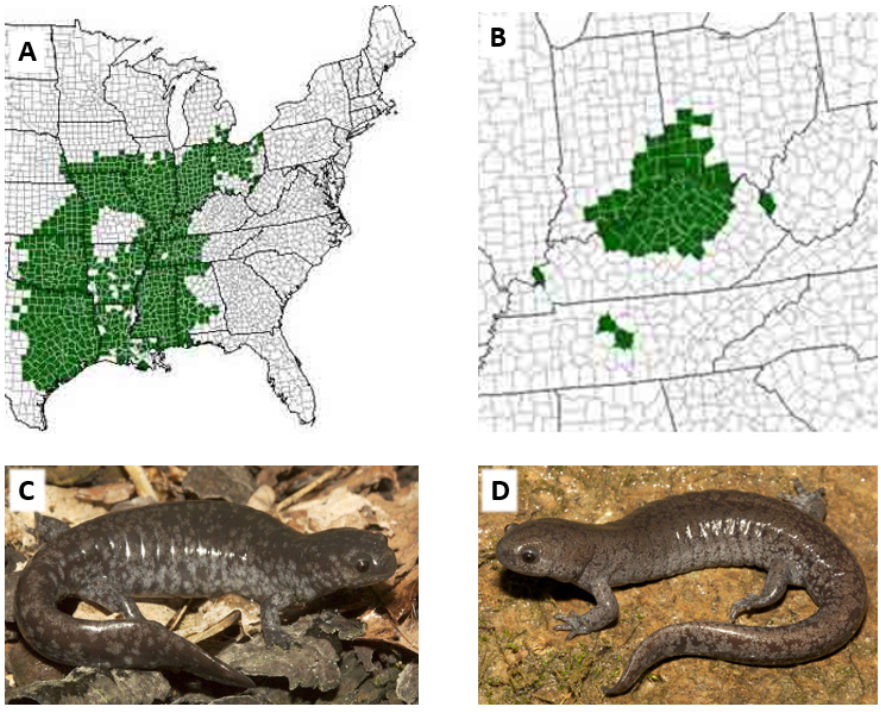

FIG. 3. (A) Range of smallmouth salamanders (courtesy of USGS and Ohio Amphibians.com). (B) Range map of streamside salamanders (courtesy of USGS and Ohio Amphibians.com). (C) Smallmouth salamander from central Indiana (photo courtesy of Todd Pierson). (C) Range map of streamside salamanders (courtesy of USGS and Ohio Amphibians.com). (D) Streamside salamander from southeastern Indiana (photo courtesy of Todd Pierson).

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Supplemental Data

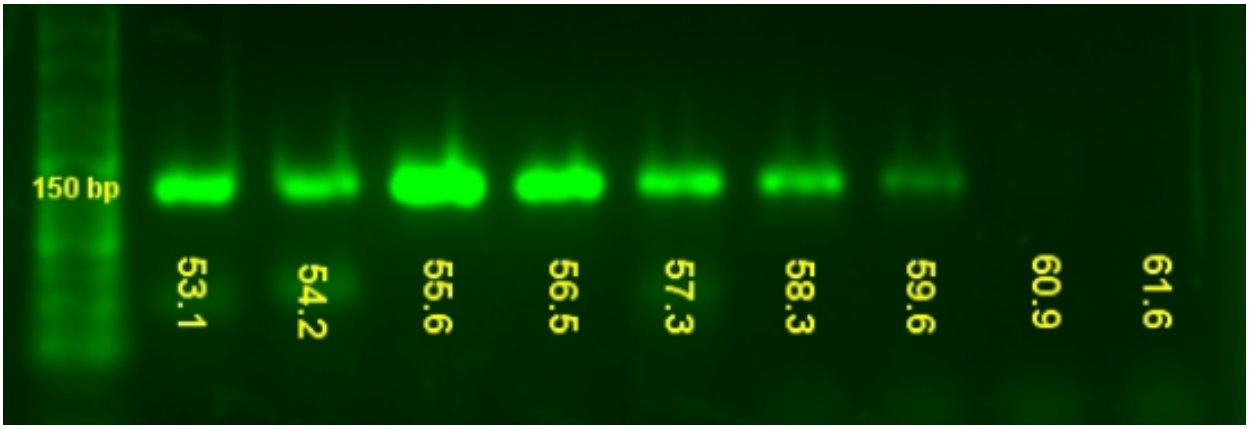

FIG. S1. Gradient reaction run to optimize annealing temperature. Template DNA consisted of water extracted DNA from smallmouth salamander tank tests. 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 6.75 µl nuclease free water, 3.75 µl filtered water extracted DNA and 2.0 µl of F and R primers. Cycling conditions consisted of an initial denaturation stage of 95.0ºC for 2 minutes followed by 55 cycles of 95.0ºC for 30 s, annealing temperature shown on figure for 30 s, 72.0ºC extension for 30 s with a final extension of 72.0ºC for 5 minutes. Ladder displayed is Hyper 25 BP ladder (Bioline).

TABLE S1. Forward and reverse primer pairs and probe developed for smallmouth salamander. Probe sequence is included here but was not tested in vitro or in laboratory water exposure tests.

Amplicon length (BP)	Oligo	Sequence (5'-3')
147	F	TCAATGAATTTGAGGCGGATTT
	R	CCTGTAGGGTTATTAGATCCTGTT
	P	TGTAGCCCATATTTGCCGAGACGT

TABLE S2. Ambystomid species and Eastern Red-Spotted Newt used in in vitro specificity test. All specimens were collected in Kentucky.

Species	**Collection locality**	**Cyt b sequenced**	**Length**	**G.B. accession #**
Ambystoma texanum	Butler Co.	Y	744	OM236537
Ambystoma talpoideum	Logan Co.	N	-	-
Ambystoma jeffersonianum	Powell Co.	Y	749	MZ962318
Ambystoma barbouri	Madison Co.	Y	935	OL456142
Ambystoma maculatum	Rowan Co.	N	-	-
Ambystoma opacum	Powell Co.	Y	720	KT780868.1
Ambystoma tigrinum	Warren Co.	Y	782	OM289824
Notophthalmus viridescens	Powell Co.	Y	272	MZ962319

TABLE S3. Amplicon produced with *A. texanum* primers from water samples taken during the laboratory tanks tests. Bold blue bases represent F and R primers.

Length	Sequence
147 BP	**TCAATGAATTTGAGGCGGATTT**TCAGTTGACAAAGCTA CCTTAACTCGATTCTTTGCCTTCCACTTCTTATTTCCATTC TTAATTGCAGGAACAAGCATTATTCATCTCCTTTTTCTTCA CGA**AACAGGATCTAATAACCCTACAGG**