Tennessee Journal of Herpetology, Vol. 6, pp. 4-17, 2023
ISSN 2576-9340

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

Elizabeth K. Strasko*, Rebecca R. Piche, Ben F. Brammell

Department of Science and Health, Asbury University, Wilmore, KY 40390
*Corresponding Author email: elizabeth.strasko214@topper.wku.edu

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

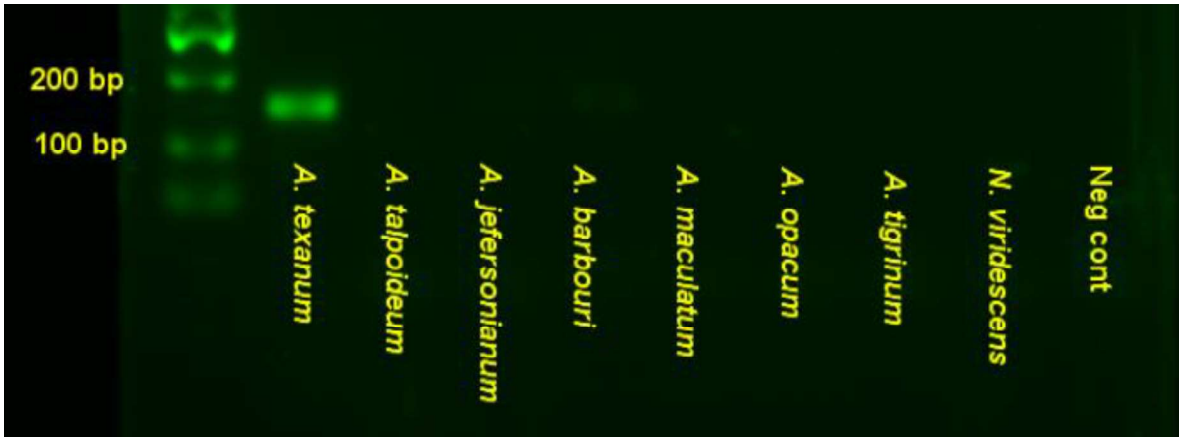

FIG. 1. Tissue extracted DNA specificity tests for smallmouth salamander (*A. texanum*) primers using tissue extracts of six sympatric or potentially sympatric species of *Ambystoma* species and eastern newt (*Notophthalmus viridescens*). 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 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).

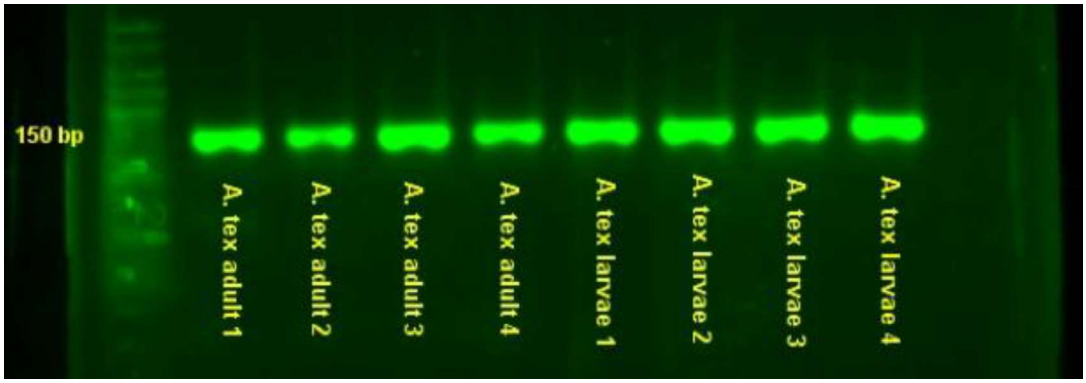

FIG. 2. Filtered water extracted DNA reactions run with smallmouth salamander (*A. texanum*) primers. Lanes 2 – 5 are replicates of the adult water test and Lanes 6 – 9 are replicates of the larval water test. 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 6.75 µl nuclease free water, 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.

Acknowledgements.—We sincerely thank Bret Kuss for providing *A. texanum* and *A. tadpoideum* specimens for this project. Additionally, we thank Jarrett Johnson (WKU) and Cy Mott (EKU) for providing sympatric species tissue for use in in vitro testing. Salamander tissue was collected under Kentucky Department of Fish and Wildlife Service Permit Number SC2111188. Research was funded by an internal faculty development grant from Asbury University (Ben Brammell, Fall 2021). The Asbury University Department of Science and Health provided additional support.

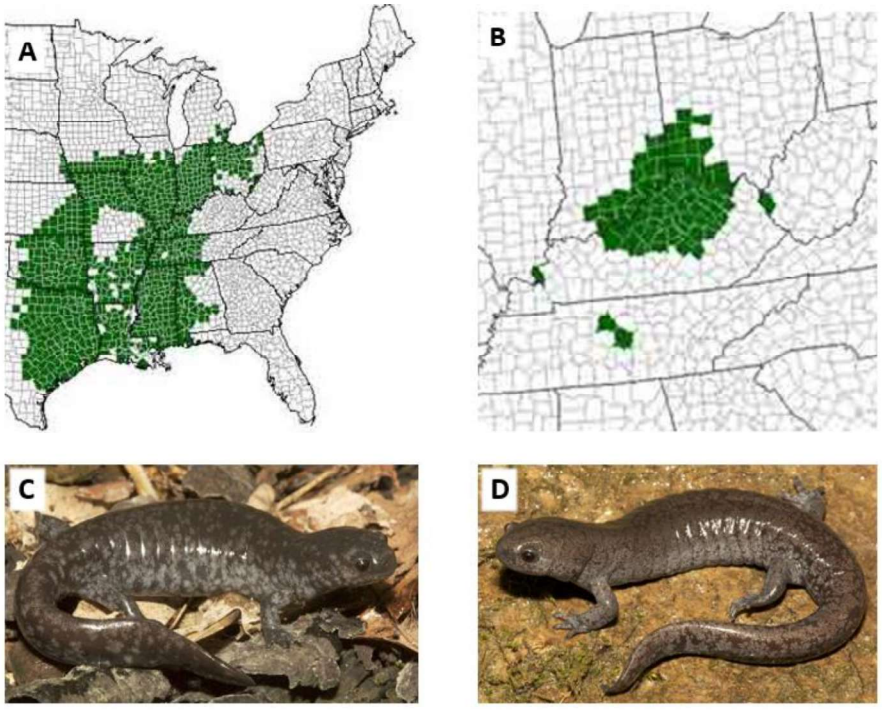

FIG. 3. (A) Range of smallmouth salamanders (courtesy of USGS and Ohio Amphibians.com). (B) Range map of streamside salamanders (courtesy of USGS and Ohio Amphibians.com). (C) Smallmouth salamander from central Indiana (photo courtesy of Todd Pierson). (C) Range map of streamside salamanders (courtesy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

LITERATURE CITED

Adrian-Kalchhauser, I., and P. Burkhardt-Holm. 2016. An eDNA assay to monitor a globally invasive fish species from flowing freshwater. PLoS ONE 11.

Bell, F.F., A.F. Flores, K.L. Sena, T.A. Maigret, C.J. Leow, R.B. Sams, D.K. Peyton, and Ben.F. Brammell. 2022. Development and validation of qPCR assays for use in eDNA detection of southern two-lined (Eurycea cirrigera) and northern dusky (Desmognathus fuscus) salamanders. Herpetological Conservation and Biology In Press, April 2022.

Bi, K., and J.P. Bogart. 2010. Time and time again: Unisexual salamanders (genus Ambystoma) are the oldest unisexual vertebrates. BMC Evolutionary Biology 10.

Brian T. Miller, and Joyce L. Miller. 2019. Gross morphology of teeth on the premaxillae of Streamside Salamanders (Ambystoma barbouri) and Small-mouthed Salamanders (Ambystoma texanum) from middle Tennessee. Tennessee Journal of Herpetology 2:20–24.

Carim, K.J., K.R. Christianson, K.M. McKelvey, W.M. Pate, D.B. Silver, B.M. Johnson, B.T. Galloway, M.K. Young, and M.K. Schwartz. 2016. Environmental DNA marker development with sparse biological information: A case study on opossum shrimp (Mysis diluviana). PLoS ONE.

Coghlan, S.A., C.A. Currier, J. Freeland, T.J. Morris, and C.C. Wilson. 2021. Community eDNA metabarcoding as a detection tool for documenting freshwater mussel (Unionidae) species assemblages. Environmental DNA 3.

Collins, J.P. 2010. Amphibian decline and extinction: What we know and what we need to learn. Diseases of Aquatic Organisms 92.

Czechowski, P., M. de Lange, M. Heldsinger, W. Rayment, C. Hepburn, M. Ladds, and M. Knapp. 2021. Environmental DNA analysis needs local reference data to inform taxonomy-based conservation policy – A case study from Aotearoa / New Zealand. bioRxiv 2021.10.22.465527.

Darling, J.A., and A.R. Mahon. 2011. From molecules to management: Adopting DNA-based methods for monitoring biological invasions in aquatic environments. Environmental Research 111:978–988.

Davy, C.M., A.G. Kidd, and C.C. Wilson. 2015. Development and Validation of Environmental DNA (eDNA) Markers for Detection of Freshwater Turtles. PLOS ONE 10:e0130965-.

Dejean, T., A. Valentini, C. Miquel, P. Taberlet, E. Bellemain, and C. Miaud. 2012. Improved detection of an alien invasive species through environmental DNA barcoding: The example of the American bullfrog Lithobates catesbeianus. Journal of Applied Ecology.

Dougherty, M.M., E.R. Larson, M.A. Renshaw, C.A. Gantz, S.P. Egan, D.M. Erickson, and D.M. Lodge. 2016. Environmental DNA (eDNA) detects the invasive rusty crayfish Orconectes rusticus at low abundances. Journal of Applied Ecology.

Dysthe, J.C., T.W. Franklin, K.S. McKelvey, M.K. Young, and M.K. Schwartz. 2018. An improved environmental DNA assay for bull trout (Salvelinus confluentus) based on the ribosomal internal transcribed spacer. PLoS ONE 13.

Eichmiller, J.J., P.G. Bajer, and P.W. Sorensen. 2014. The Relationship between the Distribution of Common Carp and Their Environmental DNA in a Small Lake. 9:1–8.

Fisher, M.C., and T.W.J. Garner. 2020. Chytrid fungi and global amphibian declines. Nature Reviews Microbiology. Volume 18.

Garcia, T.S., and A. Sih. 2003. Color change and color-dependent behavior in response to predation risk in the salamander sister

species Ambystoma barbouri and Ambystoma texanum. Oecologia 137.

Garcia, T.S., R. Straus, and A. Sih. 2003. Temperature and ontogenetic effects on color change in the larval salamander species Ambystoma barbouri and Ambystoma texanum. Canadian Journal of Zoology 81.

Gargan, L.M., T. Morato, C.K. Pham, J.A. Finarelli, J.E.L. Carlsson, and J. Carlsson. 2017. Development of a sensitive detection method to survey pelagic biodiversity using eDNA and quantitative PCR: a case study of devil ray at seamounts. Marine Biology.

Goldberg, C.S., C.R. Turner, K. Deiner, K.E. Klymus, P.F. Thomsen, M.A. Murphy, S.F. Spear, A. McKee, S.J. Oyler-McCance, R.S. Cornman, et al. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution. Volume 7.

Guivas, R.A., and B.F. Brammell. 2020. 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.

Hinz, S., Jennifer Coston-Guarini, Michael Marnane, and Jean-Marc Guarini. 2022. Evaluating eDNA for Use within Marine Environmental Impact Assessments. Journal of Marine Science and Engineering 3:375.

Ikeda, K., H. Doi, K. Tanaka, T. Kawai, and J.N. Negishi. 2016. Using environmental DNA to detect an endangered crayfish Cambaroides japonicus in streams. Conservation Genetics Resources.

Jerde, C.L., A.R. Mahon, W.L. Chadderton, and D.M. Lodge. 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters.

Kaganer. 2021. Development and application of molecular methods to enhance understanding of the amphibian-emerging infectious disease pathobiome, Thesis. Cornell .

Klymus, K.E., N.T. Marshall, and C.A. Stepien. 2017. Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. PLoS ONE 12.

Klymus, K.E., C.M. Merkes, M.J. Allison, C.S. Goldberg, C.C. Helbing, M.E. Hunter, C.A. Jackson, R.F. Lance, A.M. Mangan, E.M. Monroe, et al. 2020. Reporting the limits of detection and quantification for environmental DNA assays. Environmental DNA 2:271–282.

Kraus, F., and J.W. Petranka. 1989. A New Sibling Species of Ambystoma from the Ohio River Drainage. Copeia 1989.

Kuchta, S.R., M. Haughey, A.H. Wynn, J.F. Jacobs, and R. Highton. 2016. Ancient river systems and phylogeographical structure in the spring salamander, Gyrinophilus porphyriticus. Journal of Biogeography 43.

Langlois, V.S., M.J. Allison, L.C. Bergman, T.A. To, and C.C. Helbing. 2020. The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. Environmental DNA 3:519–527.

Laramie, M.B., D.S. Pilliod, and C.S. Goldberg. 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. Biological Conservation.

Loeza-Quintana, T., C.L. Abbott, D.D. Heath, L. Bernatchez, and R.H. Hanner. 2020. Pathway to Increase Standards and Competency of eDNA Surveys (PISCeS)—Advancing collaboration and standardization efforts in the field of eDNA. Environmental DNA 2:255–260.

Maruyama, A., K. Nakamura, H. Yamanaka, M. Kondoh, and T. Minamoto. 2014. The release rate of environmental DNA from juvenile and adult fish. PLoS ONE 9.

Mauvisseau, Q., S. Tönges, R. Andriantsoa, F. Lyko, and M. Sweet. 2019. Early detection of an emerging invasive species: EDNA monitoring of a parthenogenetic crayfish in freshwater systems. Management of Biological Invasions 10.

McCallum, M.L. 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. Journal of Herpetology 41.

Miller, B.T. and J. L. Miller. 2019. Gross morphology of teeth on the premaxillae of Streamside Salamanders (*Ambystoma barbouri*) and Small-mouthed Salamanders (*Ambystoma texanum*) from middle Tennessee. Tennessee Journal of Herpetology 2:20–24.

Niemiller, M.L., and R.G. Reynolds. 2011. The amphibians of Tennessee. University of Tennessee Press.

Page, R.B., C. Conarroe, D. Quintanilla, A. Palomo, J. Solis, A. Aguilar, K. Bezold, A.M. Sackman, and D.M. Marsh. 2020. Genetic variation in Plethodon cinereus and Plethodon hubrichti from in and around a contact zone. Ecology and Evolution 10.

Paine, R.T.R., C.R. Hurt, and H.T. Mattingly. 2021. Monitoring a minuscule madtom: Environmental DNA surveillance of the endangered pygmy madtom (Noturus stanauli Etnier & Jenkins 1980) in the Duck and Clinch rivers, Tennessee. Environmental DNA 3.

Petranka, J.W. 1988. Salamanders of the United States. Smithsonian Institution Press, Washington, D.C.

Petranka, J.W., and A. Sih. 1987. Habitat Duration, Length of Larval Period, and the Evolution of a Complex Life Cycle of a Salamander, Ambystoma texanum. Evolution 41.

Piaggio, A.J., R.M. Engeman, M.W. Hopken, J.S. Humphrey, K.L. Keacher, W.E. Bruce, and M.L. Avery. 2014. Detecting an elusive invasive species: A diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. Molecular Ecology Resources.

Pierson, T.W., A.M. McKee, S.F. Spear, J.C. Maerz, C.D. Camp, and T.C. Glenn. 2016. Detection of an Enigmatic Plethodontid Salamander Using Environmental DNA. Copeia 2016:78–82.

Robertson, A. v., C. Ramsden, J. Niedzwiecki, J. Fu, and J.P. Bogart. 2006. An unexpected recent ancestor of unisexual Ambystoma. Molecular Ecology 15.

Robson, H.L.A., T.H. Noble, R.J. Saunders, S.K.A. Robson, D.W. Burrows, and D.R. Jerry. 2016. Fine-tuning for the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystems. Molecular ecology resources 16.

Roe, B.A., D.P. Ma, R.K. Wilson, and J.F.H. Wong. 1985. The complete nucleotide sequence of the Xenopus laevis mitochondrial genome. Journal of Biological Chemistry 260:9759–9774.

Roussel, J.M., J.M. Paillisson, A. Tréguier, and E. Petit. 2015. The downside of eDNA as a survey tool in water bodies. Journal of Applied Ecology 52:823–826.

Rusch, J.C., M. Mojžišová, D.A. Strand, J. Svobodová, T. Vrålstad, and A. Petrusek. 2020. Simultaneous detection of native and invasive crayfish and Aphanomyces astaci from environmental DNA samples in a wide range of habitats in Central Europe. NeoBiota 58.

Sawaya, N.A., A. Djurhuus, C.J. Closek, M. Hepner, E. Olesin, L. Visser, C. Kelble, K. Hubbard, and M. Breitbart. 2019. Assessing eukaryotic biodiversity in the Florida Keys National Marine Sanctuary through environmental DNA metabarcoding. Ecology and Evolution 9:1029–1040.

Schmidt, B.C., S.F. Spear, A. Tomi, and C.M.B. Jachowski. 2021. Evaluating the efficacy of environmental DNA (eDNA) to

detect an endangered freshwater mussel Lasmigona decorata (Bivalvia:Unionidae). Freshwater Science 40.

Secondi, J., T. Dejean, A. Valentini, B. Audebaud, and C. Miaud. 2016. Detection of a global aquatic invasive amphibian, Xenopus laevis, using environmental DNA. Amphibia Reptilia.

Sigsgaard, E.E., H. Carl, P.R. Møller, and P.F. Thomsen. 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biological Conservation.

Spear, S.F., J.D. Groves, L.A. Williams, and L.P. Waits. 2015. Using environmental DNA methods to improve detectability in a hellbender (Cryptobranchus alleganiensis) monitoring program. Biological Conservation.

Stadhouders, R., S.D. Pas, J. Anber, J. Voermans, T.H.M. Mes, and M. Schutten. 2010. The effect of primer-template mismatches on the detection and quantification of nucleic acids using the 5′ nuclease assay. Journal of Molecular Diagnostics 12.

Sweet, S.S., and E.L. Jockusch. 2021. A New Relict Species of Slender Salamander (Plethodontidae: Batrachoseps) with a Tiny Range from Point Arguello, California. Ichthyology and Herpetology 109.

Takahara, T., T. Minamoto, H. Yamanaka, H. Doi, and Z. Kawabata. 2012. Estimation of fish biomass using environmental DNA. PLoS ONE 7.

Whiley, D.M., and T.P. Sloots. 2005. Sequence variation in primer targets affects the accuracy of viral quantitative PCR. Journal of Clinical Virology 34.

Wilcox, T.M., K.S. McKelvey, M.K. Young, S.F. Jane, W.H. Lowe, A.R. Whiteley, and M.K. Schwartz. 2013. Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. PLoS ONE 8.

Witzel, N.A., A. Taheri, B.T. Miller, R.H. Hardman, D.I. Withers, S.F. Spear, and W.B. Sutton. 2020. Validation of an environmental DNA protocol to detect a stream-breeding amphibian, the Streamside Salamander (Ambystoma barbouri). Environmental DNA 2.

Wright, E.S., L.S. Yilmaz, S. Ram, J.M. Gasser, G.W. Harrington, and D.R. Noguera. 2014. Exploiting extension bias in polymerase chain reaction to improve primer specificity in ensembles of nearly identical DNA templates. Environmental Microbiology 16.

Xia, Z., A. Zhan, M.L. Johansson, E. Deroy, G. Douglas, and H. Hugh. 2021. Screening marker sensitivity : Optimizing eDNA-based rare species detection. Diversity and Distributions 1–8.

Xia, Z., A. Zhan, Y. Gao, L. Zhang, G.D. Haffner, and H.J. MacIsaac. 2018. Early detection of a highly invasive bivalve based on environmental DNA (eDNA). Biological Invasions.

Xing, Y., Wanru Gao, Zhixin Shen, Yuanyuan Zhang, Jie Bai, Xingwei Cai, Jilong Ouyang, and Yahui Zhao. 2022. A Review of Environmental DNA Field and Laboratory Protocols Applied in Fish Ecology and Environmental Health. Frontiers in Environmental Science 73.

Supplemental Data

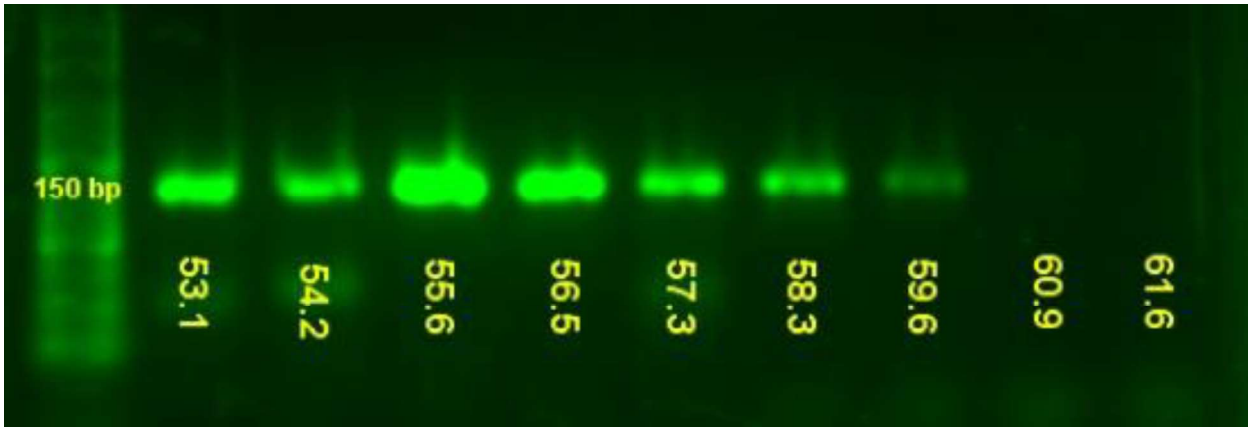

FIG. S1. Gradient reaction run to optimize annealing temperature. Template DNA consisted of water extracted DNA from smallmouth salamander tank tests. 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 6.75 µl nuclease free water, 3.75 µl filtered water extracted DNA and 2.0 µl of F and R 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**