Herpetological Conservation and Biology 17(2):398–412.
Submitted: 6 September 2021; Accepted: 26 March 2022; Published: 31 August 2022.

Development and Validation of qPCR Assays for Use in eDNA Detection of Southern Two-lined (*Eurycea cirrigera*) and Northern Dusky (*Desmognathus fuscus*) Salamanders

Florene G. Bell[1], Angie F. Flores[1], Kenton L. Sena[2], Thomas A. Maigret[3], Chi Jing Leow[1], Ronald B. Sams[1], David K. Peyton[4], and Ben F. Brammell[1,5]

[1]*Department of Science and Health, 1 Macklem Drive, Asbury University, Wilmore, Kentucky 40390, USA*
[2]*Lewis Honors College, 1120 University Drive, University of Kentucky, Lexington, Kentucky 40526, USA*
[3]*Department of Biology, 101 T.H. Morgan Building, University of Kentucky, Lexington, Kentucky 40506, USA*
[4]*Department of Biology & Chemistry, 103 Lappin Hall, Morehead State University, Morehead, Kentucky 40351, USA*
[5]*Corresponding author, e-mail: ben.brammell@asbury.edu*

Abstract.—**Environmental DNA (eDNA) uses DNA shed from organisms into the environment to detect their presence and provides an effective, non-invasive method to survey organisms in an efficient manner. Recent works have emphasized the need for careful development and both laboratory and field validation of eDNA assays. We developed species specific qPCR assays for Southern Two-lined (*Eurycea cirrigera*) and Northern Dusky (*Desmognathus fuscus*) salamanders, two salamanders found throughout much of the eastern USA. We designed primers and probes based on sequences obtained from locally collected specimens and screened for specificity against 19 salamander species that occur sympatrically with these species in various parts of their range. We collected 38 water samples from streams in a minimally disturbed forest in eastern Kentucky, USA, and we analyzed samples for both *E. cirrigera* and *D. fuscus* DNA. There were 16 samples that were positive for *E. cirrigera* and 21 for *D. fuscus* DNA. We cloned and sequenced four *E. cirrigera* and five *D. fuscus* amplicons from filtered water to verify identity. These data add to the growing pool of knowledge concerning eDNA monitoring of salamander species and provide useful reference data as well as valuable molecular tools for future monitoring and range delineation studies.**

Key Words.—Appalachia; *Desmognathus fuscus*; eDNA; *Eurycea cirrigera*; salamander

Introduction

In the past decade, environmental DNA (eDNA) has become firmly established as an effective method for detecting the presence of 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. 2018; Sawaya et al. 2019). The number of studies using species-specific markers to detect macroorganisms from water samples increased from one in 2008 to 56 in 2019 (Xia et al. 2021). Although the largest percentage of these studies conducted to date address fish populations (50.3%, 195 studies), the second most frequent target were amphibians (20.4%, 79 studies), followed by mussels, crustaceans, and then other invertebrates (Xia et al. 2021).

Recent works have brought to the forefront the need for high quality, carefully tested assays for use in species-specific marker studies (Klymus et al. 2020; Xia et al. 2021). Validation of primers and probes *in silico*, *in vitro*, and *in situ* is essential to confirm specificity of markers before use (Darling and Mahon 2011; Goldberg et al. 2011; Roussel et al. 2015; Klymus et al. 2020; Langlois et al. 2020). Given the time required to design and validate these markers, the availability of pre-validated markers should greatly facilitate eDNA studies. Xia et al. (2021) report that only 30.4% of specific marker studies published between 2008 and 2019 use previously developed markers, likely a result of the paucity of available markers. Additionally, the availability of multiple markers for a single species is advantageous given the genetic variability in sympatric species, and therefore potential for false positives (amplification of DNA of a sympatric species) and false negatives (failure to amplify target species DNA as a result of regional variation) at any given location (Wilcox et al. 2013). Markers developed for members of a species in one location may not function with members of that same species from other parts of their range (Kaganer 2022). Clearly, species-specific markers that have been through a rigorous screening and validation process to prevent both false positives and negatives are of great value that extends well beyond their time and location of origin (Xia et al. 2021).

We investigated the use of eDNA in the detection of two widely distributed salamander species in the eastern U.S.: the Southern Two-lined Salamander (*Eurycea cirrigera*) and the Northern Dusky Salamander (*Desmognathus fuscus*) through the development and testing *in silico*, *in vitro*, and *in situ* of primers and probes specific to these species. Both salamander species frequently occur in high densities throughout much of their range (Bank et al. 2006; Nowakowski and Maerz 2009). Their widespread distribution and abundance make them likely candidates for much needed studies examining anthropogenic impacts on salamander communities, ranging from local effects to climate change induced range alterations. Currently published studies report local impacts on both salamander species (Bank et al. 2006; Barrett and Guyer 2008; Price et al. 2006, 2011; Munshi-South et al. 2013). Successful development and validation of molecular tools for eDNA detection of these species will greatly facilitate future studies.

Eurycea cirrigera is a widely distributed salamander found from northern Indiana and Illinois throughout the southeast to northern Florida and eastern Louisiana (Muenz et al. 2008). *Eurycea cirrigera* is often the most abundant salamander species in low order streams where larval densities have been reported to reach 72 individuals/m^2 (Nowakowski and Maerz 2009). Adult and juvenile *E. cirrigera* specimens occupy stream margins and nearby terrestrial environments (MacCulloch and Bider 1975; Crawford and Semlitsch 2007) while larvae are completely aquatic (Duellman and Wood 1954) with a 2–3 y larval period (Richmond 1945). Although no widespread declines in *E. cirrigera* have been reported, declines in adult or larval abundance in localized areas have been reported in correlation with altered hydrology associated with urban development (Barrett and Guyer 2008; Price et al. 2011), increasing impervious surface cover (Miller et al. 2007), and other forms of habitat disturbance (Willson and Dorcas 2003), highlighting their sensitivity to anthropogenic impact.

Desmognathus fuscus is a widespread species found in suitable habitat throughout eastern North America northward from eastern Tennessee and South Carolina (Bank et al. 2006). Preferred habitat includes seeps, runs, and small streams that are free of predatory fish (Hulse 2001). *Desmognathus fuscus* remains closely tied to the water throughout their life, following an aquatic larval period of 9–14 mo (Petranka 1988). They do forage nocturnally outside water but are typically found within 2 m of the water source (Grover 2000; Hulse 2001). Although no widespread declines in *D. fuscus* populations are reported, local declines associated with the stocking of non-native salmonids, fungal pathogens, substrate impactedness, urbanization, and pollution are reported or suspected (Bank et al. 2006; Price et al. 2006, 2011; Munshi-South et al. 2013).

The molecular tools provided by this work will facilitate quantification of the presence and perhaps the abundance of these organisms, enabling future conservation efforts related to these widespread and ubiquitous species which serve as effective sentinels of anthropogenic impact. These assays have been designed based on sequences obtained from organisms within the study area and validated *in silico*, *in vitro*, and *in situ* as universally recommended (Darling and Mahon 2011; Roussel et al. 2015; Klymus et al. 2017; Langlois et al. 2020; Xia et al. 2021), providing ready-made tools which will save future researchers time and effort.

Materials and Methods

Tissue collection and sequencing.—We collected tissue from an adult *E. cirrigera* and *D. fuscus* captured at our field validation site in Robinson Forest (Breathitt and Knott counties, Kentucky, USA). Additionally, we collected DNA from 14 other sympatric or potentially sympatric salamander species captured either in Robinson Forest or central Kentucky. 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. We amplified portions of cyt b from Robinson Forest collected target species using published primers (Roe et al. 1985; *E. cirrigera*, GenBank ID: MZ485475, 380 BP; *D. fuscus*, GenBank ID: MZ485476, 633 BP). Sequences were run in triplicate and completed by ACGT, Inc. (www.actginc.com).

Assay development and testing.—We aligned these partial cytb sequences with 19 potential sympatric Kentucky salamander species using MegaX and Clustal W. We designed F and R primer pairs using PrimerQuest software (IDT) and aligned these with sympatric or potentially sympatric species to verify specificity (Tables 1 and 2). All primers have at least three mismatches in the F or R primer. We assigned accession numbers of cyt b sequences used in alignments (Tables 1 and 2).

We evaluated F and R primers via a temperature gradient approach to determine optimal annealing temperature (57–60° C for both primer sets). For *in vitro* testing, we ran end-point PCR on tissue extracts of eleven sympatric salamander species. Twenty-five 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 95.0° C for 2 min followed by 40 cycles of 95.0° C for 45 s, 57.0° C for 60 s, and 72.0° C for 60 s.

Table 1. Mismatches in the Southern Two-lined Salamander (*Eurycea cirrigera*) oligos and sympatric or potentially sympatric salamander species in Kentucky, USA. Abbreviations are FP = forward primer, RP = reverse primer, P = probe, % sim. = percentage similarity of the *E. cirrigera* cytb sequence obtained in this project (Accession# MZ485475) to the sequence indicated by the accession # in the table, Symp. = the species does or may occur in the study area, *In vitro* = the primers were screened in laboratory tissue tests with this species, Y = yes, and N = no. Species are the Cave Salamander (*Eurycea lucifuga*), the Longtail Salamander (*Eurycea longicauda*), the Northern Two-Line Salamander (*Eurycea bislineata*), the Northern Dusky Salamander (*Desmognathus fuscus*), the Seal Salamander (*Desmognathus monticola*), the Allegheny Mountain Dusky Salamander (*Desmognathus ochrophaeus*), the Black Mountain Salamander (*Desmognathus welteri*), the Spotted Dusky Salamander (*Desmognathus conanti*), the Northern Red Salamander (*Pseudotriton ruber*), the Mud Salamander (*Pseudotriton montanus*), the Spring Salamander (*Gyrinophilus porphyriticus*), the Four-toed Salamander (*Hemidactylium scutatum*), the Northern Slimy Salamander (*Plethodon glutinosus*), the Northern Zigzag Salamander (*Plethodon dorsalis*), the Ravine Salamander (*Plethodon richmondi*), the Streamside Salamander (*Ambystoma barbouri*), the Marbled Salamander (*Ambystoma opacum*), the Jefferson Salamander (*Ambystoma jeffersonianum*), the Spotted Salamander (*Ambystoma maculatum*), and the Eastern Newt (*Notophthalmus viridescens*).

Sympatric species	FP mismatches	RP mismatches	P mismatches	% sim.	Seq. accession #	Symp.	*In vitro*
Eurycea cirrigera	0	0	1	99.2	NC_035494.1	-	-
Eurycea lucifuga	3	0	1	88.7	KT873718.1	N	Y
Eurycea longicauda	2	1	2	88.1	AY528403.1	Y	N
Eurycea bislineata	4	2	1	91.8	AY528402	N	N
Desmognathus fuscus	3	5	4	80.6	MZ485476	Y	Y
Desmognathus monticola	5	6	4	78.8	MZ418126	Y	N
Desmognathus ochrophaeus	5	6	6	78.8	EU314289	Y	N
Desmognathus welteri	6	6	5	79.1	EU314293	Y	N
Desmognathus conanti	5	6	5	79.7	EU314275.1	Y	N
Pseudotriton ruber	2	4	6	83.5	AY728220	Y	Y
Pseudotriton montanus	3	3	6	82.4	KR054760.1	Y	Y
Gyrinophilus porphyriticus	4	4	5	79.1	AY728230	Y	Y
Hemidactylium scutatum	5	4	2	79.6	AY728231	Y	Y
Plethodon glutinosus	6	4	7	78.0	MN723529.1	Y	Y
Plethodon dorsalis	5	4	6	79.4	GQ464404	N	Y
Plethodon richmondi	4	8	9	75.5	AY378072	Y	N
Ambystoma barbouri	6	6	3	81.7	GU078513.1	N	Y
Ambystoma opacum	5	8	1	81.4	KT780868.1	Y	Y
Ambystoma jeffersonianum	7	6	4	80.6	KT780869.1	N	Y
Ambystoma maculatum	6	8	5	77.9	EF036637.1	Y	Y
Notophthalmus viridescens	6	6	2	80.1	AY691731	Y	Y

Once primer specificity was verified *in vitro*, we designed probes using PrimerQuest and assays (F and R primer and probe) were ordered (Table 3). Probes contained a 5'FAM reporter dye and 3' ZEN/Iowa Black FQ quencher and were ordered from Integrated DNA Technologies (IDT; Coralville, Iowa, USA). We determined assay sensitivity using five-fold serial dilutions of target species tissue extracted DNA (5,000, 500, 50, 5, and 0.5 pg/l); three technical replicates were run for each DNA concentration (Supplemental Information Table S1). We used synthetic DNA to further assess assay sensitivity as recommended to enable standardization and interlaboratory comparison (Hobbs et al. 2019; Klymus et al. 2020). We ordered double-stranded synthetic gBlocks DNA from IDT consisting of the full amplicon from our locally sequenced salamander DNA as well as additional bases added to exceed the minimum recommended lengths (Supplemental Information Table S2). We ran both tissue and synthetic DNA standard curves once independent of the field collected sample qPCR analysis.

We resuspended stock gBlocks in IDTE, pH 8 (IDT) and serially diluted them in nuclease free water (IDT) to produce a range of eight synthetic DNA concentrations from 10^8 to 0.1 copies/μL. We ran 2 μL of each dilution in qPCR reactions with four technical replicates. The final range tested per reaction was therefore 0.2 to 2×10^8 copies/μL. Each 20 μl reaction contained the following: TaqMan EMM (10 μL), nuclease free water (7 μL), resuspended gBlock (2 μL), and assay (1 μL). Thermocycler conditions were as follows: 95° C for 10 min, 55 cycles of 95° C for 15 s, and 60° C for 1 min.

Table 2. Mismatches in *D. fuscus* oligos and sympatric species. FP = forward primer, RP = reverse primer, P = probe, % sim. = percent similarity of the *D. fuscus* cytb sequence obtained in this project (Acc #: MZ485476) to the sequence indicated by the accession # in the table, Symp. = the species does or may occur in the study area, *In vitro* = the primers were screened in laboratory tissue tests with this species, Y = yes, and N = no. Species are the Seal Salamander (*Desmognathus monticola*), the Allegheny Mountain Dusky Salamander (*Desmognathus ochrophaeus*), the Black Mountain Salamander (*Desmognathus welteri*), the Spotted Dusky Salamander (*Desmognathus conanti*), the Southern Two-lined Salamander (*Eurycea cirrigera*), the Cave Salamander (*Eurycea lucifuga*), the Longtail Salamander (*Eurycea longicauda*), the Northern Red Salamander (*Pseudotriton ruber*), the Mud Salamander (*Pseudotriton montanus*), the Spring Salamander (*Gyrinophilus porphyriticus*), the Four-toed Salamander (*Hemidactylium scutatum*), the Northern Slimy Salamander (*Plethodon glutinosus*), the Northern Zigzag Salamander (*Plethodon dorsalis*), the Ravine Salamander (*Plethodon richmondi*), the Streamside Salamander (*Ambystoma barbouri*), the Marbled Salamander (*Ambystoma opacum*), the Jefferson Salamander (*Ambystoma jeffersonianum*), the Spotted Salamander (*Ambystoma maculatum*), and the Eastern Newt (*Notophthalmus viridescens*).

Sympatric species	FP mismatches	RP mismatches	P mismatches	% sim.	Seq. accession #	Symp	*In vitro*
Desmognathus monticola	2	2	1	89.6	AY691738	Y	Y
Desmognathus ochrophaeus	5	2	2	91.1	EU314289	Y	N
Desmognathus welteri	1	2	3	90.0	EU314293	Y	N
Desmognathus conanti	4	2	2	90.9	EU314275.1	Y	N
Eurycea cirrigera	4	6	6	80.0	NC_035494.1	Y	Y
Eurycea lucifuga	6	7	5	78.2	JQ920623	N	Y
Eurycea longicauda	7	6	6	78.8	AY528403.1	Y	N
Pseudotriton ruber	5	4	5	82.3	AY728220	Y	Y
Pseudotriton montanus	5	5	5	80.2	KR054760.1	Y	Y
Gyrinophilus porphyriticus	4	5	5	81.6	AY728230	Y	Y
Hemidactylium scutatum	6	4	3	80.2	AY728231	Y	Y
Plethodon glutinosus	6	2	8	78.5	MN723529.1	Y	Y
Plethodon dorsalis	4	1	5	77.7	GQ464404	N	Y
Plethodon richmondi	4	6	8	75.0	AY378072	Y	N
Ambystoma barbouri	3	6	4	80.1	GU078513.1	N	Y
Ambystoma opacum	4	7	5	79.2	KT780868.1	Y	Y
Ambystoma jeffersonianum	5	6	4	79.2	KT780869.1	N	Y
Ambystoma maculatum	3	7	6	78.7	EF036637.1	Y	Y
Notophthalmus viridescens	5	6	5	79.5	AY691731	Y	Y

We plotted the resulting data against copy number per reaction to determine the limits of detection (LOD) and quantitation (LOQ). We defined LOD as the lowest dilution of a standard curve that resulted in a detection of target DNA with at least one qPCR replicate at a threshold cycle (Ct) of < 45. We defined the LOQ as the last standard dilution in which targeted DNA was detected and quantified in a minimum of 90% of qPCR replicates of the standard curve under a Ct of 45 (Mauvisseau et al. 2019).

Field sampling and processing of eDNA samples.— We collected stream-water samples from Robinson Forest, a minimally disturbed research forest in eastern Kentucky managed by the Department of Forestry and Natural Resources of the University of Kentucky. Robinson Forest hosts a variety of basic and applied research projects, including research investigating the environmental impacts of timber harvest and surface mining on aquatic ecosystems (Maigret et al. 2014; Price et al. 2016; Witt et al. 2016). Both *E. cirrigera* and *D. fuscus* are abundant in Robinson Forest streams

Table 3. Quantitative PCR assays developed for Southern Two-lined Salamanders (*Eurycea cirrigera*; *Ec*) and Northern Dusky Salamanders (*Desmognathus fuscus*; *Df*) from Kentucky, USA. The abbreviation AL is amplicon length.

Target species	AL (BP)	Oligo	Sequence (5'-3')
Ec	96	F	CAGATACCACCTCCGCATTC
		R	TAGAGGCTCCGTTGGTATGA
		P	TGTAGCCCATATTTGCCGAGACGT
Df	115	F	GCACATATTTGCCGTGATGTAG
		R	CGTGATAGATTCCTCGTCCAAT
		P	TCACGCAAACGGAGCATCTTTCTTCT

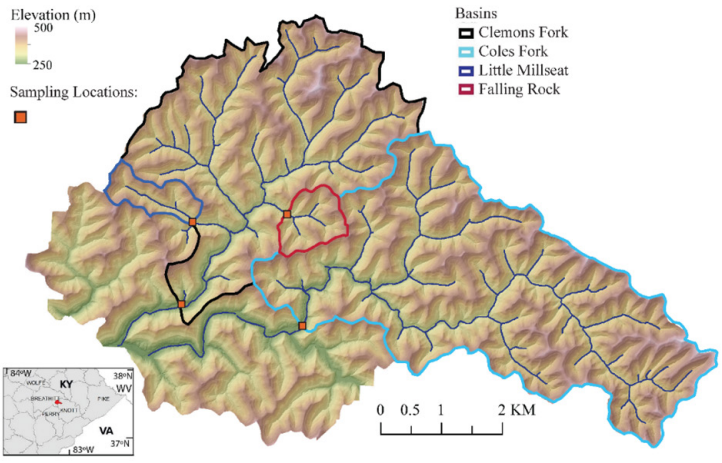

Figure 1. Sample locations on Clemons Fork, Coles Fork, Little Millseat, and Falling Rock creeks in Robinson Forest, Breathitt and Knott counties, Kentucky, USA.

(Maigret et al. 2014). For this analysis, we collected 1 L water samples from four watersheds in Robinson Forest: Clemons Fork, Coles Fork, Falling Rock, and Little Millseat. These watersheds vary in size (Clemons, 1,660 ha; Coles, 1,409 ha; Falling Rock, 88 ha; Little Millseat, 79 ha) and impact because some subwatersheds of Clemons Fork have been logged as part of timber harvest experiments, and others have been impacted by surface mining activity, while the other three watersheds are managed as controls with minimal anthropogenic disturbance (Johnson et al. 2010; Witt et al. 2016). We collected water samples at the same site on each creek (Fig. 1) either nine (Coles Fork and Little Millseat) or 10 (Clemons Fork and Falling Rock) times between January and later October or November 2016 (Table 4). We transported stream-water samples on ice and stored them under refrigeration until they could be processed. We stored samples at 4° C 1–86 d (mean = 26.8 d; Supplemental Information Table S3). We filtered samples by vacuum filtration using Nucleopore filters (3 μm pore size, 47 mm diameter) and we stored filters in -4° C for further analysis.

Stream-water chemistry, streamflow, and stream-water temperature for the four sampled Robinson Forest watersheds were recently released via U.S. Geological Survey ScienceBase (Sena et al. 2021). Briefly, stream-water samples collected on the same dates and at the same locations (but collected independently of the samples filtered and screened using PCR analyses) were analyzed by the Forest Hydrology Laboratory of the University of Kentucky using standard methods (described in Sena et al. 2021) for Cl, NO_3, SO_4, NH_4, pH, Alkalinity, Ca, Mg, K, Na, Conductivity, and Total Organic Carbon (Table 5). Streamflow data included mean, minimum, and maximum daily streamflow. We used these data to calculate mean, minimum, and maximum streamflow values for the week leading up to each sampling date (Table 6). Water temperature data also included mean, minimum, and maximum daily water temperature, and were similarly used to calculate mean, minimum, and maximum temperatures for the week leading up to each sampling date (Table 6). We analyzed water chemistry, streamflow, and water temperature data using Student's *t*-tests ($\alpha = 0.05$) to evaluate patterns that might be predictive of PCR screening results by grouping data based on PCR assay results (positive vs. negative, independently for each species screened; Tables 5 and 6).

In addition to these samples, we used three additional sites in central Kentucky (Supplemental Information Table S4) to obtain additional water collected amplicons of *E. cirrigera* and *D. fuscus* eDNA for sequencing. We extracted eDNA using a DNeasy blood and tissue kit (Qiagen), demonstrated to provide superior yields relative to other extraction methods (Hinlo et al. 2017), and a modified version of a published protocol (Goldberg et al. 2011). Briefly, whole filters were cut into 30–40 pieces and incubated at 56° C overnight in 720 μl ATL buffer and 80 μl Proteinase K. We performed final elutions twice (total 400 μl of AE buffer), and we stored the extracted DNA at -20° C until analysis.

We quantified Environmental DNA using a Step One PlusTM Real-Time PCR system (Life Technologies, Carlsbad, California, USA) in optical 96-well PCR plates. Each plate contained tissue extracted DNA as a positive control and we ran all samples in triplicate. Each 20 μl reaction contained the following: TaqMan EMM (10 μL), nuclease free water (2 μL), eDNA extract (7 μL), and assay (1 μL). Thermocycler conditions were as follows: 95° C for 10 min, 55 cycles of 95° C for 15 s, and 60° C for 1 min. Each plate contained a positive control (tissue extracted DNA from target species) but no negative controls.

Table 4. Quantitative PCR (primer/probe) detection of Southern Two-lined Salamanders (*Eurycea cirrigera*) and Northern Dusky Salamanders (*Desmognathus fuscus*) in water samples collected from Robinson Forest, Kentucky, USA. Shown are the number of positive samples detected out of the three qPCR replicates conducted.

Site	Date	*E. cirrigera*	*D. fuscus*
Clemons Fork	27 January 2016	0	0
Clemons Fork	17 February 2016	0	0
Clemons Fork	16 March 2016	0	0
Clemons Fork	4 May 2016	0	0
Clemons Fork	10 May 2016	0	0
Clemons Fork	13 June 2016	0	0
Clemons Fork	19 July 2016	0	0
Clemons Fork	14 September 2016	0	3
Clemons Fork	1 November 2016	0	2
Coles Fork	27 January 2016	0	3
Coles Fork	2 February 2016	0	3
Coles Fork	16 March 2016	3	3
Coles Fork	10 May 2016	3	3
Coles Fork	13 June 2016	2	3
Coles Fork	19 July 2016	1	3
Coles Fork	14 September 2016	1	3
Coles Fork	11 October 2016	1	2
Coles Fork	1 November 2016	0	1
Falling Rock	17 February 2016	3	3
Falling Rock	10 March 2016	0	0
Falling Rock	16 March 2016	3	3
Falling Rock	30 March 2016	3	3
Falling Rock	4 May 2016	0	0
Falling Rock	13 June 2016	2	2
Falling Rock	19 July 2016	3	3
Falling Rock	10/5/2016	0	0
Falling Rock	1 November 2016	1	0
Little Millseat	27 January 2016	1	3
Little Millseat	17 February 2016	3	2
Little Millseat	10 March 2016	0	0
Little Millseat	16 March 2016	0	0
Little Millseat	4 May 2016	3	3
Little Millseat	13 June 2016	1	3
Little Millseat	19 July 2016	2	1
Little Millseat	5 October 2016	0	1
Little Millseat	25 Ocotber 2016	2	2

Amplicon sequencing.—We reanalyzed select positive samples from both Robinson Forest and three collection sites in central Kentucky (Table 7) using end point PCR in the manner previously described. We ligated PCR products to pGEM-T Easy plasmid vectors (Promega.com) and transformed plasmids into DH5-alpha *E. coli* cells. We grew cells on tryptic soy agar plates containing ampicillin and X-gal and we selected white colonies to grow in tryptic soy broth. Plasmid DNA was subsequently purified from multiple bacterial clones using Qia-prep spin columns (Qiagen.com) and sequenced by ACTG, Inc. (www.actginc.com).

Per sample detection probability.—Detection is likely to be imperfect in eDNA field studies, and thus we sought to estimate our probability of detection for each watershed and target species. Because previous studies have conclusively demonstrated the presence of our target species in the sampled watersheds (Maigret et al. 2014; Muncy et al. 2014; Price et al. 2016), we were able to estimate overall per-sample detection probability in a manner similar to Pierson et al. (2016) as the proportion of samples from each sampled watershed in which we detected DNA from our target species. We calculated and plotted the cumulative probability of detection (*p* after n samples) using the function dbinom in R version 3.1.1 (R Core Team 2014).

Results

In silico *testing*.—F and R primers pairs of *E. cirrigera* have a minimum of five total mismatches with sympatric species with the exception of the Cave Salamander (*E. lucifuga*) and the Longtail Salamander (*E. longicauda*; three each; Table 1). In the case of these two species, the probes contain one (*E. lucifuga*) and two (*E. longicauda*) mismatches (Table 1). Interestingly the probe also has a single mismatch with some published *E. cirrigera* cytb sequences (Table 1) but not others, including a sequence generated from a specimen collected within our study area (GenBank ID: MZ485475). *Desmognathus fuscus* F and R primers have a minimum of two mismatches each with all sympatric species while the probe has one with the Seal Salamander (*D. monti*cola) and two or more with all others (Table 2).

Assay validation.—Tissue standard curves showed consistent amplification at all concentrations, down to 0.5 pg/l (*E. cirrigera,* mean CT = 37.3 and *D. fuscus,* mean CT = 40.3, Supplemental Information Table S1). Synthetic DNA standard curves (Fig. S1) indicated an amplification efficiency of 91.6 (*E. cirrigera*) and 96.8% (*D. fuscus*). The limit of quantification for both assays was 20 copies/μl and limit of detection was two copies/μl. Coefficients of Determination (r^2-values) for the curves were 0.998 (*E. cirrigera*) and 0.960 (*D. fuscus*; Supplemental Information Fig. S1).

Specificity testing.— End point reactions with *E. cirrigera* and *D. fuscus* primer pairs and 14 sympatric or potentially sympatric salamander species demonstrated

Table 5. Mean (± 1 SD) water quality metrics for stream-water samples screened using PCR assays for Southern Two-lined Salamanders (*Eurycea cirrigera*; E. cir.) and Northern Dusky Salamanders (*Desmognathus fuscus*; D. fus.) from four streams in Robinson Forest, Breathitt, Knott and Perry Counties, Kentucky, USA. †Stream-water samples for chemical analysis were collected from the same location at the same time as stream-water samples collected and screened using PCR assays. Stream-water chemistry data accessed from Sena et al. (2021). Notes are ‡Cond = Electrical Conductivity, TOC = Total Organic Carbon **P*-values indicate probability associated with a Student's *t*-test for difference in water chemistry values between negative and positive screening results, considered significant if $P < 0.05$.

	Cl	NO_3	SO_4	NH_4	pH	Alkalinity	Ca	Mg	K	Na	Cond‡	TOC
PCR Result	mg/L	mg/L	mg/L	mg/L	-	mg HCO_3^-/L	mg/L	mg/L	mg/L	mg/L	mS/cm	mg/L
Clemons Fork												
E. cir - (n = 10)	0.77 ± 0.21	0.04 ± 0.04	14.5 ± 2.1	0.03 ± 0.05	5.44 ± 0.28	34.1 ± 19.1	4.39 ± 2.1	2.79 ± 0.94	1.46 ± 0.51	1.63 ± 1.0	64.3 ± 25.8	2.30 ± 0.67
E. cir + (n = 0)	-	-	-	-	-	-	-	-	-	-	-	-
P (*t*-test) *	-	-	-	-	-	-	-	-	-	-	-	-
D. fus - (n = 8)	0.77 ± 0.23	0.05 ± 0.03	14.6 ± 2.3	0.03 ± 0.06	5.4 ± 0.26	27.5 ± 14.7	3.76 ± 1.85	2.54 ± 0.88	1.30 ± 0.39	1.32 ± 0.86	55.8 ± 21.2	2.33 ± 0.73
D. fus + (n = 2)	0.78 ± 0.10	0.00 ± 0.00	14.3 ± 1.85	0.05 ± 0.06	5.60 ± 0.38	60.4 ± 6.12	6.93 ± 0.93	3.76 ± 0.15	2.12 ± 0.45	2.88 ± 0.11	97.9 ± 1.56	2.09
P (*t*-test)	0.905	**0.003**	0.890	0.800	0.597	**0.006**	**0.033**	**0.006**	0.190	**0.001**	**0.001**	-
Coles Fork												
E. cir - (n = 4)	0.77 ± 0.05	0.06 ± 0.05	7.81 ± 1.24	0.02 ± 0.03	5.28 ± 0.20	25.9 ± 13.8	2.57 ± 1.15	1.67 ± 0.48	1.43 ± 0.54	1.29 ± 0.60	43.7 ± 18.4	2.23 ± 0.37
E. cir + (n = 5)	0.72 ± 0.16	0.02 ± 0.03	8.92 ± 1.42	0.02 ± 0.04	5.43 ± 0.24	27.8 ± 12.0	2.88 ± 0.92	1.88 ± 0.49	2.03 ± 1.70	1.65 ± 0.63	53.0 ± 13.5	1.63 ± 0.18
P (*t*-test)	0.514	0.340	0.253	0.983	0.329	0.835	0.676	0.540	0.487	0.403	0.431	**0.039**
D. fus - (n = 1)	0.76	0	7.07	0	5.22	45	3.94	2.28	2.08	2.16	64.2	1.78
D. fus + (n = 8)	0.74 ± 0.13	0.04 ± 0.04	8.59 ± 1.38	0.02 ± 0.04	5.38 ± 0.23	24.7 ± 10.6	2.59 ± 0.92	1.73 ± 0.46	1.73 ± 1.37	1.40 ± 0.59	47.0 ± 15.5	1.95 ± 0.45
P (*t*-test)	-	-	-	-	-	-	-	-	-	-	-	-
Falling Rock												
E. cir - (n = 3)	0.77 ± 0.13	0.11 ± 0.03	7.12 ± 0.37	0.08 ± 0.05	5.02 ± 0.34	19.2 ± 13.1	2.24 ± 1.37	1.54 ± 0.56	1.30 ± 0.51	1.32 ± 0.59	42.2 ± 14.8	3.26 ± 0.12
E. cir + (n = 7)	0.77 ± 0.13	0.13 ± 0.10	8.27 ± 1.51	0.02 ± 0.04	5.15 ± 0.19	21.1 ± 8.3	2.41 ± 0.97	1.66 ± 0.45	1.29 ± 0.44	1.06 ± 0.33	45.5 ± 11.4	2.83 ± 1.23
P (*t*-test)	0.988	0.699	0.098	0.109	0.571	0.830	0.856	0.767	0.995	0.529	0.757	0.442
D. fus - (n = 4)	0.80 ± 0.12	0.09 ± 0.05	7.26 ± 0.41	0.06 ± 0.06	5.04 ± 0.28	23.8 ± 14.2	2.72 ± 1.47	1.76 ± 0.62	1.47 ± 0.54	1.41 ± 0.52	47.9 ± 16.5	2.73 ± 0.91
D. fus + (n = 6)	0.75 ± 0.13	0.14 ± 0.10	8.37 ± 1.63	0.02 ± 0.04	5.16 ± 0.21	18.3 ± 4.16	2.12 ± 0.66	1.54 ± 0.34	1.18 ± 0.33	0.95 ± 0.19	42.2 ± 8.24	3.06 ± 1.22
P (*t*-test)	0.546	0.302	0.162	0.267	0.487	0.500	0.492	0.561	0.380	0.171	0.561	0.683
Little Millseat												
E. cir - (n = 5)	0.75 ± 0.09	0.08 ± 0.08	7.11 ± 0.40	0.01 ± 0.02	5.18 ± 0.20	21.9 ± 10.1	2.19 ± 1.10	1.71 ± 0.73	1.26 ± 0.45	1.35 ± 0.81	41.9 ± 15.4	2.62 ± 1.53
E. cir + (n = 4)	0.73 ± 0.23	0.06 ± 0.07	8.44 ± 2.30	0.03 ± 0.05	4.90 ± 0.18	23.8 ± 15.9	2.42 ± 1.54	1.66 ± 0.66	1.55 ± 0.60	1.13 ± 0.58	43.2 ± 18.2	2.51 ± 0.050
P (*t*-test)	0.904	0.709	0.335	0.480	0.069	0.847	0.810	0.912	0.459	0.652	0.907	0.904
D. fus - (n = 4)	0.86 ± 0.17	0.07 ± 0.08	7.2 ± 0.42	0.00 ± 0.00	5.23 ± 0.19	22.8 ± 11.6	2.35 ± 1.18	1.74 ± 0.79	1.41 ± 0.58	1.58 ± 0.79	46.8 ± 15.6	1.99 ± 0.20
D. fus + (n = 5)	0.65 ± 0.04	0.08 ± 0.08	8.11 ± 2.12	0.03 ± 0.05	4.92 ± 0.16	22.7 ± 13.9	2.25 ± 1.40	1.64 ± 0.62	1.37 ± 0.51	1.00 ± 0.53	39.1 ± 16.5	3.01 ± 1.38
P (*t*-test)	0.085	0.840	0.390	0.199	**0.043**	0.987	0.919	0.837	0.912	0.267	0.498	0.235

Table 6. Mean (± 1 standard deviation) streamflow and water temperature metrics for stream-water samples screened using PCR assays for Southern Two-lined Salamanders (*Eurycea cirrigera*) and Northern Dusky Salamanders (*Desmognathus fuscus*) from Kentucky, USA. The abbreviation SF = streamflow with mean streamflow = mean daily streamflow averaged across the week ending on the sampling day; minimum and maximum streamflow = the minimum and maximum streamflow recorded for the week ending on the sampling day. Mean temp = mean daily water temperature averaged across the week ending on the sampling day. Minimum and maximum temperature = minimum and maximum water temperature recorded for the week ending on the sampling day. Streamflow and water temperature data accessed from Sena et al. (2021). *P*-values indicate probability associated with a Student's *t*-test for difference in streamflow and water temperature values between negative and positive screening results with significance if $P < 0.05$ (bold).

	Mean SF	Min SF	Max SF	Mean temp	Min temp	Max temp
PCR Result	CFS	CFS	CFS	°C	°C	°C
Clemons Fork						
E. cir - (n = 10)	18 ± 29.0	3.02 ± 4.30	61.5 ± 92.6	13.1 ± 5.9	12.6 ± 6.0	13.7 ± 5.8
E. cir + (n = 0)	-	-	-	-	-	-
P (*t*-test)*	-	-	-	-	-	-
D. fus - (n = 8)	22.4 ± 31.2	3.74 ± 4.56	76.7 ± 98.4	12.1 ± 6.0	11.5 ± 6.1	12.7 ± 5.9
D. fus + (n = 2)	0.46 ± 0.28	0.18 ± 0.08	0.60 ± 0.42	17.3 ± 3.87	17.0 ± 3.8	17.6 ± 3.78
P (*t*-test)	0.087	0.063	0.065	0.247	0.227	0.264
Coles Fork						
E. cir - (n = 4)	8.51 ± 11.2	3.97 ± 6.39	24.6 ± 26.8	10.8 ± 6.2	10.4 ± 6.2	11.1 ± 6.2
E. cir + (n = 5)	4.84 ± 7.35	2.20 ± 2.83	12.5 ± 22.7	14.3 ± 3.8	13.7 ± 4.2	14.8 ± 3.52
p (t-test)	0.597	0.634	0.499	0.367	0.408	0.334
D. fus - (n = 1)	0.18	0.17	0.23	14.4	14	14.8
D. fus + (n = 8)	7.26 ± 9.09	3.34 ± 4.67	20.0 ± 24.5	12.5 ± 5.32	12.0 ± 5.44	13.0 ± 5.23
P (*t*-test)	-	-	-	-	-	-
Falling Rock						
E. cir - (n = 3)	-	-	-	12.2 ± 5.12	9.89 ± 6.37	15.7 ± 2.06
E. cir + (n = 7)	-	-	-	12.3 ± 6.39	9.14 ± 6.40	14.8 ± 6.53
p(t-test)	-	-	-	0.983	0.873	0.757
D. fus - (n = 4)	-	-	-	12.2 ± 4.18	10.1 ± 5.22	15.2 ± 2.02
D. fus + (n = 6)	-	-	-	12.3 ± 6.99	8.87 ± 6.96	15.1 ± 7.13
P (*t*-test)	-	-	-	0.978	0.757	0.974
Little Millseat						
E. cir - (n = 5)	0.48 ± 0.43	0.17 ± 0.22	1.77 ± 2.41	10.6 ± 7.28	7.81 ± 6.11	13.6 ± 8.0
E. cir + (n = 4)	2.00 ± 2.59	0.12 ± 0.13	30.7 ± 43.5	12.8 ± 8.95	10.4 ± 8.56	15.2 ± 9.5
p(t-test)	0.327	0.679	0.275	0.694	0.626	0.791
D. fus - (n = 4)	0.33 ± 0.32	0.21 ± 0.23	0.71 ± 0.45	14.1 ± 6.45	11.4 ± 7.41	17.4 ± 4.40
D. fus + (n = 5)	1.82 ± 2.28	0.10 ± 0.12	25.8 ± 39.3	9.55 ± 8.56	7.02 ± 6.65	11.9 ± 10.1
P (*t*-test)	0.219	0.443	0.226	0.394	0.389	0.318

no amplification of non-target species DNA following 40 cycles (Supplemental Information Fig. S2). In addition to the 14 species included in the initial test, *D. fuscus* primers were later tested in an identical manner with DNA from a locally (eastern Kentucky) collected *D. monticola* (GenBank ID:MZ418126) and also proved specific.

Field testing.—No samples were positive for *E. cirrigera* DNA in any water sample in Clemons Fork (Table 4). In contrast, Coles Fork (6/9), Falling Rock (6/9), and Little Millseat (6/9) all had numerous detections of *E. cirrigera* (Table 4). In a similar manner, Clemens Fork had only two samples positive for *D. fuscus* DNA while Coles Fork (9/9), Falling Rock (5/9), and

Table 7. Sequences of amplicons cloned and sequenced from a total of 11 water samples collected either in the Robinson Forest study site or central Kentucky, USA. The identity of the amplicon to the sequence of the target is shown in the right-hand column; either Southern Two-lined Salamanders (*Eurycea cirrigera*) or Northern Dusky Salamanders (*Desmognathus fuscus*). Samples listed in the same shaded area represent one amplicon, cloned, and sequenced in duplicate.

Collection locality	Primers used to create amplicon	% similarity to target species
Clemons Fork	*E. cirrigera*	100
Clemons Fork	*E. cirrigera*	100
Falling Rock	*E. cirrigera*	100
Falling Rock	*E. cirrigera*	100
Asbury Creek	*E. cirrigera*	98.2
Asbury Creek	*E. cirrigera*	100
Stoney Run	*E. cirrigera*	99.0
Falling Rock	*D. fuscus*	99.2
Falling Rock	*D. fuscus*	99.2
Little Millseat	*D. fuscus*	99.2
Little Millseat	*D. fuscus*	99.2
Clemons Fork	*D. fuscus*	99.2
Clemons Fork	*D. fuscus*	97.5
Falling Rock	*D. fuscus*	87.5
Falling Rock	*D. fuscus*	87.5
Highbridge Seep	*D. fuscus*	99.2
Asbury Creek	*D. fuscus*	98.3
Asbury Creek	*D. fuscus*	99.2
Stoney Run	*D. fuscus*	98.3
Stoney Run	*D. fuscus*	99.2

Little Millseat (7/9) all had numerous positive samples (Table 4). Stream-water chemistry was significantly different for samples testing positive for *D. fuscus* than samples testing negative for *D. fuscus* in Clemons Fork (NO_3, Alkalinity, Ca, Mg, Na, and Conductivity) and Little Millseat (pH), but samples from Coles Fork and Falling Rock showed no significant differences by *D. fuscus* screening results (Table 5). Stream-water chemistry was similar across samples testing positive for *E. cirrigera* versus samples testing negative for *E. cirrigera* for all metrics and watersheds, except for total organic carbon in Coles Fork (Table 5). Stream-water temperature and streamflow metrics demonstrated no significant differences across screening results for either species in any watershed (Table 6).

Amplicon sequencing.—Sequenced *E. cirrigera* amplicons obtained from stream water were 98.2–100% similar to our published *E. cirrigera* sequence (GenBank ID: MZ485475; Table 7). Six of the seven sequenced *D. fuscus* amplicons were between 98.3 and 99.2%, similar to our published sequence (GenBank ID: MZ485476; Table 7). One amplicon, from Falling Rock, was only 87.5% similar to this sequence (Table 7).

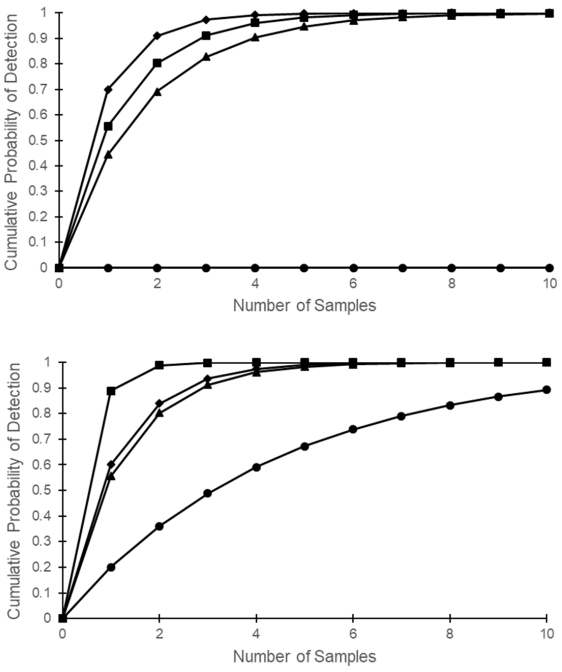

Figure 2. Cumulative detection probabilities for eDNA assays for (Top) Southern Two-lined Salamanders (*Eurycea cirrigera*) and (Bottom) Northern Dusky Salamanders (*Desmognathus fuscus*) in four streams in Robinson Forest, Breathitt, Knott, and Perry counties, Kentucky, USA. Circles = Clemons Fork, squares = Coles Fork, diamonds = Falling Rock, and triangles = Little Millseat.

Per sample detection probability.—We detected *E. cirrigera* in 0/10 (Clemons Fork), 6/9 (Coles Fork), 6/9 (Falling Rock), and 6/9 (Little Millseat) samples, and for *D. fuscus* obtained positive detections in 2/10 (Clemons Fork) 9/9 (Coles Fork), 5/9 (Falling Rock), and 7/9 (Little Millseat) samples (Table 4). We estimated the overall per sample detection probability after 9 –10 repeated samples to be 0.99 for *E. cirrigera* in Coles Fork, 0.99 in Falling Rock, and 0.97 in Little Millseat. We obtained no positive *E. cirrgera* samples from Clemons Fork. For *D. fuscus* the detection probabilities were estimated as 0.89 (Clemons Fork), 1.0 (Coles Fork), 0.99 (Falling Rock), and 1.0 (Little Millseat). Cumulative detection probabilities for eDNA surveys differed between *E. cirrigera* and *D. fuscus* in four habitats (Fig. 2).

Discussion

We are aware of only two other assays developed for *D. fuscus* (Beauclerc et al. 2019; Hernandez et al.

2020) and none for *E. cirrigera*, despite the widespread distribution of these salamanders. In a manner perhaps illustrative of the need for the availability of multiple primers for a single species, we suggest that our *D. fuscus* primers offer advantages over the previously published *D. fuscus* assays, at least in the southern portion of the *D. fuscus* range. Of the two assays proposed for *D. fuscus* in Canada, one (Hernandez et al. 2020) has no mismatches in any oligo with the Allegheny Mountain Duscky Salamander (*D. ochrophaeus*) and the other (Beauclerc et al. 2019) has mismatches in the probe (seven) but none in the F and R primer with *D. ochrophaeus* (Supplemental Information Tables S5 and S6). Our F and R primers have five and two mismatches respectively with published *D. ochrophaeus* sequences, enabling their use in end-point PCR as well as qPCR. *Desmognathus ochrophaeus* distribution in Canada is limited to two disparate populations in two small areas (Committee on the Status of Endangered Wildlife in Canada 2018) in contrast to the much more widespread distribution of *D. ochrophaeus* in the USA. Our *D. fuscus* assay enables the detection of this species using either PCR approach, throughout the large portion of their range in the U.S. where they occur sympatrically with *D. ochrophaeus*.

Five species of salamanders of the genus *Desmognathus* are found in Kentucky (Meade 2000). Although we were only able to test against one of these species (*D. monticola*) *in vitro*, *in silico* tests indicate our *D. fuscus* primers should be specific against all three other *Desmognathus* species found in Kentucky. *Desmognathus monticola*, the one species we were able to test *in vitro*, is most similar to *D. fuscus* (five total mismatches in all oligos). We would therefore not expect non-specific binding with the other three *Desmognathus* species which all have greater numbers of total mismatches in all oligos (Black Mountain Dusky salamander, *D. welteri,* six; Spotted Dusky Salamander, *D. conanti*, eight; *D. ochrophaeus*, nine).

One interesting trend noted in the field collected samples was the absence of *E. cirrigera* and the appearance only twice of *D. fuscus* in the Clemons Fork water samples collected between 27 January and 1 November 2016. In contrast *E. cirrigera* eDNA appeared in 67% of the water samples collected from the three other creeks while *D. fuscus* eDNA appeared in 67–78% of the samples from the remaining creeks. Our detection probabilities in Clemons Fork were likewise zero (*E. cirrigera*) and 0.89 (*D. fuscus*), the lowest for any watershed. Although we concede the lack of internal positive controls leaves open the possibility of inhibitor influence, these results are consistent with field observations indicating the presence of native fish in the larger Clemons Forks, versus their absence in the sampled areas of the other streams. Kuehne (1962) reported collection of 17 fish species from Clemons Fork including Green Sunfish (*Lepomis cyanellus*), a known salamander larvae predator (Sih et al. 1992). As expected, the presence of fish has been associated with declines in abundance or absence of stream dwelling salamanders (Sih et al. 1992; Bank et al. 2006). Additionally, Clemons Forks has been impacted by historic surface mining (early 1990s), an activity associated with declines in stream salamander populations in the Appalachians (Schorr et al. 2013; Muncy et al. 2014) and maintains elevated sulfate concentrations (Sena et al. 2021) also known to impact stream amphibian populations. Consistent with this general reported pattern, mean stream-water sulfate concentrations for Clemons Fork (14.3–14.5 mg/L) were higher than those of the other streams (7.07–8.92 mg/L) for samples analyzed in our study. While sulfate was not significantly different within any of the watersheds across screening results for either species, this dramatic difference across watersheds could certainly be a factor contributing to low salamander detection in Clemons Fork.

Water chemistry metrics that were significantly different across screening results including NO_3, Alkalinity, Ca, Mg, Na, Conductivity (Clemons Fork), TOC (Coles Fork), and pH (Little Millseat). While results from Clemons Fork must be interpreted with the caveat that only two samples screened positive for *D. fuscus* (and none for *E. cirrigera*), patterns across these metrics were similar; samples testing positive for *D. fuscus* tended to have higher concentrations of the given constituent than samples testing negative. While electrical conductivity has been identified as a significant predictor of impacts of surface mining on aquatic macroinvertebrates (Pond et al. 2008) and salamanders (Hutton et al. 2020), the differences observed in our study are contrary to what would be expected. Given that these samples were from low-flow periods (mean streamflow = 0.46 and maximum streamflow = 0.60 CFS for samples testing positive for *D. fuscus* in Clemons Fork), collected 14 September and 1 November 2016, it is likely that the observed elevated concentrations of various constituents are related to a concentration effect caused by low flow, rather than an abnormal influx of water with high TDS. Overall, the fact that we detected only these few differences in stream-water chemistry (and no differences in streamflow and stream-water temperature) across screening results suggests that these assays are robust enough for use in streams across a range of stream conditions. Further analysis with higher sample sizes, and ideally, controlled predictor variables will be necessary to conclusively evaluate differences in detection probability across stream conditions.

Previous studies have noted increases in eDNA associated with breeding seasons in both fish (Laramie

et al. 2015, Erickson et al. 2016) and stream breeding salamanders (Spear et al. 2015). *Eurycea cirrigera* females typically attach 10–150 eggs to the underside of stones in lotic habitats in the winter and spring and metamorphosis occurs 1–3 y later in the spring and summer (Petranka 1998). Pierson and Miele (2019) reported no nests after 14 April and first hatchling appearance on 1 May in eastern Tennessee in the closely related Northern Two-Lined Salamander (*E. bislineata*; Pierson and Miele 2019). Jakubanis et al. (2008) reported the start of oviposition in April and appearance of larvae in May in *E. cirrigera* in eastern Illinois (Jakubanis et al. 2008). *Desmognathus fuscus* nesting sites are reportedly found either in or near small headwater streams (Organ 1961; Meade 2000) with oviposition reported from June to August (Danstedt 1975; Meade 2000) and larval transformation occurring at 9–14 mo of age (Danstedt 1975; Petranka 1998). We were unable to identify differences in detection of either *E. cirrigera* or *D. fuscus* associated with sampling when breeding of these species would be expected. Additionally, both species would be expected to have larvae present in the stream year-round, with the possible exception of *D. fuscus* in late summer, likely making the detection of eDNA trends associated with metamorphosis difficult.

Although 10 of the 11 water-obtained amplicons confirmed the identity of the target species with a high degree of certainly (≥ 97.5%), one amplicon produced from water samples using *D. fuscus* primers was less similar to our *D. fuscus* sequence (87.5%). This amplicon (provided in Supplemental Information Table S7) had 12 mismatches with our *D. fuscus* sequence (Accession# MZ485476) in the 70 BP region between the F and R primers. Additionally, these 70 BP are 96.4% similar to two published Northern Zigzag Salamander (*Plethodon dorsalis*) cytb sequences (DQ994930.1, AY378077.1), but only 87.6% similar to the third published *P. dorsalis cytb* sequence (GQ464404.1) (Table S8). Interestingly, the *D. fuscus* F and R primers had five mismatches with the published *P. dorsalis* sequence and six with these two additional *P. dorsalis* sequences (DQ994930.1, AY378077.1) seemingly making it less likely, although still possible, than non-specific binding occurred. Regardless of the origin of this amplicon, it has six mismatches with our *D. fuscus* probe, which should eliminate any possibility of the probe binding to this amplicon if it was produced in qPCR analysis, perhaps providing an example of the advantage of qPCR use in eDNA studies.

We found one mismatch of our probe for *E. cirrigera* with a number of published *E. cirrigera* cytb sequences. Interestingly, this is the result of a C as the 47th base in the amplicon produced by the *E. cirrigera* primers that is a T in many published sequences (NC_035494.1 and others). The sequence from the *E. cirrigera* specimen collected in our study area and *E. cirrigera* specimens collected in central Kentucky (Jessamine County) both had C at this locus. A total of 15 other *E. cirrigera* cyt b sequences appear in Gen Bank, the similarity with the two published sequences from our study range from 99.2–89.4%. In the case of *D. fuscus*, 25 cyt b sequences, in addition to the sequence contributed by us, appear in Gen Bank and the similarity to the sequence in our study ranges from 99.5–89.7%. Variation in mitochondrial genes and cytochrome b, in particular, is consistently observed throughout the range of salamander species and is commonly used deterministically in phylogenetic studies (Kutcha et al. 2016; Page et al. 2020; Sweet et al. 2021). Certainly, this presents a challenge to the use of eDNA to species detection and highlights the importance of the use of locally obtained sequences in assay development, and the need for multiple assays validated for various portions of a species range.

We recognize the importance of the use of both field blanks and laboratory negative controls and concede their use would have been ideal in our study; however, we note that of the 36 field samples analyzed in triplicate, 18 (50%) were negative for all replicates for *E. cirrigera* and 13 (36%) were negative for all replicates for *D. fuscus*. We believe the presence of a significant number of fully negative samples for both assays indicates that contamination, either in the field or lab, was unlikely in this analysis. Additionally, our primary objective was to demonstrate that these assays are effective in detecting target species in a field setting, not determine specific eDNA levels.

The labor-intensive nature of the development and validation of novel eDNA assays is widely recognized (Wilcox et al. 2020) as well as the need for thorough specificity testing and standardization of assay validation (Goldberg et al. 2016; Klymus et al. 2020; Loeza-Quintana et al. 2020). Additionally, our data demonstrate the need to validate assays, both *in silico* and *in vitro*, with locally collected specimens and perhaps highlight the importance of the inclusion of detailed locality information with sequence submissions. The assays we developed and tested both *in silico* and *in vitro* against all or nearly all sympatric species found in the region should serve as valuable tools enabling the detection and therefore monitoring of these widespread salamander species.

Acknowledgments.—We sincerely thank Rebecca Piche for her assistance in the laboratory work for this project and Cierla McGuire and Rebecca Blackburn for their assistance in the field and laboratory. We thank John McGregor (Kentucky Department of Fish and Wildlife Resources) for generously sharing his knowledge and providing advice. We also thank David Collett (Robinson Forest), Millie Hamilton (UK

Department of Forestry and Natural Resources), and Alysia Kohlbrand for their invaluable assistance with sample collection and processing. We thank Tanja Williamson, U.S. Geological Survey, for her assistance in the preparation of Figure 1. Salamander tissue was collected under Kentucky Department of Fish and Wildlife Service Permit Number SC1811153. Research was funded by an internal faculty development grant from Asbury University (Ben Brammell, Fall 2019). The Asbury University Department of Science and Health provided additional support.

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Supplemental Information: http://www.herpconbio.org/Volume_17/Issue_2/Bell_etal_2022_Suppl

Florene Gabriella Bell holds a B.A. in Biology from Asbury University, Wilmore, Kentucky, USA. She currently works as the Lead Laboratory Scientist at Solaris Diagnostics in Nicholasville, Kentucky, USA. Her goal is to pursue a Ph.D. in molecular or cancer biology. In addition to molecular biology her interests include aquatic ecology, wetland conservation, and phylogenetics. (Photographed by Steven Bell).

Angie Flores Madrid holds a B.A. in Biochemistry from Asbury University in Wilmore, Kentucky, USA. She is currently pursuing an M.D. at La Plata National University in La Plata, Argentina, where she plans to specialize in obstetrics. She developed a love for marine life during her childhood while spending time near her home on the beautiful Caribbean Coast of Honduras. Her interests include molecular biology and the conservation of marine organisms, and she hopes to continue doing research in the future. (Photographed by Amber Holzschuh).

Kenton Sena holds a B.A. in Biology from Asbury University, Wilmore, Kentucky, USA, an M.S. in Forestry and a Ph.D. in Integrated Plant and Soil Sciences from the University of Kentucky, Lexington, USA. He is currently a Lecturer in the Lewis Honors College, University of Kentucky, where he teaches the Honors Foundations Seminar as well as additional Honors courses in Ecology and the Environment. His research program is particularly interested in ecological restoration in sites influenced by resource extraction and urbanization. (Photographed by Meghan Arrell).

Thomas A. Maigret received his Ph.D. in Biology from the University of Kentucky, Lexington, USA, in 2020. His research focuses on the effects of habitat fragmentation and the use of genetic and genomic approaches to address fundamental questions in Landscape Ecology. (Photographed by Neva Williams).

Chi Jing Leow received his B.A. in biology from Asbury University, Wilmore, Kentucky, USA. He is currently pursuing an M.S. in biology at Southeastern Louisiana University, Hammond, USA, focusing on transcriptome of killifishes as potential model organisms. His interests include ecology, ichthyology, conservation biology, and the use of molecular techniques to address ecological questions. (Photographed by Chi Xin Leow).

Ronald B. Sams received a B.A. in Biology from Asbury University, Wilmore, Kentucky, USA, and a commission into the U.S. Army Reserve Medical Service Corps through the University of Kentucky. He is a graduating Doctor of Veterinary Medicine candidate at Auburn University, Alabama, USA, and completed a research internship at the National Cancer Institute, Bethesda, Maryland, USA, through the National Institutes of Health Summer Internship Program in Biomedical Research. Ronald is passionate about animal health and the intersection of veterinary medicine with public health, comparative medicine, and translational research. (Photographed by Auburn Veterinary Business Management Association).

David Keith Peyton is a Professor in the Department of Biology & Chemistry at Morehead State University in Morehead, Kentucky, USA. He received a B.S. in Biology and a Ph.D. in Molecular Genetics from the University of Kentucky, Lexington, USA. He teaches courses in Genetics and Biomedical Ethics. His research interests include mechanisms of pigmentation in vertebrates and molecular phylogeny of fishes. (Photographed by David Eisenhour).

Ben Frederick Brammell is a Professor of Biology in the Department of Science and Health in the Shaw School of Sciences at Asbury University in Wilmore, Kentucky, USA. He received a B.S. in Aquatic Biology from Eastern Kentucky University, Richmond, USA, an M.S. in Biology from Tennessee Technological University, Cookeville, USA, and holds a Ph.D. in Biology from the University of Kentucky, Lexington, USA. His primary research interests include the ecology, natural history, phylogenetics, and conservation of aquatic vertebrates. (Photographed by Carlos Daniel Gonzales Cobos).