

SUPPLEMENTAL INFORMATION

**DEVELOPMENT AND VALIDATION OF QPCR ASSAYS FOR USE IN
EDNA DETECTION OF SOUTHERN TWO-LINED (*EURYCEA CIRRIGERA*)
AND NORTHERN DUSKY (*DESMOGNATHUS FUSCUS*) SALAMANDERS**

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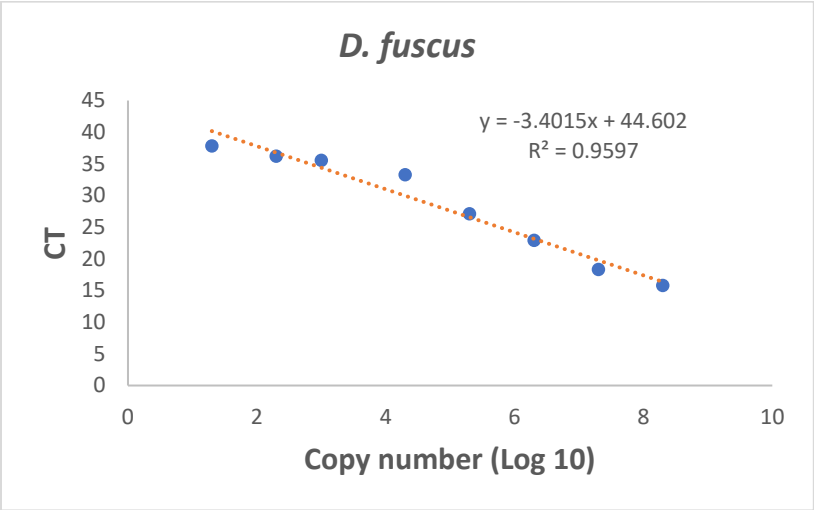
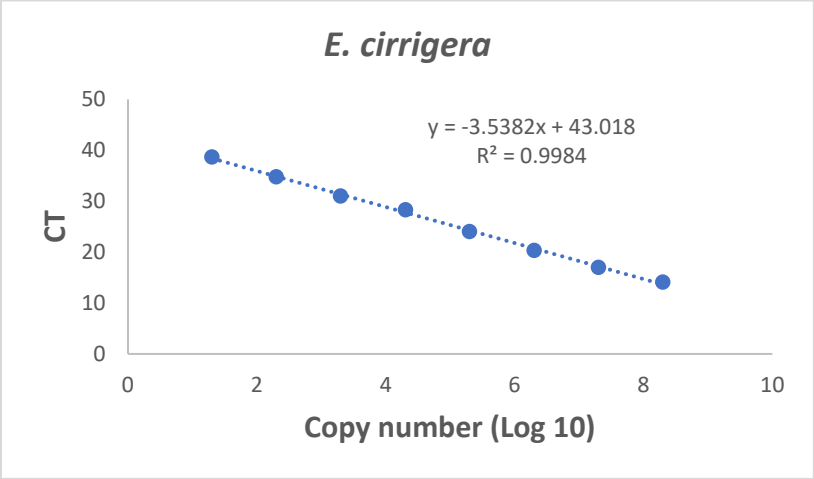


Figure S1. Copy number standard curves (gBlock) for *E. cirrigera* and *D. fuscus*.

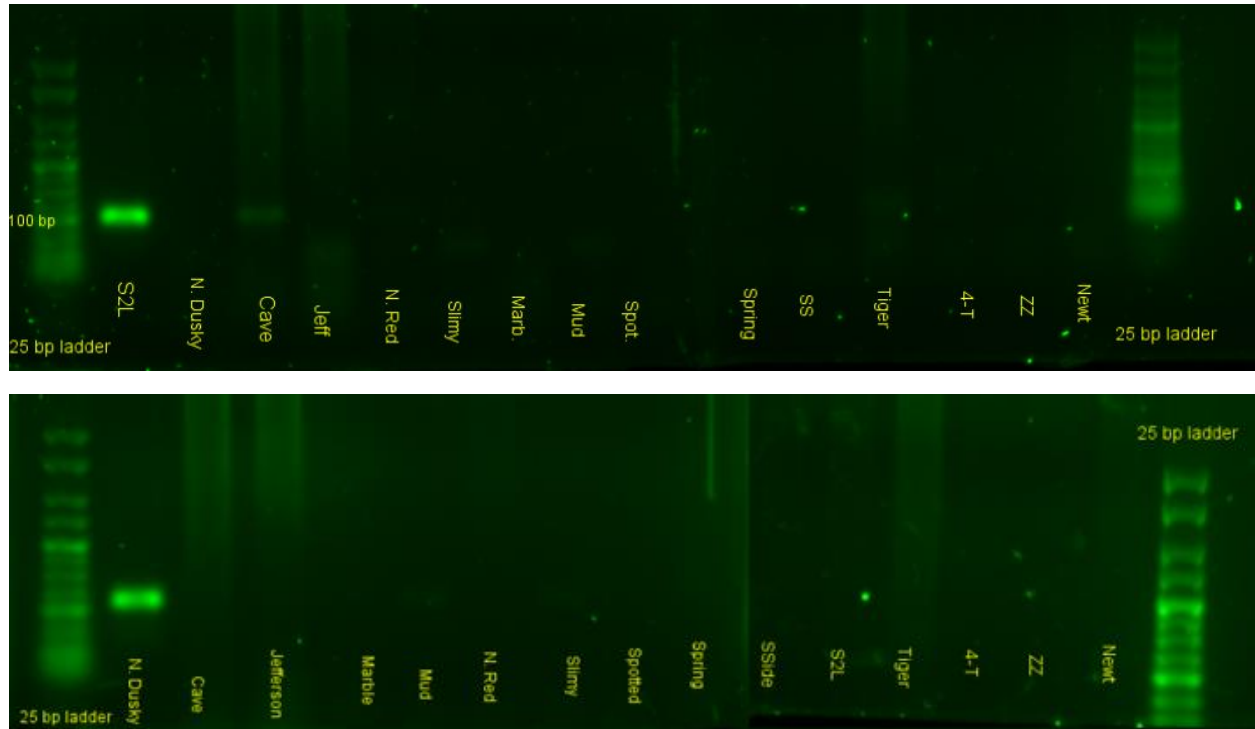


Figure S2. Tissue extracted DNA specificity tests for *E. cirrigera* and *D. fuscus*. end-point PCR on tissue extracts of eleven sympatric salamander species. 25 μ l reactions included: 12.5 μ l GoTaq Master Mix (Promega), 9 μ l nuclease free water, 2 μ l tissue extracted DNA and 1.5 μ l of F and R primers. Cycling conditions consisted of an initial denaturation stage of 95.0°C for 2 minutes followed by 40 cycles of 95.0°C for 45 s, 57.0°C for 60 s, 72.0°C for 60 s. Scientific names of the species tested that correspond to abbreviated names that appear on the graph, from left to right: *E. cirrigera* reactions (top): N. dusky = *D. fuscus*, Cave = *E. lucifuga*, Jeff = *A. jeffersonianum*, N. red = *P. ruber*, Slimy = *P. glutinosus*, Marb = *A. opacum*, Mud = *P. montanus*, Spot = *A. maculatum*, Spring = *G. porphyriticus*, SS = *A. barbourin*, Tiger = *A. tigrinum*, 4-T = *H. scutatatum*, ZZ = *P. dorsalis*, Newt = *N. viridescens*. *D. fuscus* reactions (bottom): Cave = *E. lucifuga*, Jeff = *A. jeffersonianum*, Marb. = *A. opacum*, Mud = *P. montanus*, N. red = *P. ruber*, Slimy = *P. glutinosus*, Spot = *A. maculatum*, Spring = *G. porphyriticus*, SS = *A. barbourin*, S2L = *E. cirrigera*, Tiger = *A. tigrinum*, 4-T = *H. scutatatum*, ZZ = *P. dorsalis*, Newt = *N. viridescens*.

Table S1. qPCR data for tissue extracted DNA standard curves for *D. fuscus* and *E. cirrigera*.

DNA pg/l	<i>D. fuscus</i>		<i>E. cirrigera</i>	
	CT values	Mean CT	CT values	Mean CT
0.5	36.73288	37.2733	38.3962	40.31711
0.5	36.74088		40.96551	
0.5	38.34614		41.58963	
5	33.94893	34.22221	36.01592	35.79301
5	34.4515		35.76144	
5	34.26619		35.60168	
50	30.77755	30.89042	31.16729	31.27421
50	30.94105		31.25116	
50	30.95267		31.40418	
500	27.46117	27.64107	28.60127	28.62828
500	27.733		27.70223	
500	27.72904		29.58134	
5000	23.84374	23.19064	26.38923	24.14533
5000	21.97378		22.77238	
5000	23.75441		23.27438	

Table S2. gBlock sequences used in qPCR assay sensitivity testing for *E. cirrigera* and *D. fuscus*. The black bases represent the amplicons excluding the F and R primers, blue bases represent the F + R primers, pink bases represent probes, red bases represent extra bases added to exceed the minimum length requirement (125 BP). The highlighted C in the *E. cirrigera* gBlock represents a base that is a T in many published *E. cirrigera* sequences but was a C in ours and a number of others.

Species	Total length	Target amplicon length	gBlock sequence
<i>E. cirrigera</i>	149	96	CAATACACTACACTGCAGATACCACCTCCGCATTCTCCTCTGTAGCCCATATTTGC CGAGACGTA AATTATGGCTGACTAGTACGTAATATTCATACCAACGGAGCCTCTA TTTTCTTTATTTGTATTTATCTTCATATTGGCCGAGGC
<i>D. fuscus</i>	143	115	AAGTCAGCCTGCTGCACATATTTGCCGTGATGTAGATTATGGGTGAATTATACGA AATATCACGCAAACGGAGCATTTTCTTTCTTTATCTGTATTTATATACATATTGGA CGAGGAATCTATCACGACGCTGTGTCGTAAC

Table S3. CT values from qPCR data from field collected samples. D.S. = days stored at 4°C before filtering.

Site	Coll. date	D.S.	<i>E. cirrigera</i>			<i>D. fuscus</i>		
Clemons Fork	1/27/2016	16	0	0	0	0	0	0
Clemons Fork	2/17/2016	1	0	0	0	0	0	0
Clemons Fork	3/16/2016	64	0	0	0	0	0	0
Clemons Fork	5/4/2016	58	0	0	0	0	0	0
Clemons Fork	5/10/2016	23	0	0	0	0	0	0
Clemons Fork	6/13/2016	86	0	0	0	0	0	0
Clemons Fork	7/19/2016	15	0	0	0	0	0	0
Clemons Fork	9/14/2016	7	0	0	0	42.7	39.7	45.6
Clemons Fork	11/1/2016	9	0	0	0	43.9	45.01	0
Coles Fork	1/27/2016	16	0	0	0	34.7	33.2	34.9
Coles Fork	2/2/2016	10	0	0	0	35.0	35.24	39.5
Coles Fork	3/16/2016	16	38.5	37.2	39.2	35.00	34.0	35.0
Coles Fork	5/10/2016	58	38.5	39.2	34.5	35.0	34.7	37.0
Coles Fork	6/13/2016	23	36.4	46.3	0	33.8	35.2	35.4
Coles Fork	7/19/2016	86	44.0	0	0	35.9	33.8	34.6
Coles Fork	9/14/2016	15	37.1	0	0	45.0	45.7	0
Coles Fork	10/11/2016	9	38.6	0	0	36.268	38.1	0
Coles Fork	11/1/2016	9	0	0	0	47.3	0	0
Falling Rock	2/17/2016	1	35.7	35.3	36.9	34.2	32.6	34.1
Falling Rock	3/10/2016	22	0	0	0	0	0	0
Falling Rock	3/16/2016	16	36.3	38.0	38.6	39.8	36.3	37.6
Falling Rock	3/30/2016	2	41.2	42.2	45.0	42.0	38.8	40.7
Falling Rock	5/4/2016	2	0	0	0	0	0	0
Falling Rock	6/13/2016	64	37.7	47.5	0	37.1	40.0	0
Falling Rock	7/19/2016	23	42.4	42.8	39.9	36.3	38.2	34.8
Falling Rock	10/5/2016	7	0	0	0	0	0	0
Falling Rock	11/1/2016	9	37.8	0	0	0	0	0
Little Millseat	1/27/2016	16	38.7	0	0	35.7	35.2	37.7
Little Millseat	2/17/2016	1	36.8	35.0	44.8	35.5	35.6	0
Little Millseat	3/10/2016	22	0	0	0	0	0	0
Little Millseat	3/16/2016	16	0	0	0	0	0	0
Little Millseat	5/4/2016	64	34.5	34.3	33.9	34.7	33.7	33.9
Little Millseat	6/13/2016	23	47.0	0	0	33.9	35.8	37.2
Little Millseat	7/19/2016	86	43.5	36.9	0	34.7	0	0
Little Millseat	10/5/2016	7	0	0	0	48.1	0	0
Little Millseat	10/25/2016	16	41.0	41.4	0	335.0	35.9	0

Table S4. Location of three additional collection sites utilized to obtain additional water collected amplicons of *E. cirrigera* and *D. fuscus* eDNA.

Site	County (Kentucky)	Coordinates
Asbury Creek	Jessamine	37.853112, -84.685317
Highbridge Seep	Jessamine	37.818530, -84.719434
Stoney Run	Madison	37.856335, -84.241870

Table S5. Oligo sequences for previously published *D. fuscus* eDNA oligos (Beauclerc et al. 2019) targeting COX1 and similarity to *Desmognathus* species of the southeastern U.S.

F- CCACGTATAAATAATATAAGCTTCTGATTATTACC
R- TGAAGTGAAAAGATGGTTAAATCTACAGA
P- TATTTCCGGCTAAGGGC

Species	Probe mismatches	F primer mismatches	R primer mismatches	Accession number (COX1 genes)
<i>Desmognathus monticola</i>	2	5	2	MK037300.1
<i>Desmognathus fuscus</i>	0	0	0	NC_006339.1
<i>Desmognathus ochrophaeus</i>	7	0	0	MH403754.1
<i>Desmognathus welteri</i>	7	2	0	MH403623.1
<i>Desmognathus conanti</i>	3	2	1	MN692665.1
<i>Desmognathus quadramaculatus</i>	0	4	1	MN692645.1
<i>Desmognathus auriculatus</i>	0	1	1	MG426212.1
<i>Desmognathus ocoee</i>	3	2	3	MH404022.1

Table S6. Oligo sequences for previously published *D. fuscus* eDNA oligos (Cecilia Hernandez et al. 2020) targeting COX1 and similarity to *Desmognathus* species of the southeastern U.S.

F- AATATCACAATATCAAACACCATTATTTGTC
R- GTTAGAAGTATTGTAATTCCTGCTGCTAAA
P- CCGCTATTTTACTATTATTATCACTACC

Species	Probe mism.	Accession number	FP mism.	Accession number	RP mism.	Accession number
<i>Desmognathus fuscus</i>	0	NC_006339.1	0	NC_006339.1	0	NC_006339.1
<i>Desmognathus monticola</i>	5	MK037300.1	3	MK037300.1	3	MK037300.1
<i>Desmognathus ochrophaeus</i>	0	KU985633.1	0	MH403686.1	0	KU985633.1
<i>Desmognathus welteri</i>	12	MH403623.1	1	MH403623.1	12	MH403623.1
<i>Desmognathus conanti</i>	10	MN692665.1	4	MN692665.1	13	MN692665.1
<i>Desmognathus quadramaculatus</i>	2	MK037325.1	0	MK037325.1	3	MK037325.1
<i>Desmognathus auriculatus</i>	0	EU311687.1	0	KX764610.1	0	MG426212.1
<i>Desmognathus ocoee</i>	11	MH404022.1	0	MH404022.1	13	MH404022.1

Table S7. Amplicon produced with *D. fuscus* primers (end point PCR) from water sample collected from Falling Rock Creek (Robinson Forest) (Table 7, main publication).

Length	Sequence
118 BP	TGCACATATTTGCCGTGATGTAGATTATGGCTGATTAATCCGAACC ATTCATACTAATGGAGCTTCTCTCTTTATTTGTATTTATATACAC ATTGGACGAGGAATCTATCACGAA

Table S8. Alignment of amplicon produced with *D. fuscus* primers (end point PCR) from water sample collected from Falling Rock Creek (Robinson Forest) (Table 7, main publication) and the *D. fuscus* sequence produced from this project (87.5% similar) and three published *Plethodon dorsalis* cytb sequences ([DQ994930.1](#) = 96.4% similar, AY378077.1 = 96.4% similar, and GQ464404.1 = 87.6% similar).

1. Table 6 amplicon	G C A C A T A T T T G C C G T G A T G T A G A T T A T G G C T G A T T A A T C C G A A C C A T T C A T A C T A A T G G A G C T C T C T T T C T T T A T T T G T A T T T A T A T A C A C A T T G G A C G G G A A T C T A T C A C G
2. Dusky sequence	G C A C A T A T T T G C C G T G A T G T A G A T T A T G G G T G A A T T A T C G A A A T T T C A C G C A A C G G A G C A T C T T C T T T A T C T G T A T T T A T A T A C A T A T T G G A C G G G A A T C T A T C A C G
3. DQ994930.1	G C A T A T C T G C C G C A T G T A A T T A T G G C T G A T T A A T C C G A A C C A T T C A T A C T A A T G G A G C T C T C T T T C T T T A T T T G T A T T T A T A T A C A C A T T G G A C G G G A A T C T A C T A C G
4. AY378077.1	G C A C A T A T C T G C C G C A T G T A A A T T A T G G C T G A T T A A T C C G A A C C A T T C A T A C T A A T G G A G C T C T C T T T C T T T A T T T G T A T T T A T A T A C A C A T T G G A C G G G A A T C T A C T A C G
5. GQ464404.1	G T A C A C A T T T G C C G C A T G T A A A T T A T G G C T G A C T A A T C C G A A G T A T T C A T G C C A A C G G C G C T T C T T A T T C T T T A T C T G T A T T T A T A T A C A C A T T G G A C G A G G A A T C T A T T A C G