eDNA detection of northern red (*Pseudotriton ruber*) and spring (*Gyrinophilus porphyriticus*) salamanders in eastern Kentucky streams

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Introduction

Environmental DNA (eDNA) utilizes DNA that is released from aquatic organisms into the environment to detect their presence and provides an effective, non-invasive method to determine organism presence or absence in an efficient manner[1,2,3]. We developed species-specific oligos to detect two semiaquatic salamander species.

Figure 1. (A) *Pseudotriton ruber* (northern red salamander, photo by Brianna Wilson) (B) *Gyrinophilus porphyritcus* (spring salamander), photo by Todd Pierson.

Of the 35 different salamander species in Kentucky, we selected two for this project: *Pseudotriton ruber* (northern red) and *Gyrinophilus porphyritcus* (spring). Although neither of these salamanders are threatened in Kentucky, both are listed as a species of concern in other portion of their range.

We designed species-specific primers and probes for these two salamander species and tested them *in silico*, *in vitro*, and *in situ*. *In situ* tests consisted of 36 water samples collected over a one-year period in Robinson Forest (Breathitt and Knott Counties, KY).

Methods

Sequencing

Previously published[4] or in house designed primers were utilized to amplify and sequence cytochrome b. GenBank® accession numbers appear in Table 1.

Table 1. Cytochrome b amplicons obtained from local specimens and used in primer development.

Species		Collection Location	Amplicon Length	GenBank Accession #
Pseudotriton ruber	N. red	Madison County, KY	861	OQ376719.1
Gyrinophilus porphyriticus	Spring	Breathitt County, KY	363	MZ507696.1

Primer Design

Potential primers and probes were designed using IDT's PrimerQuest software, primers pairs were evaluated for specificity using MEGAX.

Table 2. Quantitative PCR assays developed for the two salamander species.

Target Species	Amplicon Length (BP)	Oligo	Sequence (5'-3')
Pseudotriton ruber	90	F	GTCTGCCTCATTGCACAAATC
		R	GTGGGCTACTGAGGAGAATG
		P	TACACTATACCGCAGACACCACCTCA
Gyrinophilus porphyriticus	117	F	ACAGGCCTCTTCTTAGCTATAC
		R	GTTGGCGTGAATATTTCGTACT
		P	TTCAGTAGCACACATCTGCCGAGA

Methods

In Situ Testing

Water samples were collected periodically from four eastern Kentucky streams located in Robinson Forest (Little Millseat, Falling Rock, Clemons Fork, and Coles Fork) over an approximate one-year period. Approximately 10 samples were collected from each stream, 36 samples total.

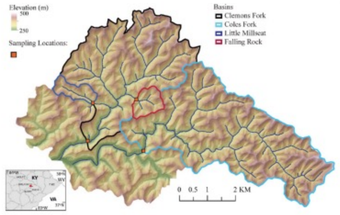

Figure 3. Sample locations on Clemons Fork, Coles Fork, Little Millseat, and Falling Rock creeks in Robinson Forest, (Breathitt and Knott Counties, KY), USA.

Water eDNA Extraction

Environmental DNA extraction was performed using a modified version of an established protocol[5]. The extraction was conducted using a DNeasy® Blood & Tissue Kit (Qiagen).

eDNA quantification

Extracted DNA was quantified using a StepOnePlus™ Real-Time PCR system. Standard curves were generated using synthetic DNA (gBlock™ IDT™) to both enable data reporting in copy number and assess lowest observed limits of detection and quantification[5].

Inhibition testing

All samples were run with an internal positive control (TaqMan™ Exogenous Internal Positive Control) to assess potential PCR inhibition.

Specificity Testing

In Silico Testing - Sympatric species

All primers and probes had a minimum of two mismatches with tested sympatric species. Additionally, we utilized the modeling software eDNAssay[6] which predicts amplification probabilities. *In silico* analysis of the *P. ruber* oligos is shown below (*G. porphyriticus* is not included because of space limitations).

In Silico Testing - Subspecies

Both assays were screened *in silico* against each existing subspecies (four for northern red, four for spring). Sequences of each subspecies were obtained from GenBank, specificity screening was conducted by analyzing mismatch presence/position amplification probability.

Results

Table 3. Mismatch table and amplification probability for *Pseudotriton ruber* with 21 sympatric species.

Sympatric species	FP mismatches	RP mismatches	P mismatches	Amp. prob.	Seq. accession #	Symp.	*In vitro*
Pseudotriton ruber	0	0	0	0.87	OQ376719	-	-
Pseudotriton montanus	3	3	4	0.25	MW319716.1	Y	Y
Gyrinophilus porphyriticus	7	4	2	0.30	MZ507696.1	Y	Y
Eurycea cirrigera	4	2	4	0.21	MZ485475.1	Y	Y
Eurycea lucifuga	4	1	6	0.31	KT873718.1	N	Y
Eurycea longicauda	5	1	5	0.25	AY528403.1	Y	N
Eurycea bislineata	4	4	6	0.20	AY528402	N	N
Desmognathus fuscus	7	3	5	0.16	MZ485476.1	Y	Y
Desmognathus monticola	4	4	4	0.16	MZ418126.1	Y	N
Desmognathus ochrophaeus	5	2	5	0.25	EU314289	Y	N
Desmognathus welteri	6	6	3	0.16	EU314293	Y	N
Desmognathus conanti	4	2	5	0.21	EU314275.1	Y	N
Plethodon glutinosus	6	5	7	0.16	MN723529.1	Y	N
Plethodon dorsalis	5	4	7	0.24	GQ464404	N	N
Plethodon richmondi	6	4	6	0.20	AY378072	Y	N
Ambystoma barbouri	5	5	5	0.25	OL456142.1	N	N
Ambystoma opacum	6	4	6	0.16	KT780868.1	Y	N
Ambystoma jeffersonianum	6	4	6	0.24	KT780869.1	N	N
Ambystoma maculatum	7	5	6	0.17	MZ485477.1	Y	N
Ambystoma tigrinum	5	4	6	0.23	MZ962317.1	N	N
Hemidactylium scutatum	4	3	4	0.11	AY728231	Y	Y
Notophthalmus viridescens	5	1	6	0.19	AY691731	Y	N

Results

Specificity testing

In Silico Testing

Both our *P. ruber and G. porphyriticus* assays exhibited varying numbers of mismatches across their four respective subspecies (only *P. ruber* results are included here). In brief, modeling results indicate only 9/19 *P. ruber* supspecies tested would be detected using our assay (prob. of 0.61 or greater). Of the 12 *G. porphyriticus* subspecies tested all except one have an amplification probability indicating amplification (results not shown).

Figure 4. Mismatch table and amplification probability for the four subspecies of *Pseudotriton ruber* from various locations within their range.

Subspecies	County	GB #	Clade	Pop. #	Amp. prob.	F primer	R primer	Probe
P. ruber ruber[1]	Madison Co, KY	OQ376719	-	-	0.873	0	0	0
P. ruber ruber	Rockcastle Co., KY	KR054853	B4	41	0.873	0	0	0
P. ruber ruber	Menifee Co., KY	KR054854	B4	42	0.873	0	0	0
P. ruber ruber	Clarke Co., GA	KR054858	B4	19	0.873	0	0	0
P. ruber ruber	Athens Co., OH	KR054922	B3	43	0.586	1 GTCTGCCTCATTGCACAAATC A	2 GTGGGCTACTGAGGAGAATG T A	0
P. ruber ruber	Summit Co., OH	KR054897	B3	45	0.586	1 GTCTGCCTCATTGCACAAATC A	2 GTGGGCTACTGAGGAGAATG T A	0
P. ruber ruber	Moore Co., NC	KR054924	B4	30	0.586	1 GTCTGCCTCATTGCACAAATC A	2 GTGGGCTACTGAGGAGAATG T A	0
P. ruber ruber	Franklin Co., TN	KR054916	B2	29	0.422	2 GTCTGCCTCATTGCACAAATC A A	2 GTGGGCTACTGAGGAGAATG T A	0
P. ruber ruber	Madison Co., AL	KR054845	B2	23	0.490	2 GTCTGCCTCATTGCACAAATC A A	3 GTGGGCTACTGAGGAGAATG A T A	0
P. ruber nitidus	Unicoi Co., TN	KR054871	B4	38	0.748	0	1 GTGGGCTACTGAGGAGAATG T	0
P. ruber nitidus	Watauga Co., NC	KR054882	B4	39	0.748	0	1 GTGGGCTACTGAGGAGAATG T	0
P. r. schencki	Swain Co., NC	KR054880	B4	36	0.873	0	0	0
P. r. schencki	Graham Co., NC	KR054875	B4	33	0.748	0	1 GTGGGCTACTGAGGAGAATG T	0
P. r. schencki	Fannin Co., GA	KR054864	B4	27	0.749	1 GTCTGCCTCATTGCACAAATC A	1 GTGGGCTACTGAGGAGAATG T	0
P. r. schencki	Gilmer Co., GA	KR054859	B4	24	0.749	1 GTCTGCCTCATTGCACAAATC A	1 GTGGGCTACTGAGGAGAATG T	0
P. r. vioscai	Marshall Co., KY	KR054889	A	40	0.490	2 GTCTGCCTCATTGCACAAATC A A	2 GTGGGCTACTGAGGAGAATG T A	0
P. r. vioscai	Winston Co., MS	KR054913	A	13	0.490	2 GTCTGCCTCATTGCACAAATC A A	2 GTGGGCTACTGAGGAGAATG T A	0
P. r. vioscai	Washington Co., LA	KR054911	A	1	0.490	2 GTCTGCCTCATTGCACAAATC A A	2 GTGGGCTACTGAGGAGAATG T A	0
P. r. vioscai	Covington Co., AL	KR054903	A	3	0.490	2 GTCTGCCTCATTGCACAAATC A A	2 GTGGGCTACTGAGGAGAATG T A	0
P. r. vioscai	Burke Co., GA	KR054895	B2	12	0.605	2 GTCTGCCTCATTGCACAAATC A A	2 GTGGGCTACTGAGGAGAATG T	0

Specificity testing

In Vitro Testing

End-point reactions (35 cycles, annealing temp. of 60°C) with target DNA and six closely related sympatric species demonstrated no amplification of non-target species.

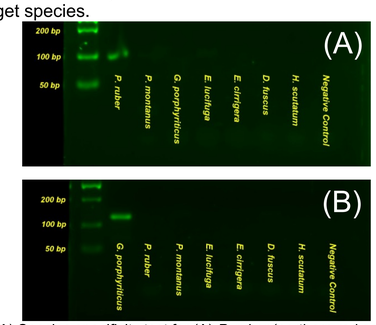

Figure 4. (A) Species specificity test for (A) *P. ruber* (northern red salamander) and (B) *G. porphyriticus* (spring salamander).

Results

Field testing

qPCR analysis of 36 field-collected samples, 15 positive detections for *P. ruber* and 10 for *G. porphyriticus* (Table 5).

Table 5. qPCR eDNA analysis from field-collected samples, tested in triplicate.

Stream	Date Collected	Northern Red Positive Detections	Northern Red copies/reaction	Spring Positive Detections	Spring copies/reaction
Clemons Fork	9/15/2015	0/3		0/3	
Clemons Fork	1/27/2016	0/3		0/3	
Clemons Fork	2/9/2016	0/3		0/3	
Clemons Fork	2/17/2016	2/3	10.5	0/3	
Clemons Fork	3/1/2016	0/3		0/3	
Clemons Fork	6/21/2016	1/3	2.0	0/3	
Clemons Fork	6/27/2016	0/3		0/3	
Clemons Fork	8/8/2016	0/3		1/3	11.3
Clemons Fork	10/5/2016	0/3		1/3	21.2
Clemons Fork	11/1/2016	0/3		1/3	48.1
Coles Fork	9/15/2015	0/3		1/3	25.1
Coles Fork	1/27/2016	1/3	10.0	0/3	
Coles Fork	2/9/2016	1/3	31.0	2/3	15.1
Coles Fork	3/1/2016	0/3		0/3	
Coles Fork	6/21/2016	0/3		3/3	18.8
Coles Fork	6/27/2016	1/3	10.1	1/3	26.3
Coles Fork	10/25/2016	1/3	7.2	0/3	
Coles Fork	2/17/2016	1/3	10.2	0/3	
Falling Rock	1/27/2016	0/3		0/3	
Falling Rock	2/9/2016	0/3		0/3	
Falling Rock	2/17/2016	0/3		0/3	
Falling Rock	3/1/2016	3/3	18.1	1/3	4.5
Falling Rock	6/21/2016	0/3		0/3	
Falling Rock	6/27/2016	0/3		0/3	
Falling Rock	10/11/2016	0/3		0/3	
Falling Rock	10/25/2016	1/3	17.7	0/3	
Little Millseat	9/15/2015	1/3		0/3	
Little Millseat	1/27/2016	0/3		0/3	
Little Millseat	2/9/2016	0/3		0/3	
Little Millseat	2/17/2016	1/3	2.7	0/3	
Little Millseat	3/1/2016	0/3		0/3	
Little Millseat	6/21/2016	2/3	13.7	0/3	
Little Millseat	6/27/2016	2/3	10.1	0/3	
Little Millseat	10/5/2016	3/3	165.3	3/3	171.7
Little Millseat	10/11/2016	0/3		1/3	13.2
Little Millseat	10/25/2016	2/3	2.1	0/3	

Conclusions

- Primers designed for *G. porphyriticus* and *P. ruber* were species-specific among the 12 sympatric species tested *in silico* and six tested *in vitro*.
- eDNA results for *G. porphyriticus* and *P. ruber* indicate a relatively low percentage of positive results, lower than that observed for a sympatric salamander species (*E. cirrigera*) in these streams[5]. These data appear consistent with the trophic status of these species.
- Previous studies in these streams have reported greater salamander abundance in Little Millseat relative to other streams[5], consistent with our observations.
- Subspecies analysis indicates assays are broadly, but not universally, effective across subspecies, emphasizing the importance of phylogenetic history in the implementation of eDNA studies.

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