The search for a rare salamander: The use of eDNA in detection of *Eurycea junaluska* in the Great Smoky Mountains National Park

Ben F. Brammell[1], Sara A. Brewer[1], Elizabeth K. Strasko[2], Jarrett R. Johnson[2], Madeline Cox[1]

[1]Department of Science and Health, Asbury University, Wilmore, KY 40390
[2]Department of Biology, Western Kentucky University, Bowling Green, KY 42101

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 a rare species of semiaquatic salamander, *Eurycea Junaluska* using eDNA.

Figure 1. *Eurycea Junaluska* (the Junaluska salamander) photo by Todd Pierson.

E. junaluska was first described in 1976 and at first only known from three creeks in Graham County, North Carolina[4]. A combination of reexamination of previously collected specimens[5] and new field work expanded the range to several locations on both the TN and NC sides of the Great Smoky Mountains National Park as well as to adjacent Polk County, TN[6]. Currently *E. junaluska* is now known to exist in a total of five counties in TN and NC[7].

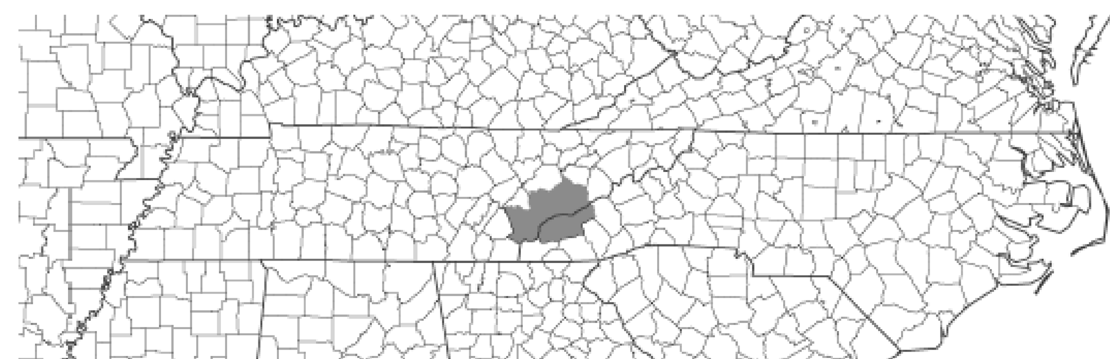

Figure 2. *Eurycea junaluska* range, from Ryan and Sever (2005)[7].

The extreme endemism of *E. junaluska* makes it vulnerable to threats that are of lesser concern to more widely distributed species[8]. Furthermore, the disjunct nature of the population means it is unlikely migration would be a means to repopulation following local extinctions[9]. Already efforts to collect specimens at the type locality on the Cheoah River have proven unsuccessful, leading to fears of extirpation resulting from upstream anthropogenic activities[7]. Chattin et al.[10] suggest that many of the current *E. junaluska* populations may occupy sub-optimal habitat and highlighted the need for studies examining this species.

Although a proposal to list *E. junaluska* as federally endangered was denied[11], the species is currently listed in TN as "In Need of Management" and as "Threatened" in NC[12]. A more thorough understanding of the range of this species would greatly enhance the understanding of exactly how rare it is and facilitate conservation efforts.

The objectives of this study were to:

A) Develop and validate a qPCR protocol to detect *E. junaluska* using eDNA.
B) Utilize this assay to survey for *E. junaluska* in the Great Smoky Mountains National Park.

Methods

Sequencing

E. junaluska DNA was acquired (Graham Co., NC) and published primers used to amplify a 515 BP region of cytochrome b (cytb). PCR products were bidirectionally sequenced via Sanger using either both the F or R primer; all sequencing was conducted by ACGT, Inc. (ACGTinc.com).

Primer Design

This sequence was aligned with a published *E. junaluska* cytb sequence (Sevier Co., TN, Acc. # KF562550.1). Primers and probes were designed using IDT's PrimerQuest software in the conserved regions between these two sequences (Figure 3).

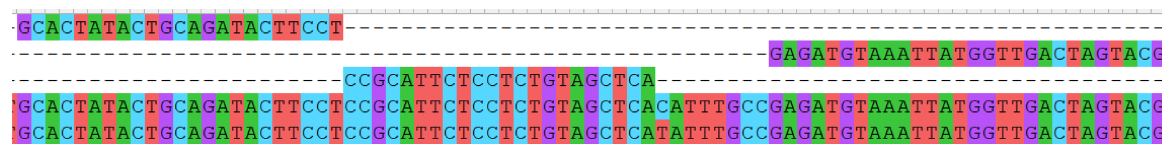

Figure 3. Two aligned *E. junaluska* sequences (bottom) with forward primer (upper left), probe (middle) and reverse primer (right). *E. junaluska* sequences are 95% similar.

In Silico Testing

Primers were tested for specificity against the 31 other salamander species found in the GSMNP (with the exception of the newly described *Desmognathus gvnigeusgwotli* (Cherokee Black-bellied salamander) for which sequences were not available. Mismatches were quantified via alignment in MEGA X and amplification probability was assessed using a machine learning model (eDNAssay)[13].

Table 1. Quantitative PCR assays developed for *E. junaluska*.

Oligo	Tm (°C)	Length (BP)	Sequence (5'-3')
Forward primer	61.6	23	GCACTATACTGCAGATACTTCCT
Reverse primer	61.7	28	CGTACTAGTCAACCATAATTTACATCTC
Probe	65.4	22	CCGCATTCTCCTCTGTAGCTCA

In Situ Testing

Water samples were collected periodically from fifty sites throughout the Great Smoky Mountains National Park over a period of two weeks in July 2023 (Figures 4 and 5). One liter water samples were collected via sterile filter funnels and vacuum filtration. Filters were field preserved in ATL buffer in 1.5-ml tubes on ice. All collection equipment in contact with samples was sterilized between sites.

Figure 4. Sample collection on the Middle Prong of the Little River, Great Smoky Mountains National Park.

Water eDNA Extraction

Environmental DNA extraction was performed using a modified version of an established protocol[14]. The extraction was conducted using a DNeasy® Blood & Tissue Kit (Qiagen). Extraction was conducted in a sterile hood known to previously have no contact with target salamander DNA.

PCR optimization

We evaluated the novel forward and reverse primers via a polymerase chain reaction temperature gradient approach to determine the optimal annealing temperature (nine temperatures between 57.1 – 66.2°C were tested).

Methods

Inhibition testing

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

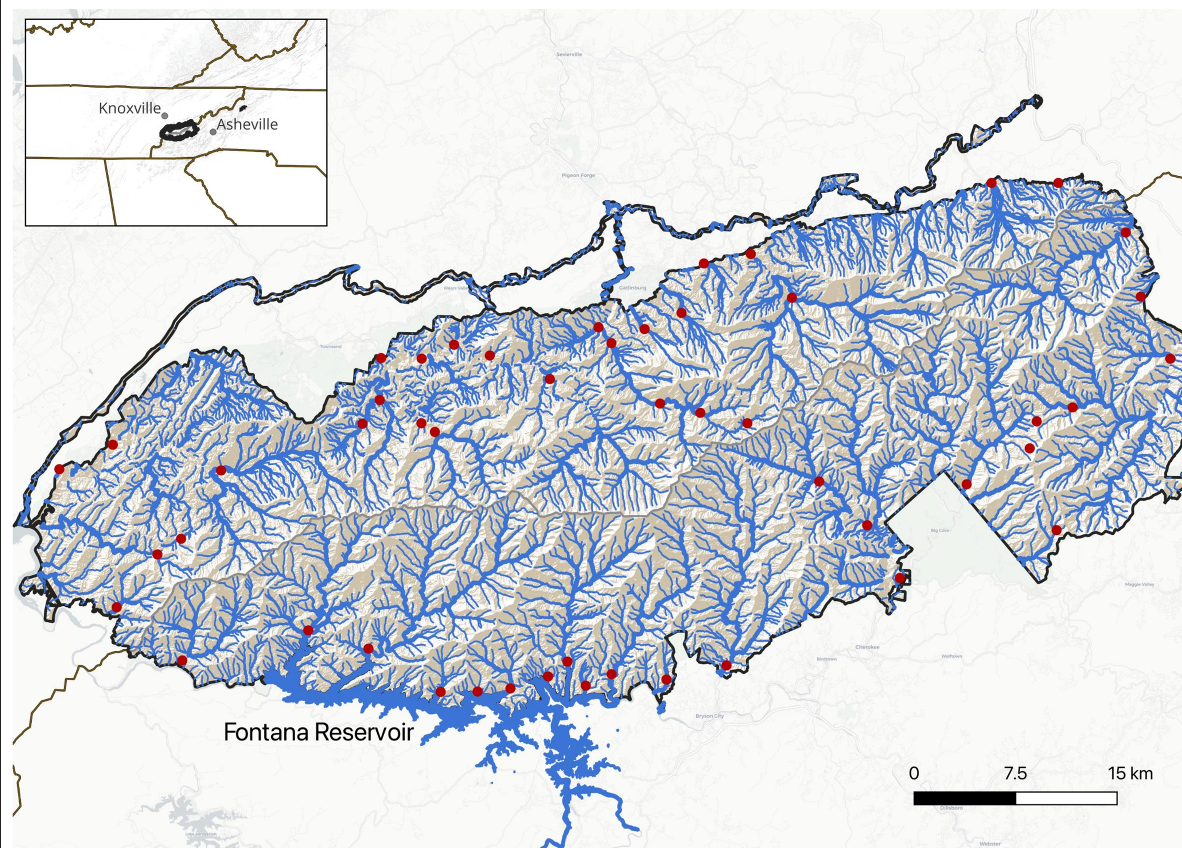

Figure 5. Sample collection sites in the Great Smoky Mountains National Park, July 2023.

eDNA quantification

Extracted DNA was quantified using a StepOnePlus™ Real-Time PCR system. Each run contained tissue-extracted target species DNA (1.0 µg/mL) as a positive control and also included a non-template negative control. Each 20.0 µL reaction contained the following: TaqMan™ EMM 2.0 (10.0 µL), nuclease-free water (IDT™) (2.0 µL), eDNA extract (7.0 µL), and assay (1.0 µL). Thermocycler conditions were as follows: 50°C for two min, 95°C for ten minutes, and 55 cycles of 95°C for 15 seconds and 60°C for one minute. Samples will be run in triplicate but only one replicate per sample has been completed at this time.

Results

In Silico Testing

All oligos (forward primer, reverse primer, and probe combined) have a minimum of seven mismatches with non-target Great Smoky Mountains National Park salamander species (Table 2). Amplification probabilities produce by the machine learning model ranged from 0.10 (six species) to 0.52 (*Eurycea wilderae*) with non target species but 0.97 for target sequences.

In Vitro Testing

End-point reactions (40 cycles, annealing temp. of 60°C) with target DNA and six closely related sympatric species demonstrated little to no amplification of seven non-target species, including three *Eurycea* species.

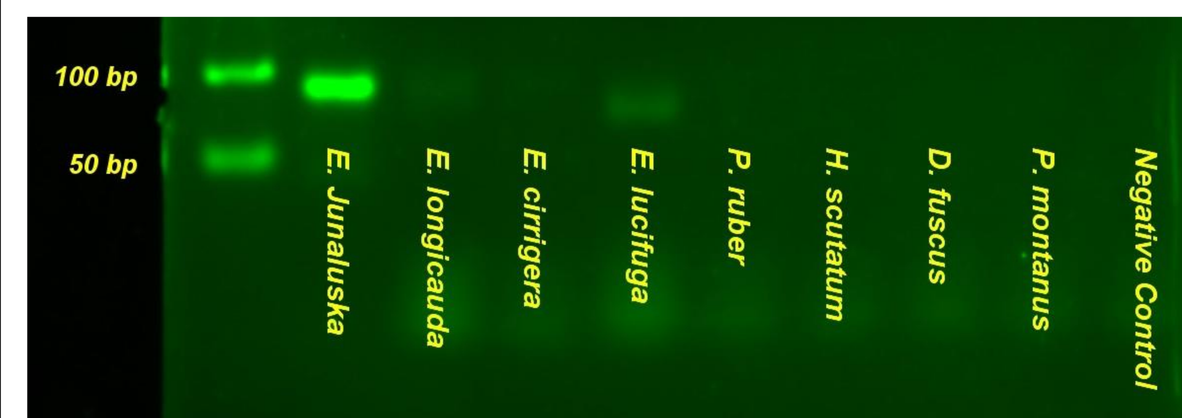

Figure 6. Species specificity test using forward and reverse primers (no probe). Reactions were run for 40 cycles at an annealing temperature of 60°C.

Results

In Situ Testing

Water sample testing is in progress. Initial results (one replicate for each of the fifty samples) indicate two amplifications. These are both in the Little River drainage and consistent with historical *E. junaluska* records.

Table 2. Mismatch table and amplification probability for *Eurycea junaluska* with all GSMNP salamander species.

	Family	Species	Common name	AP	F	R	P	Seq. accession #	Len. (BP)
1		*Ambystoma maculatum*	Spotted Salamander	0.10	6	6	4	EF036637.1	744
2	Ambystomatidae	*Ambystoma opacum*	Marbled Salamander	0.13	3	7	4	KT780868.1	720
3		*Ambystoma talpoideum*	Mole Salamander	0.11	5	6	5	EF036640.1	744
4	Cryptobranchidae	*Cryptobranchus alleganiensis*	Hellbender	0.11	5	3	7	AY691719.1	783
5		*Aneides aeneus*	Green Salamander	0.10	5	3	4	OM111015.1	365
6		*Desmognathus aeneus*	Seepage Salamander	0.18	4	8	3	EU314341.1	519
7		*Desmognathus conanti*	Spotted Dusky Salamander	0.14	7	5	4	EU314275.1	519
8		*Desmognathus imitator*	Imitator Salamander	0.14	8	7	6	EU314336.1	519
9		*Desmognathus marmoratus*	Shovel-nosed Salamander	0.15	6	5	4	EU314355.1	519
10		*Desmognathus monticola*	Seal Salamander	0.10	6	7	5	AY691738.1	783
11		*Desmognathus ocoee*	Ocoee Salamander	0.17	7	6	4	EU314297.1	519
12		*Desmognathus quadramaculatus*	Black-bellied Salamander	0.16	7	6	4	EU314374.1	519
13		*Desmognathus santeetlah*	Santeetlah Salamander	0.14	8	7	6	EU314334.1	519
14		*Desmognathus gvnigeusgwotli*	Cherokee Black-bellied Salam.	-	-	-	-	N. A.	-
15		*Desmognathus wrighti*	Pigmy Salamander	0.10	10	7	6	EU314340.1	519
16		*Eurycea guttolineata*	Three-lined Salamander	0.42	4	4	1	JQ920625.1	1012
17		***Eurycea junaluska***	**Junaluska Salamander**	**0.97**	**0**	**0**	**0**	**KF562550.1**	**674**
-	Plethodontidae	***Eurycea junaluska***	**Junaluska Salamander**	**0.97**	**0**	**0**	**0**	**Pending**	**518**
18		*Eurycea longicauda*	Long-tailed Salamander	0.51	5	3	1	JQ920624.1	1,012
19		*Eurycea lucifuga*	Cave Salamander	0.27	3	2	5	EF044248.1	982
20		*Eurycea wilderae*	Blue Ridge Two-lined Salaman.	0.52	4	1	2	MK029491.1	439
21		*Gyrinophilus porphyriticus*	Spring Salamander	0.16	6	2	5	EU336391.1	783
22		*Hemidactylium scutatum*	Four-toed Salamander	0.19	4	6	4	AY691752.1	783
23		*Plethodon glutinosus*	Northern Slimy Salamander	0.09	6	7	8	MN723529.1	1,117
24		*Plethodon jordani*	Jordan's Salamander	0.10	5	8	6	DQ994947.1	649
25		*Plethodon metcalfi*	Southern Gray-cheeked Salaman.	0.08	8	8	5	DQ994957.1	632
26		*Plethodon oconaluftee*	Southern Appalachian Salaman.	0.10	6	7	5	DQ994966.1	649
27		*Plethodon serratus*	Southern Red-backed Salamander	0.16	4	8	6	KM225293.1	1,140
28		*Plethodon ventralis*	Southern Zigzag Salamander	0.14	5	4	5	DQ994994.1	631
29		*Pseudotriton montanus*	Mud Salamander	0.16	3	3	4	KF562586.1	674
30		*Pseudotriton ruber*	Black-chinned Red Salamander	0.18	5	2	4	AY528404.1	1,118
31	Proteidae	*Necturus maculosus*	Common Mudpuppy	0.21	7	4	3	AY691724.1	783
32	Salamandridae	*Notophthalmus viridescens*	Eastern Red-spotted Newt	0.14	8	3	4	AY691731.1	783

Conclusions

- Primers designed for *E. junaluska* were species-specific among the thirty-one sympatric GSMNP species tested *in silico* and seven tested *in vitro*.
- Although all amplification probabilities predicted by the model fall below the recommended 0.55 threshold, *E. wilderae* (0.52) and *E. longicauda* (0.51) are close. Further *in vitro* qPCR testing will be completed with these species.
- Initial field eDNA results indicate a relatively low percentage of positive results, seemingly consistent with the rare status of this species.
- Future *in silico* work will examine the efficacy of this assay with *E. aquatica* given the uncertain taxonomic status of *E. junaluska* and *E. aquatica*.

Bibliography

[1] Thomsen PF, Willerslev E. Environmental DNA- An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation. 2015 [accessed 2018 Mar 30];183:4–18.
[2] Goldberg CS, Strickler KM, Fremier AK. Degradation and dispersion limit environmental DNA detection of rare amphibians in wetlands: Increasing efficacy of sampling designs. Science of the Total Environment. 2018;633:695–703.
[3] 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.
[4]Sever David M.;Dundee, H. A. ;Sullivan C. D. A New Eurycea (Amphibia: Plethodontidae) from Southwestern North Carolina. *Herpetologica* **32,** (1976).
[5]King, W. A Survey of the Herpetology of Great Smoky Mountains National Park. *American Midland Naturalist* **21,** (1939).
[6]Sever, D. Observations on the distribution and reproduction of the salamander Eurycea junaluska in Tennessee. *Journal of the Tennessee Academy of Science* **58,** 48–49 (1983).
[7]Ryan, T. J., and D. M. Sever. 2005. Eurycea junaluska,Junaluska Salamander, p. 745–746. In: AmphibianDeclines: The Conservation Status of United StatesSpecies. M. J. Lannoo (ed.). University of Califor-nia Press, Berkeley.
[8]Ryan, T. J. Larva of Eurycea junaluska (Amphibia: Caudata: Plethodontidae), with comments on distribution. *Copeia* (1997). doi:10.2307/1447861
[9]Ryan, T. J. Larval life history and abundance of a rare salamander, Eurycea junaluska. *J Herpetol* **32,** (1998).
[10]Chattin, E., Forester, D. C. & Snodgrass, J. W. Distribution of a rare salamander, Eurycea junaluska: Implications for past impacts of river channelization and impoundment. *Copeia* (2007). doi:10.1643/0045-8511(2007)7[952:DOARSE]2.0.CO;2
[11]USFWS. *Endangered and Threatened Wildlife and Plants; 12-Month Finding for a Petition To List the Junaluska Salamander as Endangered With Critical Habitat*. (1999). at <http://www.gpo.gov/fdsys/pkg/FR-1999-07-29/html/99-19425.htm>
[12]NCWRC. *Protected Wildlife Species of North Carolina*. (2014). at <http://www.ncwildlife.org/portals/0/Conserving/documents/protected_species.pdf>
[13]Kronenberger, J.A., Wilcox, T.M., Mason, D.H., Franklin, T.W., McKelvey, K.S., Young, M.K., Schwartz, M.K. (2022): eDNAssay: A machine learning tool that accurately predicts qPCR cross-amplification. Mol Ecol Resour **22:.**
[14]Bell, Florene F.*, Angie F. Flores*, Kenton L. Sena, Thomas A. Maigret, Chi Jing Leow*, Ronald Sams*, David 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 17(2):398-412.

Acknowledgements

The authors sincerely thank Bryan Stuart (North Carolina Museum of Natural Sciences) for the generous donation of *E. junalsuka* DNA. This research is part of a project funded by a Carlos C. Campbell Memorial Fellowship from the Great Smoky Mountains National Park Conservation Association (Brammell, Strasko, and Johnson 2023). Additional support was provided by the Asbury University, Shaw School of Science, Department of Science and Health. All samples were collected under permit GRSM-2023-SCI-1191 (Brammell 2023).