SUPPLEMENTAL INFORMATION

eDNA MONITORING OF A RARE SALAMANDER: THE USE OF eDNA IN DETECTION OF *EURYCEA JUNALUSKA* IN THE GREAT SMOKY MOUNTAINS NATIONAL PARK

BEN F. BRAMMELL, SARA A. BREWER, ELIZABETH K. STRASKO, MADELINE M. COX, JARRETT R. JOHNSON, AND DAVID K. PEYTON

TABLE S1. Primer sequences, annealing temperatures ("AT"), cycle numbers ("# cycles"), and expected product lengths ("Prod. len.") for primers used in amplification of *Eurycea junaluska* and *Eurycea wilderae* cytochrome b and environmentally obtained *E. junaluska* amplicons (amplified for sequencing). MVZ primers are from Moritz et al. (1992); "Eurycea cytb for." was designed for this study.

Template	Primers	Primer sequences	AT (° C)	# cycles	Prod. len.
E. junaluska	MVZ15 (F)	GAACTAATGGCCCACACWWTACGN	47.2	45	700
	MVZ18 (R)	GTCTTTGTATGAGAAGTATG			
E. wilderae	Eurycea cytb for (F)	ACTCCTTTATTGACCTCCC	40.7, 41.9 or 46.5	45	781
	MVZ16 (R)	AAATAGGAARTATCAYTCTGGTTTRAT			
Env. amplicons	Junal ENV F Gex3 B	CCGGGAGCTGCATGTGTCAGAGGACGTG CATCGATCAGCACTATACTGCAGATACTT CCT	60	40	153
	Junal ENV R Gex3 B	CCGGGAGCTGCATGTGTCAGAGGCTGCC ATCACGTACTAGTCAACCATAATTTACAT CTC	60	40	153

TABLE S2. Cytochrome b sequences generated as part of this study. "Percent similarity to existing *E. jun.*" = similarity to the previously existing *Eurycea junaluska* sequence (GenBank Accession # KF562550.1, collection locality = Blount County, TN); "AP" = assignment probability of the sequence with our *E. junaluska* assay as determined by machine learning model (Kronenberger et al. 2022).

Species	Collection locality	GenBank Acc. #	Percent similarity to existing *E. jun*	Number of mismatches with *E. junaluska* oligos			AP
				F primer	R primer	Probe	
E. junaluska	Graham Co., NC	PP277535.1	95.8	0	0	0	0.97
E. wilderae	Blount Co., TN	PP496518.1	91.8	3	1	2	0.39
E. wilderae	Cocke Co. TN	PP393048.1	91.9	*	1	2	*
E. wilderae	Cocke Co., TN	PP393049.1	92.1	3	1	2	0.39
E. wilderae	Rabon Co., GA	PP393050.1	90.8	3	2	2	0.304

* = Non-published sequences

TABLE S3. gBlocks sequences used in qPCR assay sensitivity testing for *Eurycea junaluska* (Row 1) and specificity testing (Row 2). For the *E. junaluska* gBlocks, blue bases represent amplicon, grey shading represents forward and reverse primers, and yellow shading represents probe.

	Total length (bp)	gBlocks sequence
E. junaluska	500	GCATGCAGCACTATACTGCAGATACTTCCTCCGCATTCTCCTCTGTAG CTCACATTTGCCGAGATGTAAATTATGGTTGACTAGTACGAGGATATG TTCTCCCATGAGGACAAATATCTTTCTGAGGAGCAACCGTCATCACAA ACCTACTATCCGCAATCCCATATTTAGGAGTCACAGTTGTACAGTGAA TTTGAGGTGGCTTCTCAGTGGATAAAGCAACACTAACCCGATTTTTTG CCTTTCATTTTATTTTACCATTTATAATTGCTGGCGCCAGCATTGTTCA TCTACTTTTCCTCCACGAAACAGGATCAAACAACCCAACAGGACTTA ACTCTAACCCAGATAAAATCCCATTTCATCCATATTATTCTTATAAAG ATCTGGAGCAACCGTTATCACAAATCTATTGTCCGCAATTCCATACTT GGGAGACACACTTGTTCAATGAATTTGAGGCGGCTTTTCAGTAGACA AGGCAACATTAACCGCATGCA
E. guttolineata	499	TCCCTTTTAGGAATCTGCCTAATTACACAAATCTTAACAGGATTATTT CTTGCAATGCACTACACTGCAGACACCACCTCCGCATTCTCCTCTGTA GCCCATATCTGCCGAGACGTTAATTATGGATGACTTGTACGCAGCAT TCACACCAACGGAGCCTCTATCTTCTTTATTTGTATCTATCTTCATATT GGACGAGGCTTATATTATGGCTCATACATATTTAAAGAAACCTGAAA CATTGGAGTCATTTTATTATTTTTAGTAATAGCAACAGCATTTGTAGG ATACGTTCTACCATGAGGACAAATATCTTTCTGAGGAGCAACCGTCA TCACAAATCTACTATCCGCAATCCCATATCTAGGAGACACACTTGTT CAGTGAATTTGAGGTGGCTTCTCAGTAGATAAAGCAACATTAACCCG ATTTTTCGCCTTTCATTTTATTTTACCATTTATAATTGCTGGCGCCAGC ATTGTCCATCTACTTTTCCTCC

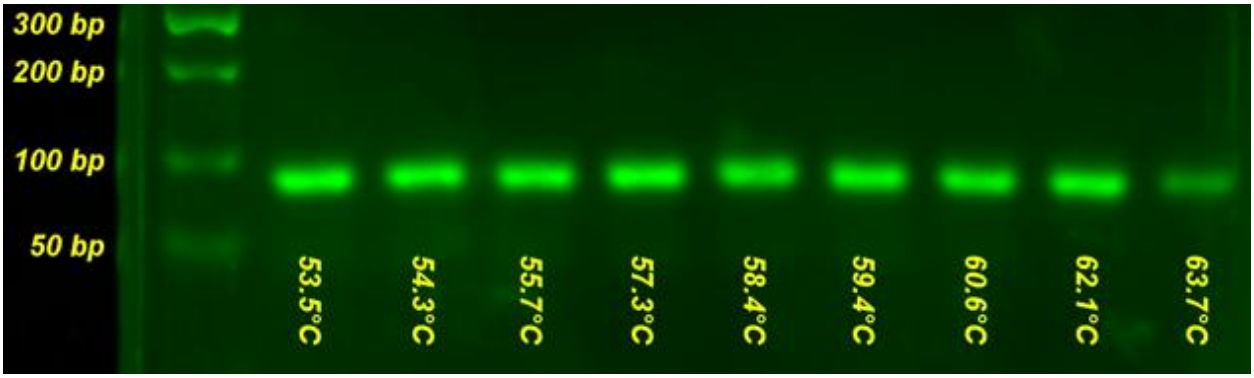

FIGURE S1. Set of gradient reactions to determine the optimal annealing temperature for the *Eurycea junaluska* primer set. Each 25.0 µl reaction included: 12.5 µl GoTaq® Master Mix (Promega), 8.5 µl nuclease-free water, 2.0 µl *E. junaluska* tissue-extracted DNA (1.0 µg/ml), and 2.0 µl of F + R primer (final reaction concentration = 0.16 µM). Cycling conditions consisted of an initial denaturation stage of 95.0° C for 2 min followed by 35 cycles of 95.0° C for 60 s, the annealing temperature (shown on the figure) for 60 s, and 72.0° C for 60 s, then a final extension of 72.0° C for 5 min. The PCR products were resolved on a 4% agarose gel with 7.0 µl of PCR product loaded per well. Ladder displayed is HyperLadder™ 50bp (Bioline).

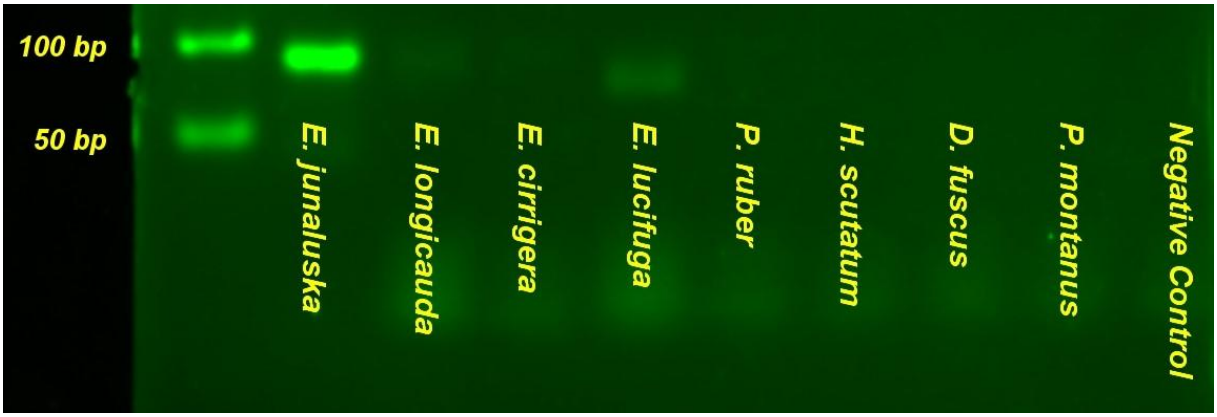

FIGURE S2. Tissue-extracted DNA specificity test for *Eurycea junaluska* primers using tissue extracts of seven sympatric or potentially sympatric salamander species. Each 25.0 µl reaction included: 12.5 µl GoTaq® Master Mix (Promega), 8.5 µl nuclease-free water, 2.0 µl tissue-extracted DNA (1.0 µg/ml), and 2.0 µl of F + R primers (final reaction concentration = 0.16 µm). Cycling conditions consisted of an initial denaturation stage of 95.0° C for 2 min followed by 40 cycles of 95.0° C for 60 s, 60.0° C for 60 s, and 72.0° C for 60 s, then a final extension of 72.0° C for 5 min. The PCR products were resolved on a 4% agarose gel with 7.0 µl of PCR product loaded per well. Ladder displayed is HyperLadder™ 50bp (Bioline).

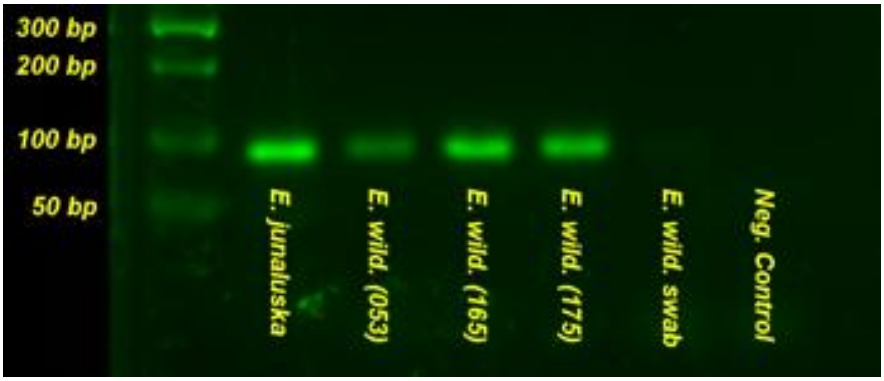

FIGURE S3. Specificity test for Eurycea *junaluska* primers using DNA extracted from three *Eurycea wilderae* (M clade) collected outside of the GSMNP (see Appendix 2 for locality information) and one *E. wilderae* (swab-collected DNA) observed inside the GSMNP. Each 25.0 µl reaction included: 12.5 µl GoTaq® Master Mix (Promega), 8.5 µl nuclease-free water, 2.0 µl tissue-extracted or swab DNA (1.0 µg/ml), and 2.0 µl of F + R primers (final reaction concentration = 0.16 µm). Cycling conditions consisted of an initial denaturation stage of 95.0° C for 2 min followed by 40 cycles of 95.0° C for 60 s, 60.0° C for 60 s, and 72.0° C for 60 s, then a final extension of 72.0° C for 5 min. The PCR products were resolved on a 3% agarose gel with 7.0 µl of PCR product loaded per well. Ladder displayed is HyperLadder™ 50bp (Bioline).

TABLE S4. Specificity testing results for our *E. junaluska* assay. "Species" = the species utilized in testing; "DNA" = the type of DNA utilized (gBlocks or plasmid clone); "Seq. infor." = the GenBank Accession number for the sequence (if available); "Coll. loc." = collection locality of the specimen; and "CPR" = copies per reaction, the number of copies present in the qPCR reaction test. "Ct value" represents the mean values, "N" signifies no amplification observed; all 10,000 copy per reaction tests were run in duplicate, and all 100,000 copy reactions run were in triplicate.

				Ct values	
Species	**DNA**	**Seq. infor.**	**Coll. loc.**	**10,000 CPR**	**100,000 CPR**
E. junaluska^{G}	gBlocks	KF562550.1	Blount Co., TN	28.5	25.0
E. junaluska^{P}	plasmid	PP277535.1	Graham Co., NC	30.0	25.6
E. wilderae[1]	plasmid	PP393049.1	Cocke Co., TN	N	N
E. wilderae[2]	plasmid	PP393050.1	Rabon Co., GA	N	N
E. longicauda	plasmid	unpublished	Pulaski Co., KY	N	N
E. lucifuga	plasmid	KT873718.1	Jessamine Co., KY	N	N
E. guttolineata	gBlocks	JQ920625.1	Berkley Co., SC	N	N
E. cirrigera	plasmid	unpublished	Jessamine Co., KY	N	N

TABLE S5. Limit of detection ("LOD"), limit of quantification ("LOQ'"), coefficient of determination (r^2-value), and amplification efficiency ("AE") for the *Eurycea junaluska* qPCR assay.

Species	**LOD**	**LOQ**	r^2	**AE**
E. junaluska	2.7	8	0.999	96.7%

TABLE S6. Sequences of *Eurycea junaluska* cytb from amplicons obtained by qPCR while validating the assay during *in vitro* testing with the 50 water samples collected from the GSMNP. Each of the four amplicons corresponds to one water sample taken within the park. Once a qPCR replicate was determined to have detected *E. junaluska* with our assay, tailed primers (designed in-house) were attached to the amplicon for sequencing purposes. "% sim. to [GenBank Accession #]" represents a comparison of the qPCR-obtained amplicon with the two *E. junaluska* cytochrome b sequences previously published in GenBank. Locality information for the two previously published sequences are: GenBank Accession # KF562550.1, collection locality = Blount County, Tennessee and GenBank Accession # PP277535.1, collection locality = Graham County, North Carolina. Each amplicon was sequenced unidirectionally by Sanger sequencing as an unpurified PCR product. Full amplicon length (without tail) is 81 base pairs (bp).

Amplicon #	% sim. to KF562550.1	% sim. to PP277535.1	Length (bp)	Sequence
1	100	98.8	81	GCACTATACTGCAGATACTTCCTCCGCATTCTCC TCTGTAGCTCACATTTGCCGAGATGTAAATTATG
	100	98.8	81	GCACTATACTGCAGATACTTCCTCCGCATTCTCC TCTGTAGCTCACATTTGCCGAGATGTAAATTATG
3	100	98.8	81	GCACTATACTGCAGATACTTCCTCCGCATTCTCC TCTGTAGCTCACATTTGCCGAGATGTAAATTATG
4	100	98.8	81	GCACTATACTGCAGATACTTCCTCCGCATTCTCC TCTGTAGCTCACATTTGCCGAGATGTAAATTATG

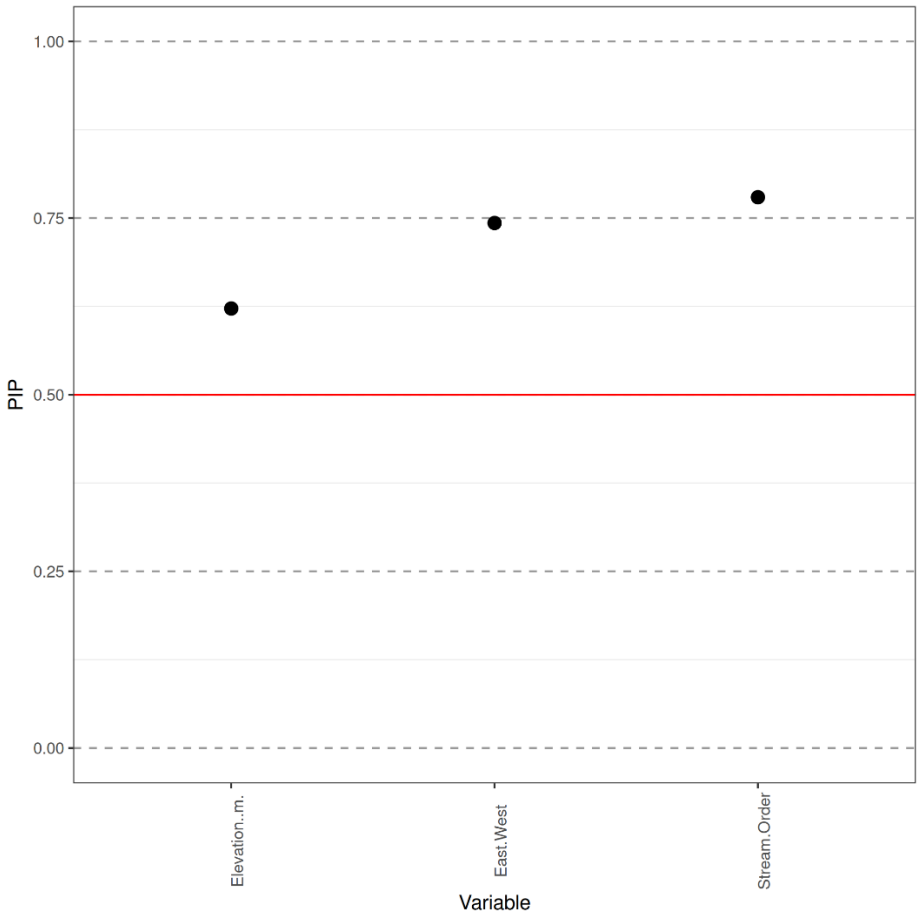

FIGURE S4. Posterior inclusion probability (PIP) values for each covariate included in the model. For each of the 50 sites included in the study, site elevation (m), Strahler stream order of the site, and whether the site was located on the east (Tennessee) or west (North Carolina) side of the GSMNP were included as covariates. All covariates had PIP scores> 0.5 and were considered important predictors for *E. junaluska* occupancy.