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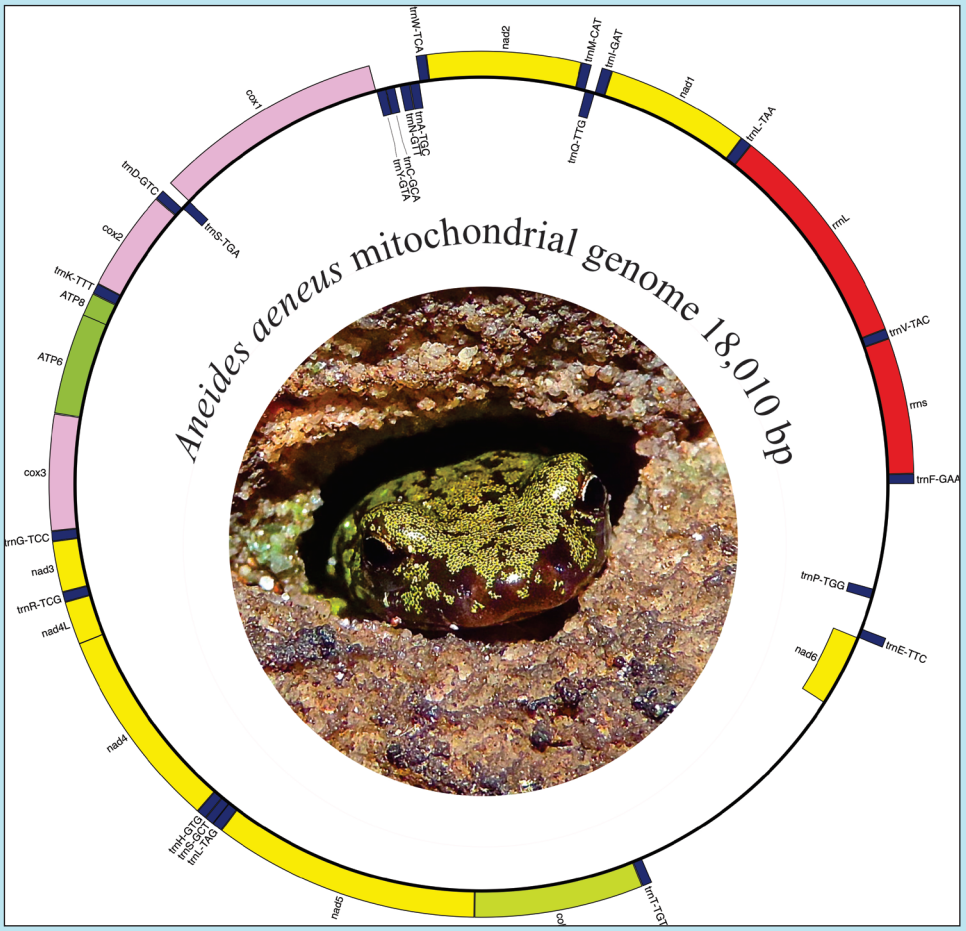

Volume 5, 2023 **eBio Notes** **No. 1**

eBio

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Cover Photograph: The Green Salamander (*Aneides aeneus*) in its native habitat, a rock outcrop in far Southwestern, VA, surrounded by a diagrammatic representation of its mitogenome. Photograph was taken by Wally Smith and the image prepared by Bruce Cahoon.

eBio (ISSN # 2165-6657), formerly the ***Eastern Biologist***, is published by the Eagle Hill Institute, PO Box 9, 59 Eagle Hill Road, Steuben, ME 04680-0009. Phone 207-546-2821 Ext. 4, FAX 207-546-3042. E-mail: office@eaglehill.us. Webpage: http://www.eaglehill.us/ebio. Published on an article by article basis. **Special issue proposals are welcome**. ***eBio*** is an open access journal. **Authors**: Submission guidelines are available at http://www.eaglehill.us/ebio. **Co-published journals:** The *Northeastern Naturalist*, *Southeastern Naturalist*, *Caribbean Naturalist*, *Urban Naturalist, Eastern Paleontologist*, and *Neotropical Naturalist*, each with a separate Board of Editors. The Eagle Hill Institute is a tax exempt 501(c)(3) nonprofit corporation of the State of Maine (Federal ID # 010379899).

The Complete Mitochondrial Genome of *Aneides aeneus* (Green Salamander)

Kendall S. Davids[1], Margie A. Tucker[1], Walter H. Smith[1], and A. Bruce Cahoon[1*]

Abstract – The complete mitochondrial genome (mtDNA) of *Aneides aeneus* Cope and Packard (Green Salamander) was sequenced. It is 18,010 bp with 13 protein coding genes (PCGs), 22 tRNA, and 2 rRNA genes. Nine of the PCGs require the addition of a polyA tail to complete the stop codons. A Maximum Likelihood phylogeny places the Green Salamander basal to *Aneides hardii* Taylor (Sacramento Mountain Salamander) and *Aneides flavipunctatus* Strauch (Speckled Black Salamander). It has the smallest mtDNA of this genus, suggesting there have been significant genome duplication events coinciding with speciation. This sequence provides genome-level resources for this species, which could aid in environmental barcoding.

Introduction. Mitochondria are essential eukaryotic organelles derived from an endosymbiotic event between a proto-eukaryotic cell and a proteobacterium (López-Garcia et al. 2017). The bacterial genome was severely reduced as it transitioned from a free-living cell to a semi-autonomous organelle and is now the eukaryotic mitochondrial genome (mtDNA). Among vertebrates, the mtDNA is usually between 15–25kb in size and encodes a small conserved set of genes primarily on one strand (the heavy strand), with a few genes occurring on the complementary light strand (Boore 1999). This genome is transcribed by a viral-like RNA polymerase from a single promoter, forming a primary transcript the length of the entire chromosome. These genes are tightly packed onto the transcript, with tRNAs found between protein coding mRNAs. The release of tRNAs by endonucleases simultaneously releases mRNAs (reviewed in Rackham and Filipovska 2022). The resulting mRNAs often have no leader sequence, which means the assembly of the ribosome occurs on the start codon. This could be a canonical AUG or non-canonical AUN, where the third nucleotide is variable but still recognized by $tRNA^{MET}$ due to modification of the wobble-base position (Haag et al. 2016; reviewed in Kummer and Ban 2021). Primary transcript processing also leaves mRNAs with truncated stop codons that are only completed when stabilizing polyA tails are added to the transcripts (Chang and Tong 2012). An additional non-canonical stop codon phenomenon found in mitochondria is the "hungry" stop codon where an arginine codon is converted to a stop by ribosome slippage (Temperley et al. 2010a, b).

Aneides aeneus Cope and Packard (Green Salamander) is a lungless salamander distributed across portions of the Appalachian Mountain region of North America. Unlike most other plethodontids, which reside in and near aquatic habitats or under

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Associate Editor: Fang Ju Lin, Coastal Carolina University.

cover objects on the forest floor, the Green Salamander is a partially arboreal rock outcrop specialist, occupying crevices in vertical rock surfaces in close proximity to hardwood forest cover (Smith et al. 2017). Due to its high degree of habitat specialization and presumed rarity, the Green Salamander is receiving increasing focus in both state and federal conservation efforts, including reviews for possible US Endangered Species Act listing (Smith et al. 2019). This lineage has also recently been found to harbor cryptic, microendemic genetic diversity across the southern portion of its range (Patton et al. 2019). As a result of the aforementioned factors, the Green Salamander is earning increasing interest from researchers hoping to use molecular approaches to understand its ecology and evolution.

We report the sequencing and assembly of the mtDNA of the Green Salamander. Our goal was to increase the genome-level resources available for this species, which could aid in understanding the evolutionary history of this genus and in remote identification procedures like environmental barcoding.

Materials and Methods. Three autotomized tail tips were collected from individual salamanders residing in rock crevices in Norton, VA, USA (36° 56' 1" N/82° 37' 47" W), immediately placed in 95% ethanol, and transported to UVA Wise where they were stored at -20°C. DNA extraction was performed using a Zymo Research (Irvine, CA, USA) Quick-DNA Miniprep Plus Kit following the manufacturer's instructions. The initial approach was to PCR amplify portions of Green Salamander mtDNA for Sanger sequencing using primers predicted to anneal to homologous regions of the Aneides mitochondrial genomes archived in GenBank (*Aneides hardii* Taylor [Sacramento Mountain Salamander] AY728226 and *Aneides flavipunctatus* Strauch [Speckled Black Salamander] AY728214). The two mitogenomes were aligned, homologous regions identified, and primers designed using Primer 3 (https://bioinfo.ut.ee/primer3-0.4.0/) and synthesized by Integrated DNA Technologies (Coralville, IA, USA) (Supplemental Table 1 contains a list of primer pairs that successfully yielded an amplicon, available online at eaglehill.us/ebioonline/suppl-files/ebio-030-Cahoon-s1.pdf). Amplicons were produced using Phusion polymerase (Fisher Scientific, Asheville, NC, USA) and a BioRad C1000 thermal cycler (Hercules, CA, USA), using the program at 95°C for 3 min (95°C 30 s, 55°C/70°C 20 s, 72°C 1 min) x 40, and at 72°C for 5 min. Amplicons were purified using a DNA Clean & Concentration Kit (Zymo Research), and Sanger sequenced by GeneWiz (South Plainfield, NJ, USA) using the primers that produced each amplicon. Once end sequences were obtained, other primers were designed to sequence "walk" across the amplicon. We were unable to complete the mitogenome using the amplicon sequencing approach, so whole genome sequencing was employed. For this approach, total DNA from an autotomized tail tip was sequenced by GeneWiz using their Short-Read Non-Human WGS service, which produced 82,500,604 individual paired-end reads with an average read length of 236 nucleotides. Paired-end reads were assembled into chromosomal fragments using the de novo assembly function of Geneious Prime (2021.2.1 BioMatters, Auckland, NZ). A single 17,705 bp contig was identified by homology to the previously sequenced amplicon fragments. This contig had an average depth of coverage of 256 and was

found to contain all of the predicted genes (protein coding, tRNAs, and rRNAs), but did not represent a closed circular chromosome. Its termini, however, were marked by the presence of the genes tRNA-Thr and nad6, which suggested a portion of a repetitive region was missing. The chromosome was completed using PCR amplification of the repetitive region (primers listed in Supplemental Table 1) and sequencing the amplicon (Sanger method), which closed the loop. The completed mtDNA was annotated using Geneious Prime. PCGs were identified by locating open reading frames in the Green Salamander mitogenome, translating them, and comparing the translations to those of the Speckled Black Salamander (endemic to portions of the Pacific Coast) and the Sacramento Mountain Salamander (endemic to a small area of New Mexico). tRNAs were identified using tRNA-Scan (http://lowelab.ucsc.edu/tRNAscan-SE/; Chan and Lowe 2019). A Maximum Likelihood (ML) phylogeny was constructed using rRNA and PCG sequences from GenBank salamander records. Alignment was completed using MUSCLE (Multiple Sequence Comparison by Log-Expectation) software with default parameters (Edgar 2004), and the tree was produced with IQTree using the model finder option which chose GTR+F+I+G4 as the best fit and 1000 replicates of ultrafast bootstrapping (http://www.iqtree.org) (Hoang et al. 2018, Kalyaanamoorthy et al. 2017, Minh et al. 2020). A type specimen for this species is deposited in the Academy of Natural Sciences of Drexel University (Philadelphia, PA, USA), specimen number 10461. The mtDNA sequence is deposited in GenBank, accession number OM743432. Whole-Genome Illumina sequence data is deposited in the SRA database as BioProject PRJNA823919, BioSample SAMN27377691.

Table 1. Incomplete and Hungry* Stop Codons within the Genus Aneides

	A. flavipunctatus	A. aeneus	A. hardii
nad1		ta	
nad2			
cox1	aga	aga	ta
cox2	t	t	t
atp8	ta	ta	
atp6			
cox3	t	t	t
nad3		t	
nad4L			
nad4	t	t	t
nad5		t	
cob	t	t	t
nad6		t	aga

*Hungry stop codons are grey

Results. The mitochondrial genome was circular, 18,010 bp in length, and included 13 protein coding genes, 22 tRNA genes, 2 rRNA genes, and a putative D-Loop. The nucleotide composition was 35.4% A, 19.3% C, 11.6% G, and 33.7% T. The heavy strand codes for 12 proteins, 14 tRNAs, and 2 rRNAs. The light strand codes for 1 protein and 8 tRNAs. There were 12 standard AUG start codons and 1 nonstandard ATC (*nad3)*. Alternative start codons also occur in Speckled Black Salamanders, with *nad1*, *nad3*, and *nad4L* beginning with ATG, *cox1* with GTG, and *nad6* with AGG. Sacramento Mountain Salamanders have two alternative start codons, with *nad1* having ATT and *cox1* with GTG. There were 3 full TAA stop codons (*nad2*, *atp6*, and *nad4L*), 9 requiring polyadenylation for completion (*nad1*, *cox2*, *atp8*, *cox3*, *nad3*, *nad4*, *nad5*, *cob*, and *nad6*), and 1 AGA "hungry" stop codon (*cox1*). Truncated stop codons are a common feature of vertebrate mitogenomes, but have been primarily described in mammalian mitochondria (reviewed in Rackham and Filipovska 2022). These transcripts are processed from the primary transcript with one or two adenines missing from the 3' terminus, and become full stop codons once the transcript is polyadenylated. Among members of *Aneides*, the Speckled Black Salamander requires polyadenylation to complete five mRNAs (*cox2*, *atp8*, *cox3*, *nad4*, and *cob*), as does the Sacramento Mountain Salamander (*cox1*, *cox2*, *cox3*, *nad4*, and *cob*) (Table 1). A hungry stop codon is the conversion of an arginine to a stop when the mitoribosome shifts upstream by -1 nucleotide, creating a stop codon. This phenomenon has been described in human mitochondria and Archaea (De Lise et al. 2021, Temperley et al. 2010a,b) and would presumably be found in other vertebrates. A hungry stop codon can be found in at least one mitochondrial gene (e.g., *cox1*, *nad5*, or *nad6*) among 15 of the 26 plethodontid mitogenomes available in GenBank. Within *Aneides*, the Speckled Black Salamander and the Green Salamander utilize a hungry stop codon for *cox1*, while the Sacramento Mountain Salamander uses one for *nad6* (Table 1).

Phylogenetic analysis placed the three available *Aneides* mtDNAs in a single clade, with the Green Salamander basal to the Speckled Black Salamander and the Sacramento Mountain Salamander (Fig. 1). This whole mtDNA analysis is consistent with single gene *cytb* and the *12S* rDNA ML trees of *Aneides* species constructed by Patton et al. (2019).

The Green Salamander mitogenome is the smallest among *Aneides* (Fig. 2), whose members have relatively large mitogenomes among the plethodontids due to expanded intergenic repeat regions. The nucleotide pairwise identity of the coding regions was 80.0% between Green Salamanders and Speckled Black Salamanders, 80.3% between Green Salamanders and Sacramento Mountain Salamanders, and 80.3% between Sacramento Mountain Salamanders and Speckled Black Salamanders. The gene order of the Green Salamander (Fig. 2) is very similar to other plethodontid mitogenomes (Mueller et al. 2004, Zhang and Wake 2009). The Green Salamander matches that of the Speckled Black Salamander but not the Sacramento Mountain Salamander, because *nad6*, tRNA(Glu), tRNA(Pro), and the putative control region were repositioned and some tRNAs and the putative control region were copied (Fig. 2). This suggests that Green Salamander and Speckled

Black Salamander synteny represents the ancestral gene order for this genus. The specific rearrangements and intergenic expansions seen among *Aneides* are uncommon among the plethodontid salamanders but not among vertebrates in general. A recent study of 100 vertebrate mitogenomes demonstrated that over 50% of them had insertions/duplications and repetitive intergenic sequences, suggesting that the lineage-specific presence of these elements is common (Formenti et al. 2021).

Discussion. Due to its high conservation concern and presumed rarity, the Green Salamander is receiving increasing focus in the literature, particularly involving its evolutionary history and genetic characteristics. Recent work has identified the presence of microendemic genetic diversity within the Green Salamander (Patton et al. 2019), resulting in the description of one new species (*A. caryaensis* Pat-

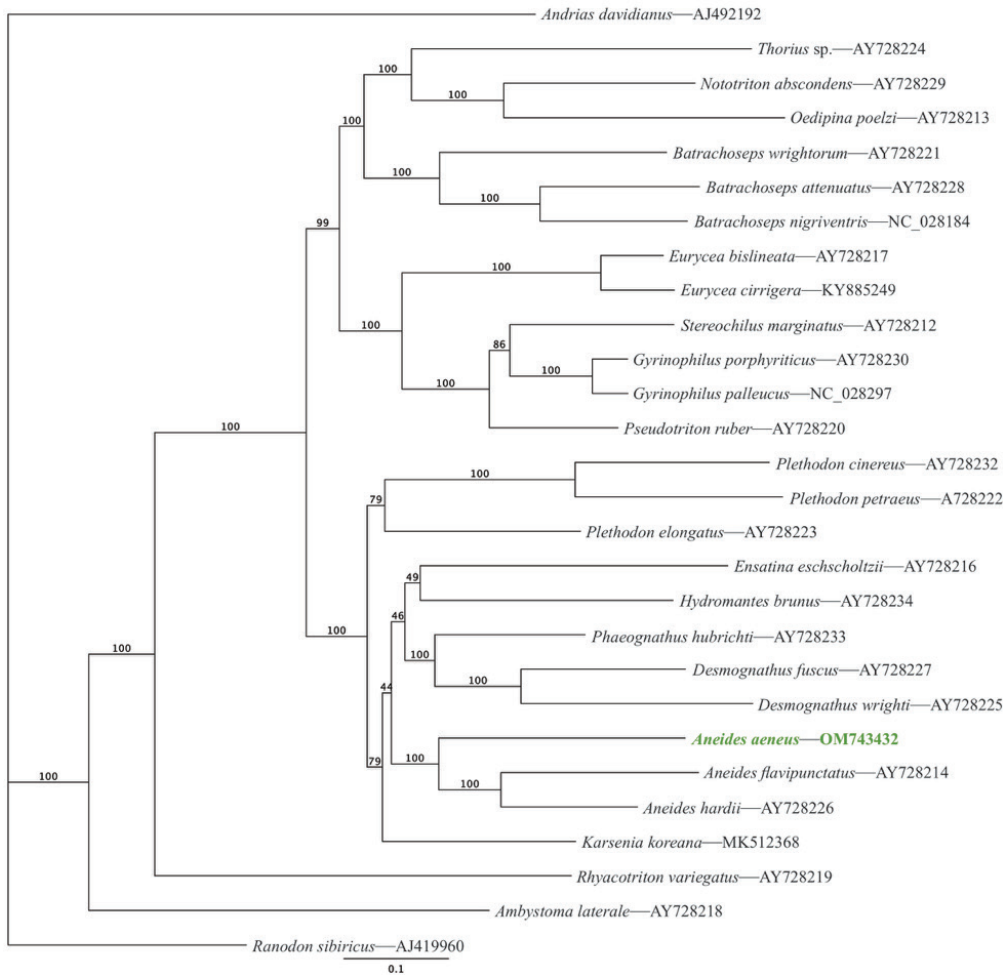

Figure 1. Unrooted Maximum Likelihood analysis (GTR+F+I+G4 model) of all rDNAs and PCGs from available Plethodontidae mtDNAs. Green Salamander is shown in green. Numbers on nodes are bootstrap values (1000 replicates, Ultrafast Bootstrapping).

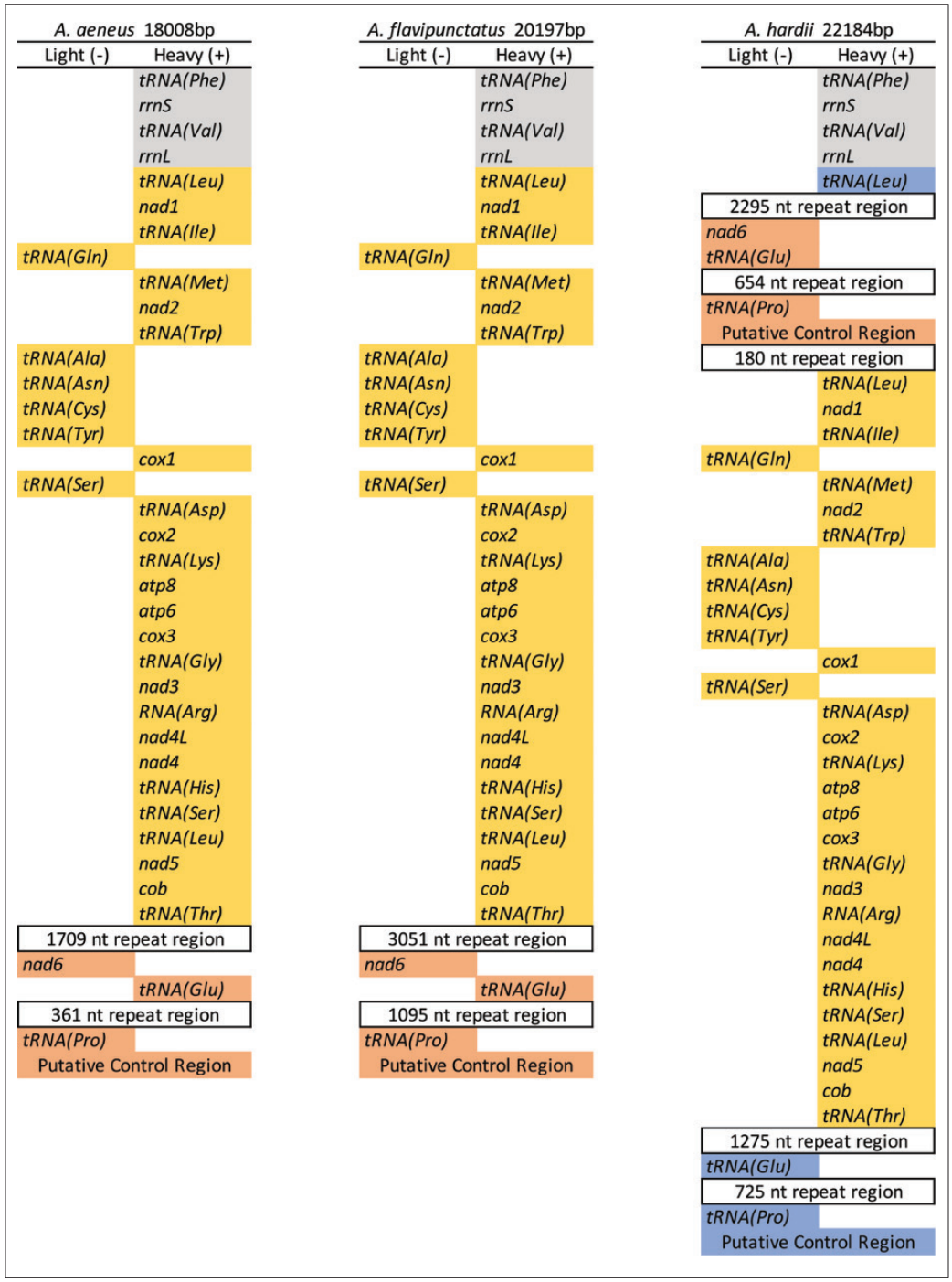

Figure 2. Gene order, mitogenome size, repeat regions, and gene duplications within the genus Aneides. Blocks of conserved genes are color coded. Genes that have been duplicated are highlighted with blue.

ton, Apodaca, Corser, Wilson, Williams, Cameron, and Wake [Hickory Nut Gorge Green Salamander]) and support for the possibility of other cryptic lineages being present across the southeastern United States. Accordingly, recent recommendations for future research on the Green Salamander have stressed the need for increased molecular studies of the species (Soto et al. 2021). In addition, increasing focus is being placed on developing new approaches that can improve the efficiency of detecting and monitoring Green Salamander populations in the field (Smith and Mullins 2022). The mitogenome data reported here can help inform these ongoing efforts by aiding in future investigations of the evolutionary history of the Green Salamander and the potential development of novel field detection tools, such as environmental barcoding.

Disclosure Statement

The authors declare no conflicts of interest.

Funding

Funding for this project was provided by the Buchanan Chair of Biology Endowment at UVA Wise. Tissue collections were performed under Virginia Department of Wildlife Resources Permit Number 051336.

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