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Range-wide Genomics Reveals Deep Divergence in Peripheral Populations of the Streamside Salamander (Ambystoma barbouri)

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Abstract

Peripheral populations often harbor unique evolutionary diversity yet face disproportionate conservation risk. The streamside salamander (*Ambystoma barbouri*) occurs primarily in the Ohio River basin in Kentucky, Indiana, and Ohio, but persists as isolated, disjunct populations in central Tennessee and western West Virginia, where rapid urbanization threatens remaining habitat. We combined mitochondrial DNA sequencing (~ 1,200 bp of the D-loop) and genome-wide SNP data to assess range-wide phylogeographic structure and fine-scale population differentiation across 89 populations throughout the contemporary geographic range of the species. Mitochondrial analyses revealed multiple deeply divergent lineages, whereas SNP-based analyses demonstrated strong nuclear differentiation between Tennessee populations and the species' northern distribution in Kentucky, Indiana, Ohio, and West Virginia. Within Tennessee, genomic data identified three well-defined genetic clusters structured by watershed boundaries, with hierarchical AMOVA supporting significant differentiation among watersheds and among populations within watersheds. Together, these results indicate that Tennessee populations of *A. barbouri* represent a distinct and evolutionarily significant component of the species' range and support their recognition as distinct conservation units.

Introduction

The streamside salamander (*Ambystoma barbouri*) is of conservation concern in the state of Tennessee as its geographic distribution encompasses rapidly developing areas of the Nashville Basin (Anderson et al. 2014; Fig. 1). The majority of *A. barbouri* breeding populations occur across a single, continuous geographic area in Kentucky, Indiana, and Ohio. However, disjunct populations are found in central Tennessee and western West Virginia (Petranka 1998, Kraus & Petranka 1989, Niemiller & Reynolds 2011). Genetic and life history studies show that Tennessee populations of *A. barbouri* are distinct from populations in the central part of *A. barbouri's* range (Eastman et al. 2009). The known distribution of A. barbouri within Middle Tennessee is now broader than was recognized when this genetic survey was completed, and genetic information for the disjunct *A. barbouri* populations in West Virginia is lacking. These peripheral populations provide an opportunity to further evaluate the central-marginal hypothesis, which predicts that population size, genetic diversity, and genetic connectivity are highest in core populations and decline toward range margins (Micheletti & Storfer 2015). Comparing genetic diversity and population structure among core, Tennessee and West Virginia populations will help determine whether disjunct peripheral populations of *A. barbouri* represent genetically depauperate range-edge populations or evolutionarily distinct lineages that warrant separate conservation consideration.

Specialized habitat preferences and dependence on undeveloped riparian zones make *A. barbouri* susceptible to habitat fragmentation and stream degradation. Adult *A. barbouri* are fossorial, residing in moist soil near breeding streams, and only emerging briefly in the winter to breed (Petranka 1998, Niemiller & Reynolds 2011). Suitable *A. barbouri* breeding habitat in Tennessee is quickly being lost, as the Nashville Basin is experiencing rapid increases in anthropogenic development (UTCBER 2023). The *A. barbouri* populations in Tennessee are currently listed as state endangered (Withers et al. 2009,

Tennessee Wildlife Resources Agency 2018), and the species is under consideration for listing at the federal level under the Endangered Species Act (ESA).

Decisions regarding the conservation status of *A. barbouri* depend on understanding the geographic structure of genetic variation within the species. Further evidence that Tennessee populations are genetically distinct could support their recognition as a Distinct Population Segment (DPS), potentially qualifying the species for special protection under federal conservation policy (USFWS 2006c; USFWS 2018). A more recent genetic survey identified clear differentiation between Tennessee and Kentucky populations at both mitochondrial and nuclear loci; however, this analysis was limited to 13 localities in middle Tennessee and five in Kentucky (Hubbs et al. 2022). Populations recently discovered in the Western Highland Rim and Western Pennyroyal Karst ecoregions of Tennessee were not included, limiting our interpretation of these genetic relationships.

Environmental differences may contribute to genetic and phenotypic divergence across *A. barbouri's* distribution. Tennessee populations experience shorter, warmer winters than populations in the species' northern range, potentially favoring local adaptations in breeding timing and larval development (Thulander and Hall 2025; Beer et al. 2022). Populations of *A. barbouri* in Tennessee appear to exhibit faster embryonic and larval development and a shorter time to metamorphosis than populations in the core portion of the range, a pattern consistent with previous work showing that hydroperiod, temperature, and ephemeral breeding habitats can favor accelerated development and earlier metamorphosis in ambystomatid salamanders (Petranka, 1984b; Petranka & Sih, 1987; Maurer & Sih, 1996). These differences may represent adaptive responses to Tennessee's more ephemeral and variable stream hydroperiods (Lyons et al. 2023). In contrast, Kentucky populations occupy more permanent streams and have been reported to exhibit longer incubation periods, extended larval development, and larger size at metamorphosis (Niemiller et al., 2006; Venesky et al., 2009). More broadly, these patterns are consistent with classic models of amphibian metamorphosis, in which larval period and size at metamorphosis vary with growth conditions, mortality risk, and habitat duration (Wilbur & Collins, 1973; Werner, 1986). Genetic relationships between Tennessee populations and those in Ohio, Indiana, and West Virginia have not previously been assessed outside of the few populations within these states that were analyzed in Eastman et al. (2009). Comprehensive conclusions about the distinctiveness of Tennessee *A. barbouri* require expanded sampling across Tennessee and throughout the species' full geographic distribution.

This study presents the first range-wide phylogenetic and population genomic analysis of *A. barbouri*, with a focus on genetic differentiation between Tennessee and northern populations and fine-scale genetic structure within recently discovered Tennessee populations. We used mitochondrial DNA sequencing and single-nucleotide polymorphism (SNP) genotyping to address two objectives: (1) determine whether Tennessee *A. barbouri* populations are genetically distinct from populations in Kentucky, Ohio, Indiana, and West Virginia, and (2) evaluate fine-scale genetic structure within Tennessee, including newly discovered peripheral populations. Results from this study will inform

population prioritization for conservation planning and decisions regarding federal protections under the ESA.

Methods

Tissue Collections

Information on known and historical breeding localities for *A. barbouri* was obtained from the Tennessee Department of Environment and Conservation Natural Heritage Program (TNNHP). All eggs, adults, and larvae occurring within a continuous waterway were treated as a single collection site, and multiple breeding populations were not observed within the same stream. Throughout this study, populations sampled from Kentucky, Indiana, and Ohio are collectively referred to as the “Core Distribution”, and individual breeding sites within this region are referred to as Core sites. Sampling sites were selected to encompass all hydrogeographic regions and HUC-10 watersheds within the Tennessee range, with priority given to recently discovered populations along the range periphery (Fig. 2). From December 1, 2022, through December 1, 2023, *A. barbouri* tissue samples were collected from 63 sites across Bedford (1), Cheatham (1), Davidson (7), Marshall (5), Robertson (2), Rutherford (9), Sumner (20), Williamson (5), and Wilson (14) counties in Tennessee. Samples of the closely related *A. texanum* were also collected from Big Cypress State Park, Tennessee and were included to provide a comparative lineage for phylogenetic context and to evaluate patterns of divergence given previously observed discordance between mitochondrial and nuclear markers (Hubbs et al. 2022). Additional *A. barbouri* and *A. texanum* tissue samples from Kentucky, Indiana, Ohio, and West Virginia were provided by collaborators at Miami University, Marshall University, and Washington State University.

Surveys were conducted by walking upstream from the downstream end until reaching the stream terminus, a property boundary, or until approximately 20 tissue samples had been collected. To minimize the risk of pathogen transmission among sites, all equipment was sterilized with a 20% bleach solution and rinsed with deionized water before entering each stream. Sampling followed non-destructive rock-turning methods (Jung et al., 2000), with rocks replaced in their original positions. Tissue samples were collected from eggs, larvae, and adult *A. barbouri*, depending on life stage availability at the time of sampling. A single sample consisted of three eggs from one cluster, a whole larva, or a 2–5 cm tail clip from a large larva or adult (IACUC Permit Number IACUC-21-22-008: Scientific Collection Permit #1323). Only one egg per clutch was used for DNA extractions. Each sample was labeled with the site identifier, life stage, and individual number. A maximum of 25 samples were collected per site across all life stages. Tissue samples were stored in tubes containing ~ 400 µL of RNAlater Solution (Invitrogen). Samples were kept in solution at room temperature for 24–48 hours to fully absorb the solution and were stored at -20°C prior to DNA extraction. DNA extractions were performed using the EZNA Tissue Kit (Omega Biotek) following the manufacturer's instructions except that DNA was eluted in water instead of elution buffer.

Mitochondrial Sequencing

A total of 361 individuals representing 89 breeding localities were included for Sanger sequencing of the mitochondrial D-loop. Approximately 1,200 bp of the mitochondrial D-loop was amplified using primers developed by Shaffer & McKnight (1996). The D-loop region was selected for phylogenetic reconstructions as this sequence is widely used to infer population structure and phylogenetic relationships in the genus *Ambystoma* (e.g. Bogart et al. 2007; Charney et al. 2014; Church et al. 2003; Shaffer & McKnight 1996; Zamudio & Savage 2003). Thermocycler conditions for PCR amplification were as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 53°C for 15 s, and 72°C for 90 s, and concluded with a final extension of 10 min at 72°C. Amplified products were enzymatically cleaned using Exonuclease I and Shrimp Alkaline Phosphatase (New England Biolabs) prior to bidirectional Sanger sequencing on an ABI 3730 instrument (MCLAB). Chromatograms were imported and inspected in SEQUENCHER 5.2 (Gene Codes Corporation). Sequence alignment was performed using ClustalW (Thompson et al. 1994) via the multiple-alignment function in BioEdit (Hall 1999).

Phylogenetic Reconstructions

Phylogenetic analyses were performed on all newly generated sequences as well as 140 *Ambystoma* haplotypes obtained from GenBank. GenBank sequences included 26 additional *A. barbouri* haplotypes as well as outgroup haplotypes representing *A. mabeei* (n = 2), *A. maculatum* (n = 2), *A. texanum* (n = 27), and unisexual *Ambystoma* (n = 84; Supplement 1). Unisexual *Ambystoma* likely originated through hybridization between female *A. barbouri* and male *A. laterale*. (Bi & Bogart 2010; DeSalle et al. 2023). Two model-based methods, maximum likelihood and Bayesian analysis, were used for phylogenetic reconstructions. Maximum likelihood phylogenetic analysis was performed using RAxML-NG v.1.2.2 (Stamatakis 2014) with the GTR + Γ model (standard default for RAxML) and eight discrete gamma rate categories. The best-scoring tree was inferred from 10 parsimony starting trees, and branch support was assessed with 200 bootstrap replicates. Bayesian phylogenetic analyses were performed using MrBayes (Ronquist et al. 2012) under the HKY + I + Γ model as selected using BIC in MEGA11. Two independent MCMC runs of 10 million generations were conducted, sampling every 1,000 generations, and the first 25% of samples were discarded as burn-in. Convergence was assessed by comparing independent runs and confirming that the average standard deviation of split frequencies was below 0.01 and that log-likelihood values were similar between runs.

GBS Library Construction

Samples from 741 individuals representing 89 breeding localities were included in the construction of genotyping-by-sequencing (GBS) libraries. Library construction followed the method described by Elshire et al. (2011). Before library construction, genomic DNA concentrations were measured using the Quantifluor dsDNA Kit (Thermo Fisher Scientific), and all samples were normalized to ~ 8.5 ng/µL. DNA was digested with the restriction enzyme ApeKI, then ligated to adaptors containing PCR binding sites

and unique barcodes. Barcoded samples were pooled and amplified with primers complementary to the adaptor sequences. PCR products were first purified using the Qiagen PCR Purification Kit and subsequently cleaned with the AxyPrep Mag PCR Clean-up Kit (Axygen, Big Flats, NY, USA). Library quality was assessed by running on a 1.5% agarose gel. The final barcoded libraries were sequenced on an Illumina NextSeq 1000, using P2 100 bp sequencing chemistry (Illumina).

Bioinformatics

Fastq files from sequencing of the GBS libraries were demultiplexed using the *process_radtags* module in the *Stacks* software (Rochette et al. 2019). SNPs were called and assembled using *ipyrad* (v 0.9.102) (Eaton DAR & Overcast I 2020), which uses a seven-step assembly process to convert the sequence data into bi-allelic SNPs for phylogenetic and population genetic analyses (Eaton & Overcast, 2020). Sequence data was filtered to obtain two final datasets. The first dataset (AP dataset) contained samples from all collection sites in the study (Kentucky, Indiana, Ohio, Tennessee, West Virginia) to assess relationships between the disjunct Tennessee collection sites and the Core Distribution. To resolve finer-scale structure, a dataset limited to Tennessee collection sites was created (TN dataset). SNP calling in iPyrad used the *reference alignment* option; sequences were aligned to the *A. barbouri* genome assembly (Gaupp et al. unpublished data; BioProject PRJNA1454854). Quality filtering in iPyrad included the removal of sequence reads with an average Phred quality score below 33, and an average read depth less than 5. Subsequent filtering using the software VCFtools (Danecek et al, 2011) removed loci deviating from Hardy-Weinberg equilibrium ($p < 0.05$), genotypes present in fewer than 50% of individuals, SNPs with a minor allele frequency of less than 0.05, and retained only one SNP per locus to minimize linkage. Breeding localities represented by fewer than five individuals after filtering were excluded from downstream analyses.

Analyses of SNP datasets

Basic summary statistics, including pairwise F_{ST} estimates, observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficients (F_{IS}), and the proportion of polymorphic loci (PPL) were estimated using the R package *hierfstat* (Goudet 2005). Multivariate analyses using Discriminant Analysis of Principal Components (DAPC) were conducted on both the AP and TN SNP datasets to estimate the optimal number of genetically distinct clusters (K) and to visualize population structure (Jombart et al. 2010; Jombart & Collins 2015; Hubbs et al. 2022). The function *find.clusters* in the R package *adegenet* (v4.3.1) was used to determine the optimal number of clusters based on the Bayesian Information Criterion (BIC; Jombart & Ahmed 2011).

We assessed hierarchical genetic structure using analysis of molecular variance (AMOVA) implemented in the R package *poppr* (Excoffier et al 1992: Kamvar et al. 2014). AMOVA was performed on the TN dataset to partition genetic variation among watershed groups, among breeding localities within watersheds, and within each breeding locality. Groupings were defined *a priori* based on hydrologic units

(at the HUC-10 level), with breeding localities nested within watersheds. Significance of variance components was evaluated using 999 permutations, in which individuals were randomly reassigned among hierarchical levels (watersheds and breeding localities) to generate null distributions under no genetic structure.

Results

Mitochondrial Sequencing

Sanger sequencing yielded an approximately 1200 bp alignment of the mitochondrial D-loop from 361 individuals across 89 breeding localities representing Kentucky, Indiana, Ohio, Tennessee, and West Virginia. From this dataset, 180 unique haplotypes were identified; 110 haplotypes were from localities in the Core Distribution and 70 haplotypes were from breeding localities in Tennessee. Four major monophyletic clades were identified in both Bayesian and ML phylogenetic reconstructions (Fig. 3A) with high support values. Mitochondrial *Clade I* included all unisexual *Ambystoma* haplotypes from the Core Distribution, *A. barbouri* haplotypes from the Louisville, Kentucky area, and *A. barbouri* haplotypes from the Central Nashville Basin (Fig. 3B-C). Clade II included all *A. texanum* haplotypes, as well as *A. barbouri* haplotypes from the southern end of the Nashville Basin. Clade III included all *A. barbouri* haplotypes from Indiana, Ohio, and West Virginia, as well as haplotypes from Kentucky and the Eastern Nashville Basin (Wilson County, Tennessee). Lastly, Clade IV consisted exclusively of *A. barbouri* haplotypes north of the Cumberland River in Tennessee; this clade was strongly supported as sister to Clade III.

GBS

Using a reference-based assembly in ipyrad and the draft genome assembly for *A. barbouri* (Gaupp et al. unpublished data; BioProject PRJNA1454854), our initial assembly recovered 55,870 loci (1,528,613 SNPs) from 741 individuals across 89 breeding localities in the AP dataset. After filtering for low coverage, Hardy-Weinberg disequilibrium, and linkage disequilibrium and excluding individuals with fewer than 1,000,000 reads, the final AP dataset contained 867 SNPs from 434 individuals across 42 breeding localities. For the TN dataset, the initial assembly recovered 40,993 loci (993,405 SNPs) from 583 individuals across 54 breeding localities. After filtering, the final TN dataset contained 982 SNPs from 434 individuals representing 33 breeding localities (Table 1).

Population Structure

Discriminant Analysis of Principal Components (DAPC) identified K=4 as the most likely number of genetic clusters across all populations (AP dataset), based on Bayesian Information Criterion (BIC) scores and the retention of 135 principal components (PCs) (Fig. 4A). These genetic clusters were geographically structured and closely aligned with major watershed boundaries, particularly within Tennessee. Cluster 4 consisted primarily of populations from the Core Distribution (Kentucky, Indiana,

Ohio, and West Virginia). Clusters 1, 2, and 3 largely included individuals sampled exclusively in Tennessee. The intra-Tennessee structure (Fig. 4D) followed specific geographic divisions: Cluster 1 included populations located north of the Cumberland River within the Nashville Basin. Cluster 2 was comprised of Tennessee populations immediately south of the Cumberland River, including one population each in northern Kentucky and Indiana. Cluster 3 included populations from the southern and central portions of the Nashville Basin. Several populations in central Kentucky showed evidence of admixture with Cluster 2. The DAPC analysis based on the Tennessee-only dataset (Fig. 4 B,D) was largely in agreement with genetic clusters obtained from the AP dataset but further refined the boundaries among the three Tennessee clusters. This analysis also showed evidence of admixture among Clusters 1–3 within Tennessee that coincided with geographic proximity among neighboring watersheds.

Pairwise F_{ST} estimates revealed pronounced genetic differentiation between disjunct Tennessee populations and the Core populations, as well as finer-scale structure within Tennessee that closely aligns with watershed boundaries (Fig. 5). Mean pairwise F_{ST} estimates between Tennessee and Core populations were high (mean F_{ST} = 0.415), indicating strong divergence. In contrast, average pairwise F_{ST} values among populations within Tennessee (mean F_{ST} = 0.119) and within the Core Distribution (mean F_{ST} = 0.146) were substantially lower, reflecting greater genetic connectivity within regions. A subset of Core populations, including KY8, KY12, and KY14, exhibited moderate differentiation from other Core populations. Pairwise F_{ST} estimates within this subset averaged 0.167, while comparisons between this group and the remaining Core populations averaged 0.205, suggesting partial isolation within the Core Distribution.

The distance-based tree constructed from pairwise F_{ST} values resolved three well-supported genetic clades within Tennessee that corresponded closely to the three genetic clusters identified in DAPC analyses. Mean pairwise F_{ST} estimates within the Northern, Central, and Southern Tennessee clusters were 0.090, 0.049, and 0.071, respectively, indicating relatively low differentiation among populations within clusters. In contrast, pairwise F_{ST} values between Tennessee clusters averaged 0.152, consistent with long-term restricted gene flow among regional lineages. One population, Wil2, did not cluster with any of the three Tennessee groups. Located in the eastern portion of the Nashville Basin, Wil2 showed elevated differentiation from other Tennessee populations, with a mean pairwise F_{ST} of 0.185. Interestingly, individuals from Wil2 were assigned to multiple Tennessee ESUs in the DAPC analysis, a pattern that may indicate secondary gene flow between divergent clades.

Analysis of molecular variance (AMOVA) supported the population structure inferred from DAPC and pairwise F_{ST} analyses (Table 2). Most genetic variation occurred within populations (81.2%), but significant differentiation was also detected among populations within watersheds (10.2%) and among watersheds (8.6%). All hierarchical variance components were significant (Φ_{ST} = 0.188, Φ_{SC} = 0.111, Φ_{CT} = 0.086; P = 0.001), indicating that HUC-10 watershed boundaries explain a significant component of genetic structure while finer-scale differentiation persists among populations within watersheds.

Together, these results are consistent with restricted gene flow between drainages and hierarchical population structure across the Tennessee portion of the species' range.

Within Population Genetic Variation

Within-population genetic variation based on genomic SNPs was slightly higher in populations from Kentucky, Ohio, Indiana, and West Virginia than in populations from Tennessee (Table 1). Among non-Tennessee populations, Kentucky sites exhibited the highest mean observed and expected heterozygosity (H_O = 0.016, H_E = 0.019), followed by Ohio (H_O = 0.014, H_E = 0.013). In contrast, Tennessee populations showed lower average heterozygosity overall (H_O = 0.011, H_E = 0.014). The percentage of polymorphic loci was lower on average in populations from the Core Distribution than in Tennessee populations (mean PPL = 4.74 and 6.69, respectively), a pattern likely influenced by smaller sample sizes in core populations. Within Tennessee, populations in the eastern portion of the Nashville Basin (Wil1–Wil10) exhibited the highest levels of genetic variation, with site Wil1 showing the greatest H_O, H_E, and PPL values among all Tennessee populations (0.017, 0.024, and 11.2%, respectively). In contrast, populations from the southwestern portion of the Nashville Basin (Bed1, Willi1, Willi5, Mar1, Mar3, and Mar4) consistently showed the lowest levels of genetic variation (mean H_O = 0.009, H_E = 0.009, and PPL = 4.05%).

Discussion

This study provides the first range-wide phylogeographic and genomic assessment of *A. barbouri*, revealing how historical isolation and landscape features shape genetic diversity across the species' distribution. By integrating mitochondrial phylogenetics with genome-wide SNP data, we show that Tennessee populations are strongly differentiated from the species' Core Distribution and contain multiple divergent lineages structured by watershed boundaries. At broad geographic scales, both marker types support the evolutionary independence of Tennessee populations relative to Kentucky, Indiana, Ohio, and West Virginia. Within Tennessee, we identify three primary genetic clusters that align with major drainage basins, reflecting long-term limits to gene flow among watersheds. As expected for a long-lived, low-dispersal amphibian, mitochondrial clades do not map perfectly onto nuclear genetic clusters, reflecting differences in inheritance, lineage sorting, and historical introgression. Finally, fine-scale patterns of heterozygosity reveal substantial variation in genetic diversity among Tennessee populations, with several sites exhibiting reduced diversity consistent with historical isolation or recent anthropogenic disturbance. In the sections below, we first evaluate the extent of divergence between Tennessee and core populations, then examine phylogeographic structure within Tennessee, and finally discuss implications for conserving distinct evolutionary lineages under current patterns of habitat loss and land-use change.

Core Distribution and Peripheral Populations

Our range-wide analyses demonstrate that Tennessee populations of *A. barbouri* are significantly differentiated from populations in the species' Core Distribution, though the pattern of divergence differs between mitochondrial and nuclear markers. This pattern is consistent with the central-marginal hypothesis, which predicts that peripheral populations may exhibit reduced connectivity and increased genetic differentiation relative to core populations, particularly when range-edge populations are geographically isolated or occur in environmentally distinct habitats (Micheletti & Storfer 2015; Beer et al. 2022). This pattern also mirrors earlier work by Hubbs et al. (2022), which documented substantial genetic separation between Tennessee and Kentucky populations but was limited in geographic scope. In our mitochondrial phylogeny, most of the Core populations formed a monophyletic clade (Clade III) that was paraphyletic with respect to multiple Tennessee clades, indicating deep and potentially ancient divergence among Tennessee lineages.

Notably, populations in north-central Kentucky represent an exception to the broader geographic pattern and cluster with central Tennessee populations in both mitochondrial (Clade I) and nuclear SNP (Cluster 2) analyses. Previous range-wide microsatellite studies of *A. barbouri* also found nonuniform genetic structure across the core distribution, including genetic clusters separated by the Ohio and Kentucky rivers, indicating that Kentucky populations are not genetically homogeneous across the species' range (Micheletti & Storfer 2017). Thus, the affinity between north-central Kentucky and central Tennessee populations observed here may reflect a more complex history of range expansion and regional connectivity than expected under a simple core-periphery model. Collectively, these results suggest multiple northward expansions of *A. barbouri* originating from Tennessee, estimated to have begun around ~ 470,000 years ago (Hubbs et al. 2022), and are consistent with Pleistocene climatic dynamics in which populations expanded northward during interglacial periods following glacial retreat (Hewitt 2000; Clark et al. 2009).

Disjunct populations of *A. barbouri* in the northwestern corner of West Virginia were originally documented in mid-20th-century surveys but lacked confirmation for several decades (Green 1956). Although species-level range summaries continued to list West Virginia as part of the peripheral distribution, the status of these populations remained uncertain due to the absence of recent records and the lack of genetic material from the region in previous phylogeographic studies (but see GenBank EU980591). Our study confirms that these populations persist and are closely related to populations within the Core Distribution. Both mitochondrial and nuclear data indicated that the West Virginia populations surveyed here (WV1, WV2) are genetically distinct yet share recent ancestry with Core populations. Phylogenetic analyses based on mitochondrial haplotypes recovered all West Virginia haplotypes as a single, well-supported monophyletic clade nested within Core Distribution haplotypes (Clade III), suggesting that West Virginia populations were established during the same northern expansion that gave rise to populations in Kentucky, Indiana, and Ohio. Pairwise F_{ST} estimates further indicate that WV1 is among the most genetically distinct sites within the Core Distribution, likely reflecting long-term isolation at the northeastern edge of the species' range. Collectively, these results suggest that West Virginia populations represent a small, peripheral extension of the northern clade that has persisted in isolation while retaining a shared evolutionary origin with other Core populations. Its

genetic distinctiveness highlights the conservation value of these disjunct populations, which may harbor unique historical genetic variation or locally adapted alleles.

Biogeography within Tennessee.

Patterns of genetic structure within Tennessee reveal a deeply partitioned biogeographic landscape in which river systems, karst geology, and long-term landscape stability have promoted independent evolutionary trajectories among populations. Both mitochondrial and nuclear datasets indicate that Tennessee represents a historically isolated portion of the species' range, containing multiple long-persisting lineages with limited contemporary gene flow among watersheds. Hierarchical AMOVA further supported this interpretation, demonstrating significant genetic differentiation both among watersheds and among populations within watersheds, with watershed-level structure explaining a measurable proportion of total genetic variance. Similar to other SNP-based amphibian studies, modest among-group (watershed) variance components in the AMOVA are expected given high within-group diversity, yet still reflect biologically meaningful population structure when statistically significant and geographically concordant (Meirmans and Hedrick 2011; McCartney-Melstad et al. 2018). These findings reinforce results from earlier genomic work, which identified three Evolutionarily Significant Units (ESUs) within the state. Each ESU was defined on the basis of distinct mitochondrial phylogeographic patterns and divergence in nuclear allele frequencies, reflecting the same broad regional divisions recovered here (Hedrick et al. 2006, Moritz 1994). The expanded genomic dataset presented in this study provides a more detailed and spatially comprehensive picture of evolutionary structure within Tennessee than was previously possible.

Mitochondrial Clade I included populations in Davidson and Wilson counties and extends into Oldham and Jefferson counties in Central Kentucky. This clade likely represents an older lineage that once spanned upland karst landscapes prior to late-Pleistocene drainage reorganizations. Its spatial extent overlaps substantially with the previously defined Central ESU, which included eastern Davidson and Wilson counties. The new sampling reveals that Clade I is more widespread than previously recognized, forming a cohesive mitochondrial lineage across multiple adjacent watersheds. Notably, Clade I also included mitochondrial haplotypes recovered from unisexual *Ambystoma*, which clustered within this lineage in the mtDNA phylogeny, consistent with previous evidence that unisexual lineages have repeatedly captured and retained *A. barbouri* mitochondrial genomes (Hubbs et al. 2022). Nuclear data also support the integrity of this lineage; most populations assigned to Clade I are from the Central Tennessee ESU and fall within a tightly grouped genetic cluster (DAPC Cluster 2). Thus, the Central ESU remains well supported, but its boundaries should be expanded to reflect this broader distribution.

Mitochondrial Clade II populations occur across southern and central portions of the Nashville Basin and span several adjacent HUC-10 watersheds. Despite their geographic proximity to Clade I, these sites share no haplotypes and exhibit strong mitochondrial and nuclear differentiation, demonstrating long-term isolation maintained by limited dispersal across even modest watershed divides. This clade aligns most closely with the previously identified Southern ESU, which included populations from Williamson,

Rutherford, and Bedford counties. However, the new data show that Clade II extends farther north and east than previously understood, occupying a large swath of central Tennessee that includes several Wilson County sites not previously sampled. DAPC analyses reinforce this broader distribution: populations south of the Cumberland River and clustered within the Stones River and Duck River basins form a cohesive Southern genetic cluster. Accordingly, the Southern ESU should be expanded to incorporate the full geographic range of Clade II, which represents a deeply divergent and historically stable lineage confined largely to southern and central Tennessee drainages.

A small number of Tennessee individuals carried mitochondrial haplotypes belonging to Clade III, the lineage otherwise characteristic of the Core Distribution (Kentucky, Indiana, Ohio, and West Virginia). Within Tennessee, these haplotypes occurred primarily in Wilson County. Their presence likely reflects either retention of ancestral mitochondrial variation predating divergence between Tennessee and northern lineages or historical introgression associated with range expansion during interglacial periods. Importantly, individuals carrying Clade III haplotypes do not show corresponding nuclear differentiation and consistently cluster with Tennessee populations in SNP-based analyses. Accordingly, these occurrences do not support recognition of an additional Tennessee ESU, but instead provide insight into historical connectivity and mito-nuclear discordance between Tennessee and Core populations.

Mitochondrial Clade IV represents a distinct, monophyletic lineage restricted to northern Tennessee, occurring exclusively in populations north of the Cumberland River. In the mitochondrial phylogeny, Clade IV is strongly supported as sister to Clade III, the Core Distribution lineage, suggesting a shared evolutionary origin followed by subsequent isolation. The geographic confinement of Clade IV to the northern Nashville Basin indicates long-term persistence within a hydrologically and topographically distinct region, with the entrenched Cumberland River likely acting as a barrier to southward dispersal. Although closely related to Core populations at the mitochondrial level, individuals carrying Clade IV haplotypes form a cohesive Tennessee group in genome-wide SNP analyses (DAPC Cluster 1), consistent with prolonged genetic isolation. Together, these patterns indicate that Clade IV reflects an early-diverging Tennessee lineage that has remained evolutionarily independent despite its phylogenetic affinity to the Core Distribution.

Comparable patterns of isolation shaped by hydrology and environmental gradients are well documented in *A. barbouri* and other *Ambystoma* taxa. In a fine-scale genetic survey of *A. barbouri* populations in Kentucky, Micheletti and Storfer (2017) identified three genetic clusters separated by the Ohio and Kentucky rivers and showed that declining limestone availability and increasing growing-season precipitation at range margins act as cryptic habitat barriers that restrict dispersal. These findings, together with evidence that environmental gradients constrain connectivity toward range edges in *A. barbouri* (Micheletti & Storfer 2015; Beer et al. 2022), parallel the role of watershed boundaries (namely the Cumberland and Stones River systems) and karst-mediated drainage reorganization in structuring Tennessee populations. Similar patterns of landscape-mediated isolation have been observed in other ambystomatids as well; landscape genetic studies of blotched tiger salamanders and California tiger salamanders have shown that combinations of topography, hydrology, and anthropogenic features (e.g.,

roads, arid uplands) strongly influence dispersal routes and genetic subdivision (Spear et al. 2005; Wang et al. 2009). Similarly, work on marbled (*A. opacum*) and spotted salamanders (*A. maculatum*) has indicated that landcover, manmade infrastructure, and wetland geography can act as significant barriers to gene flow, even in otherwise connected forested landscapes (Greenwald et al. 2009; Bartoszek & Greenwald 2009; Whiteley et al. 2014). Collectively, these studies support the conclusion that the hydrogeographic boundaries and karst features of the Interior Low Plateaus are capable of generating the strong spatial genetic structure observed among *A. barbouri* populations in Tennessee.

Genetic Variation Within Populations

Spatial patterns of heterozygosity revealed pronounced differences in within-population genetic diversity, reflecting both historical demographic processes and contemporary habitat conditions. Overall, populations in Kentucky exhibited the highest levels of heterozygosity, whereas populations in Tennessee and Ohio showed reduced genetic diversity, consistent with expectations that anthropogenic habitat alteration can erode within-population variation. Within Tennessee, heterozygosity varied substantially among counties. Populations in Wilson County, primarily associated with the Central genetic cluster, displayed the highest average expected heterozygosity (H_e), observed heterozygosity (H_o), and the greatest proportion of polymorphic loci (PPL). Wilson County populations were recovered as basal to other Tennessee *A. barbouri* populations in both mitochondrial phylogenetic reconstructions and the F_{ST}-based distance tree, suggesting that this lineage may represent an older genetic component within the state. In addition, Wilson County and surrounding areas have experienced relatively limited or more recent habitat disturbance compared to populations nearer the center of the Nashville Basin.

In contrast, populations in Bedford, Marshall, Rutherford, and Williamson Counties exhibited markedly lower heterozygosity, consistent with a history of sustained development, population bottlenecks, or reduced connectivity among breeding sites. These patterns support the hypothesis that increasing urbanization and stream degradation within the Nashville Basin are associated with declines in genetic diversity. Notably, one population in Robertson County (Rob1) showed relatively high heterozygosity despite its proximity to a long-standing apartment complex, suggesting either recent demographic recovery or the persistence of sufficient connectivity to maintain genetic variation. Collectively, these results demonstrate substantial heterogeneity in within-population genetic diversity across Tennessee *A. barbouri* populations that may reflect the combined influence of historical lineage divergence along with contemporary landscape changes.

Management Implications and Future Directions

This study provides an updated framework for the conservation of *A. barbouri* in Tennessee and supports recognition of these populations as a Distinct Population Segment (DPS) under the U.S. Endangered Species Act. Under the joint DPS policy (USFWS & NMFS 1996), a vertebrate population must demonstrate discreteness, significance, and risk. Tennessee populations meet the criterion of discreteness by forming multiple deeply divergent mitochondrial clades and three distinct nuclear clusters that are clearly separated from the Core Distribution. They meet the significance criterion in that

these southern lineages contain unique evolutionary diversity found nowhere else in the species' range, represent long-term refugial populations, and would create a substantial gap in the species' distribution if lost. Finally, rapid urbanization, stream degradation, and reduced heterozygosity in several southern populations indicate elevated risk, consistent with ESA Section 4 criteria. Given this structure, the three Tennessee genetic clusters should be treated as separate conservation units within the DPS. Management priorities include preserving genetic variation within clusters, protecting high-diversity sites, and maintaining connectivity among headwater breeding streams through riparian buffers, reduced habitat fragmentation, and limits on development near critical habitats. Incorporating genomic structure into regional planning will help sustain the evolutionary resilience of these disjunct Tennessee lineages.

Recognizing each of the three Tennessee genetic clusters as separate conservation units within the DPS would improve management by ensuring that evolutionary potential is maintained across the full breadth of Tennessee diversity. Populations exhibiting high heterozygosity (e.g., northeastern Wilson County, Robertson County) should be prioritized for protection, while those with reduced variation may require monitoring for demographic decline. Conservation actions should focus on preserving connectivity among headwater streams, maintaining riparian buffers, minimizing further fragmentation of breeding habitats, and maintaining consistent hydrologic regimes through protection and restoration of larger watersheds to reduce the risk of population sinks. Integrating fine-scale genomic structure into management planning will strengthen efforts to protect the unique evolutionary lineages of *A. barbouri* in Tennessee and support their long-term persistence.

Conclusion

This study provides the most comprehensive genetic assessment of Tennessee *Ambystoma barbouri* to date by integrating mitochondrial phylogenetics with genome-wide SNP data to evaluate both range-wide and fine-scale patterns of population structure. Our findings confirm that Tennessee populations are genetically distinct from those in the species' Core Distribution and reveal three deeply divergent lineages within the state, each likely reflecting long-term isolation in historical refugia. These results underscore the evolutionary significance of Tennessee populations and demonstrate that they harbor unique genetic diversity shaped by long-standing barriers to gene flow. Incorporating this structure into conservation planning will be essential for preserving the species' adaptive potential through maintaining genetic diversity. As habitat loss and fragmentation intensify across the Nashville Basin, proactive and genetically informed management strategies will be critical to ensuring the long-term persistence of *A. barbouri*.

Statements and Declarations

The authors have no conflicts of interest to disclose.

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Author contributions

Jared Thompson contributed to tissue collection, molecular laboratory work, data generation, and manuscript writing. Miranda Gaupp generated the Ambystoma barbouri genome assembly and provided comments on the manuscript. David Ian Withers, William Sutton, Anna Humphrey, Morgan Page, and Tereza Jezkova contributed to tissue collection and provided comments on the manuscript. All authors reviewed and approved the final manuscript. Carla Hurt conceived and designed the study, acquired funding, supervised the project, conducted data analyses, and led manuscript preparation.the Tennessee Wildlife Resources AgenTcy for financial support of this research.

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Tables

Table 1. Summary of within-population genetic variation for *Ambystoma barbouri* based on biallelic SNP data. For each population, ***N*** is the number of individuals analyzed; **H** is mean observed heterozygosity across loci; **H** (gene diversity) is mean expected heterozygosity across loci; $\mathbf{F_{IS}}$ is the mean inbreeding coefficient within populations; and **PPL** is the percentage of polymorphic loci. Polymorphic loci were defined as loci with a minor allele frequency ≥ 0.01 within a population and genotypes called for at least three individuals.

Population	N	H□	H□	F_{IS}	PPL (%)
Tennessee Populations					
Bed1	6	0.010	0.009	−0.017	3.4
Dav1	20	0.010	0.010	−0.022	8.2
Dav2	10	0.012	0.011	−0.062	5.7
Dav3	13	0.012	0.010	−0.068	7.1
Dav4	18	0.012	0.011	−0.059	8.2
Mar1	10	0.012	0.011	−0.028	3.9
Mar3	10	0.009	0.010	0.059	5.7
Mar4	8	0.008	0.007	−0.068	3.7
Rob1	14	0.017	0.022	0.121	11.0
Rut1	7	0.012	0.017	0.198	6.0
Rut2	6	0.009	0.016	0.227	4.6
Rut4	16	0.007	0.012	0.277	7.1
Rut7	10	0.006	0.010	0.200	4.3
Rut8	8	0.008	0.007	−0.075	3.6
Sum1	18	0.010	0.016	0.270	8.9
Sum10	7	0.011	0.012	0.041	5.4
Sum11	7	0.008	0.009	0.043	3.2
Sum12	17	0.010	0.017	0.240	9.5
Sum5	17	0.011	0.012	0.054	10.1
Wil1	16	0.017	0.024	0.175	11.2
Wil10	10	0.013	0.014	0.001	6.7
Wil12	11	0.007	0.008	−0.016	4.2
Wil13	20	0.012	0.013	0.094	9.7
Wil2	6	0.017	0.032	0.207	8.6
Wil5	17	0.012	0.018	0.155	10.6
Wil6	14	0.011	0.021	0.260	8.5
Wil7	10	0.009	0.017	0.220	6.2

Population	N	H□	H□	F_{IS}	PPL (%)
Wil8	19	0.012	0.016	0.114	8.6
Wil9	14	0.013	0.011	0.050	5.8
Willi1	11	0.007	0.009	0.053	3.9
Willi5	5	0.005	0.011	0.476	3.7
Core Populations					
IN3	5	0.011	0.014	0.150	4.6
KY10	5	0.013	0.017	0.151	4.8
KY11	5	0.012	0.010	−0.188	3.2
KY12	5	0.017	0.021	0.087	5.4
KY14	5	0.018	0.021	0.020	6.1
KY8	6	0.020	0.020	−0.024	6.1
KY9	5	0.018	0.016	0.023	5.4
OH5	5	0.011	0.013	0.081	5.1
OH6	6	0.016	0.015	0.045	4.6
OH7	5	0.015	0.012	−0.183	3.3
WV1	7	0.014	0.014	−0.026	3.6

Table 2. Analysis of molecular variance (AMOVA) based on SNP data from Tennessee populations of the streamside salamander (*Ambystoma barbouri*). Genetic variation was partitioned among watersheds, among populations within watersheds, and within populations. Φ statistics are hierarchical analogs of F-statistics. Asterisks indicate statistical significance based on 999 permutations (*P* = 0.001).

Source	df	Sum of squares	Mean square	Estimated variance	%	Φ statistic
Among Watersheds	14	1326.44	94.75	1.979	8.63	Φ_{CT} = 0.086*
Among populations within groups	18	847.64	47.09	2.326	10.15	Φ_{SC} = 0.111*
Within populations	365	6794.33	18.61	18.615	81.22	Φ_{ST} = 0.188*
Total	397	8968.41	22.59	22.920	100	—

Figures



Figure 1

Image of *Ambystoma barbouri*. Photograph courtesy of David Pineros.

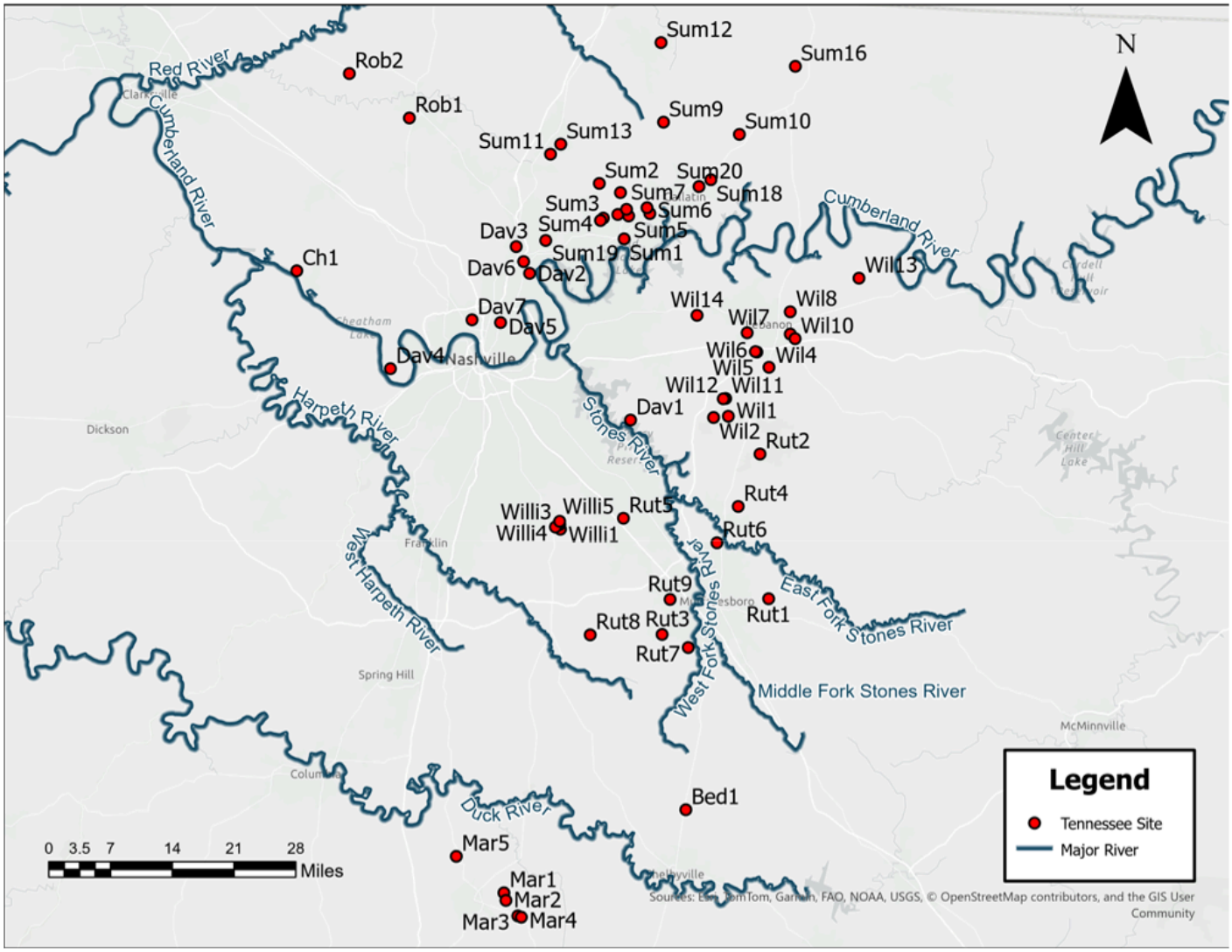


Figure 2

Map showing all sites surveyed within the disjunct Tennessee range of *Ambystoma barbouri*.

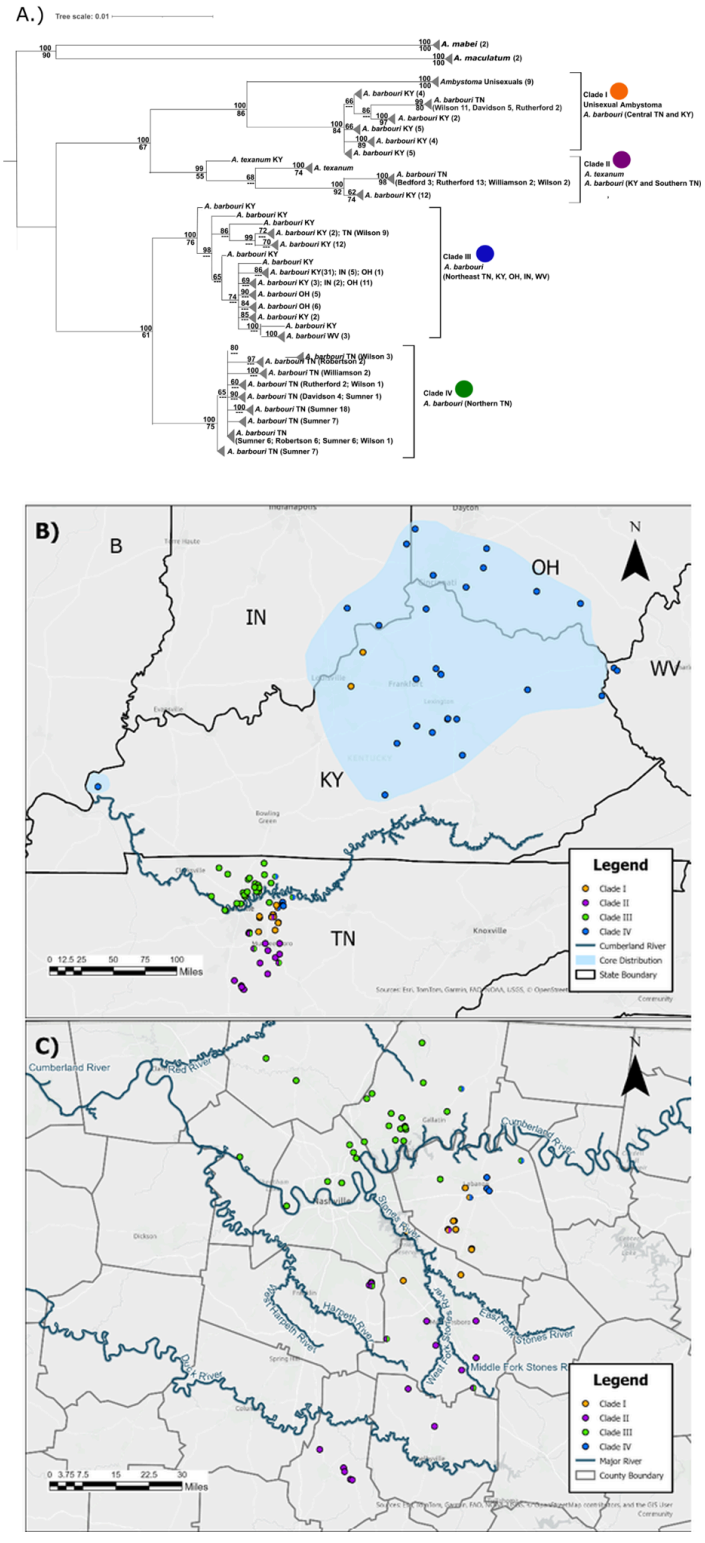


Figure 3

(A) Maximum likelihood (ML) phylogenetic reconstruction of *Ambystoma barbouri* mitochondrial haplotypes based on ~1,200 bp of the mitochondrial control region (D-loop). Numbers above nodes indicate bootstrap support from ML analyses, and numbers below nodes indicate posterior probabilities from Bayesian analyses. Colored symbols denote major mitochondrial clades. GenBank numbers at tips correspond to GenBank accession numbers listed in Supplementary Table 1. Maps displaying the geographic locations of clade assignments identified by Bayesian and maximum-likelihood (ML) for a (B) all surveyed *A. barbouri* populations with the core range highlighted in blue and (C) Tennessee *A. barbouri* populations.

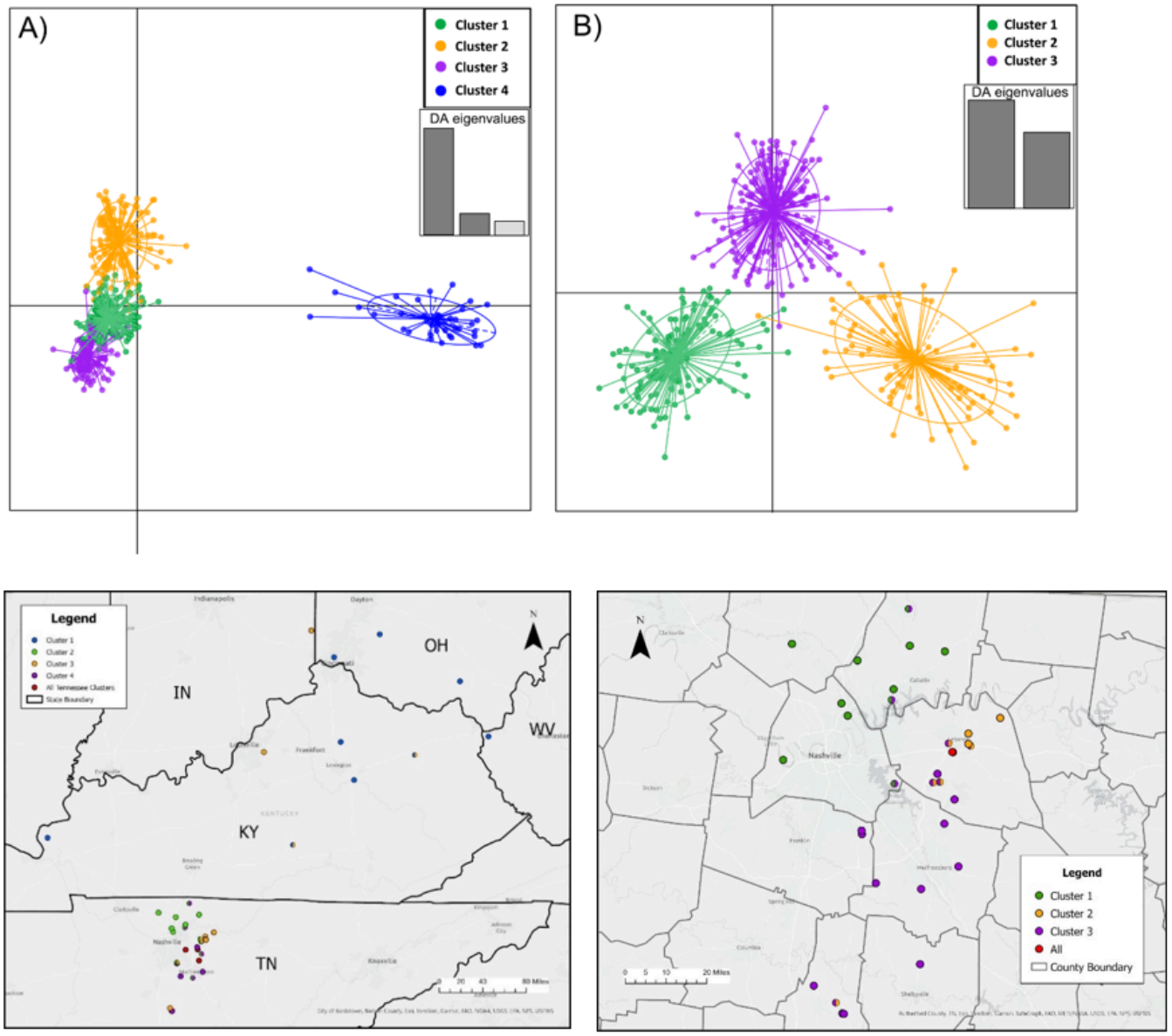


Figure 4

Discriminant Analysis of Principal Components (DAPC) results for *Ambystoma barbouri*. (A) Scatter plot of individuals from all surveyed populations (AP dataset) and (B) scatter plot of individuals from Tennessee populations only (TN dataset). Maps show the geographic distribution of inferred genetic

clusters for the (C) AP dataset and (D) TN dataset. Colors indicate genetic cluster membership as shown in the legend.

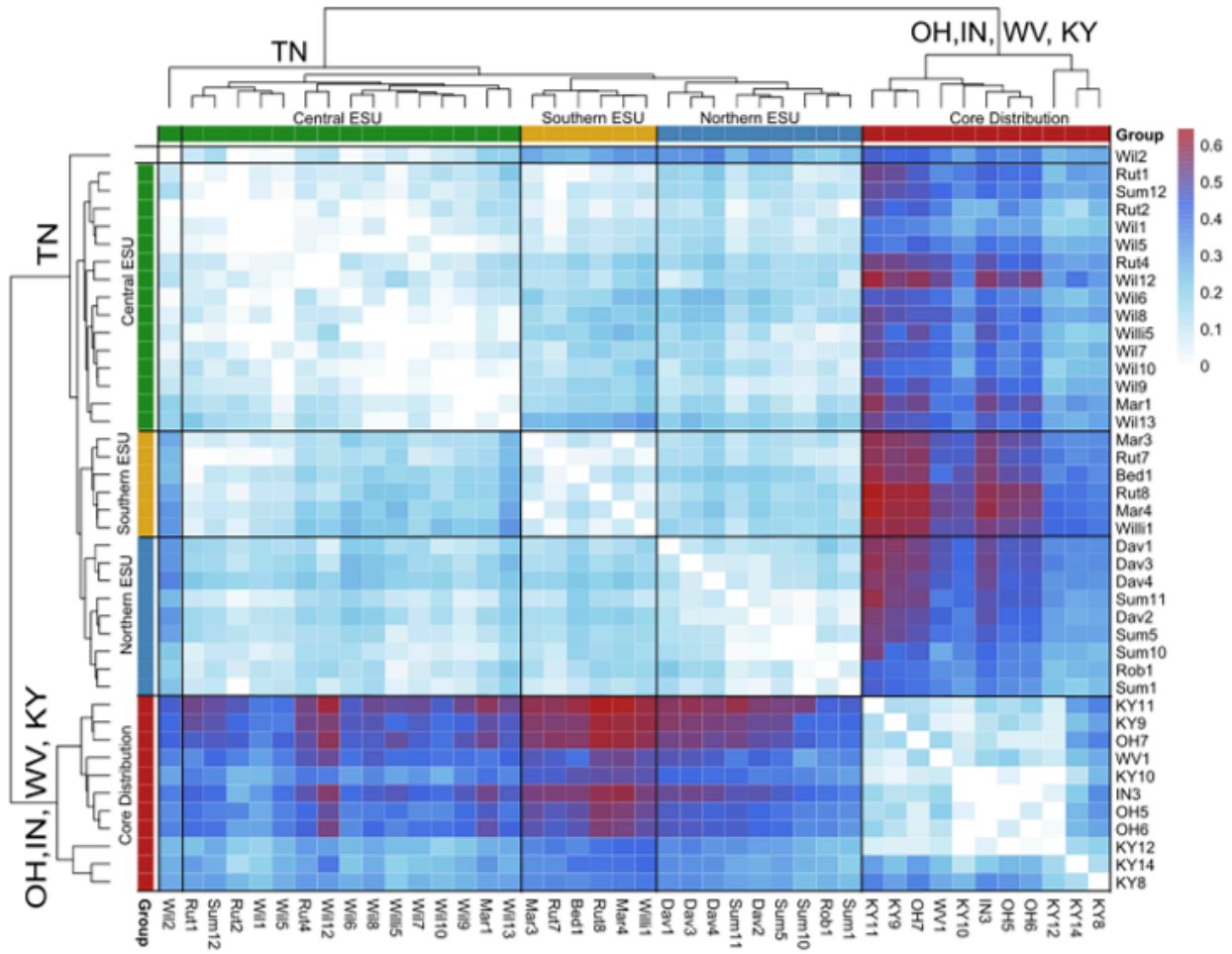


Figure 5

Heatmap and distance-based dendrogram illustrating genetic pairwise F_{ST} among sampling sites based on pairwise F_{ST} values from 867 SNP genotypes in the AP dataset.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplement1.docx