Understanding “Alexandrian” extinctions using genomic DNA from fluid-preserved museum specimens of *Desmognathus* salamanders

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Abstract

Species that went extinct prior to the genomic era are typically out-of-reach for modern phylogenetic studies. We refer to these as “Alexandrian” extinctions, after the lost library of the ancient world. This is particularly limiting for conservation studies, as genetic data for such taxa may be key to understand extinction threats and risks, the causes of declines, and inform management of related, extant populations. Fortunately, continual advances in biochemistry and DNA sequencing offer increasing ability to recover DNA from historical museum specimens, including fluid-preserved natural history collections. Here, we report on success in recovering nuclear and mitochondrial data from the apparently-extinct subspecies *Desmognathus fuscus carri* (Neill 1951), a plethodontid salamander from spring runs in central Florida. The two specimens are 50 years old and were likely preserved in unbuffered formalin, but application of a recently derived extraction procedure yielded usable DNA and partially successful Anchored Hybrid Enrichment sequencing. These data suggest that the populations of *D. f. carri* from peninsular Florida are conspecific with the *D. auriculatus* A lineage as suggested by previous authors, but likely represented an ecogeographically distinct genetic segment that has now been lost. Genetic data from this Alexandrian extinction thus confirm the geographic extent of population declines and extirpations as well as their ecological context, suggesting a possibly disproportionate loss from sandy-bottom clearwater streams compared to blackwater swamps. Success of these methods bodes well for large-scale application to fluid-preserved natural history specimens from relevant historical populations, but the possibility of significant DNA damage and related sequencing errors in additional hurdle to overcome.

Introduction

Wilson (1992) introduced the concept of “Centinelan” extinctions (see also Winchester and Ring 1996); the extinction of species due to human activities before they are ever discovered and described, named after Centinela Ridge in Ecuador that was clearcut before being surveyed. In contrast, many recent extinctions involve species that are nonetheless relatively well-known. Taxa such as the Dodo and Thylacine have been extinct for dozens or hundreds of years, yet are still well-studied (Jones and Stoddart 1998; Shapiro 2002; Roberts and Solow 2003; White et al. 2018). Scientists have even generated high-quality genome assemblies from historical specimens of pre-modern species such as the Wooly Mammoth and Neanderthal (Miller et al. 2008; Prüfer et al. 2014), allowing us to study their genetics in-depth in perpetuity, despite dying out thousands of years ago. The same is true of many species that have gone extinct in the genomic era, or are likely to go extinct in the near future; assuming they have been sequenced or at least bio-banked, we have an enduring record of their presence, the ability to study their evolutionary origins and genetic identity, and even the capacity for de-extinction (Sherkow and Greely 2013).

In contrast, there is a large class of extinctions of named taxa that occurred prior to the genomic era, about which there is little knowledge and for which there are few if any genetic resources. The sum of our knowledge of these may be little more than the brief notes from an original description, or possibly a few

historical museum specimens. We refer to this class of extinctions as “Alexandrian,” after the lost library of antiquity which contained untold knowledge from the ancient world. Thus, information on these extinct taxa survives only in a few words or specimens, as information on the Great Library survives only in a few fragments of Callimachus’ *Pinakes* (Witty 1958). For instance, hundreds of lizard and mammal species are known only from their holotype, most of which pre-date the genomic era (Amori et al. 2016; Meiri et al. 2018). Another famous example is the only salamander presumed extinct in North America, *Plethodon ainsworthi*, known only from two poorly-preserved type specimens collected in 1964 (Lazell 1998). Recent attempts to recover genetic material failed (Pierson et al. 2020), leaving open the previous question of whether this taxon is “extinct, extant, or nonexistent” (Himes and Beckett 2013).

The power of conservation genomics (Steiner et al. 2013) is of limited applicability for such taxa, despite their potential relevance to conservation management of related extant species (Moodley et al. 2017). Extinct named taxa may also be of particular relevance if they represent distinct phylogenetic lineages associated with specific traits, habitats, or other factors affecting extinction risk (Roycroft et al. 2021). Alternatively, finding that an extinct taxon is not valid but instead part of a wide-ranging species with extant populations can unify the geographic focus of conservation efforts, which may have been fragmented based on the belief that these were separate taxa (Zink 2004). This is in addition to the obvious improvement in our biodiversity knowledge and taxonomic accuracy gained from such cases (Kehlmaier et al. 2020). Genetic data from extirpated populations may thus offer substantial insight into a species’ diversity, the causes of decline, and the possibilities for effective recovery (see Shaffer et al. 2015).

Fortuitously, an increasing capacity to recover genomic resources from fluid-preserved specimens offers great promise to extract crucial data from historical specimens (Hykin et al. 2015; Ruane and Austin 2017). Increasingly-sophisticated laboratory protocols have yielded high-quality DNA extracts even from decades-old material (O’Connell et al. 2021; Straube et al. 2021). Thus, we are now potentially able to recover at least some genetic data for some Alexandrian extinctions, allowing us to assemble information from those manuscripts that were previously lost. This may be particularly relevant for taxa whose extinction is enigmatic – due to unknown or incompletely understood causes – and for which this knowledge may have conservation implications for extant populations of related, imperiled taxa.

We illustrate this here with another example from eastern Nearctic plethodontid salamanders, *Desmognathus fuscus carri* Neill, 1951, now considered a junior subjective synonym of *D. auriculatus* after Rossman (1959). It is important to note that populations historically assigned to *D. “auriculatus”* represent at least 4 candidate species, one of which was recently described as *D. valentinei* (Means et al. 2017). Of the remaining three mito-nuclear candidate species (Pyron et al. 2020), *D. auriculatus* A occurs in northern Florida and southern Georgia, while *D. auriculatus* B & C form a distantly related clade in the coastal plain of southeastern Georgia, South Carolina, and North Carolina (Beamer and Lamb 2008, 2020).

Intriguingly, *Desmognathus auriculatus* A represents one of few enigmatic amphibian declines in North America, having mysteriously disappeared from the vast majority of its historic localities in the Coastal Plain of the southeastern U.S. (Means 2015). This includes many sites with apparently pristine and relatively undisturbed habitat containing abundant populations of other amphibians and reptiles including other salamanders, even other *Desmognathus* (Dodd 1998; Means and Travis 2007; Beamer and Lamb 2008; Graham et al. 2010; Maerz et al. 2015). Some montane salamanders have apparently experienced enigmatic declines at some sites, but not rangewide (Highton 2005; Caruso and Lips 2013). Other Coastal Plain salamanders have also declined, such as the Flatwoods Salamanders *Ambystoma bishopi* & *A. cingulatum* and the Striped Newt *Notophthalmus perstriatus*, but with clearly identifiable causes such as habitat alteration or loss (Dodd and LaClaire 1995; Pauly et al. 2012). In contrast, none of the major drivers of amphibian declines such as climate change, environmental modification, infectious diseases, or invasive species (see Blaustein et al. 2011) seem to explain the sudden range-wide disappearance of *D. auriculatus* A (and only *D. auriculatus* A) from so many seemingly suitable sites in the late 1960's and early 1970's (Means 2015).

Crucially, *Desmognathus auriculatus* A persists at several sites in northern Florida and southern Georgia (Means et al. 2017; Beamer and Lamb 2020), but seems to be entirely absent from peninsular Florida with the exception of a few unverified sightings (Dodd 1998). One of us (CRH) has conducted systematic rangewide surveys and confirmed the apparent absence of *D. auriculatus* A from most of its historical localities, as initially reported by the numerous authors cited above. This includes the Marion Co. populations originally described as *D. fuscus carri* by Neill (1951), which represents an Alexandrian extinction; it was formally recognized as a distinct taxon but has gone extinct before any modern analyses could be conducted. However, it was synonymized solely on the basis of external morphological measurements (Rossman 1959), while *Desmognathus* species are often characterized by cryptic diversity and extreme morphological conservatism (Tilley et al. 2013; Camp et al. 2013). It is therefore crucial to answer the question of "extinct, extant, or nonexistent" regarding *D. f. carri* to understand i) the true diversity of amphibians in the southeastern U.S., ii) the geographic context of an enigmatic instance of the global phenomenon of amphibian declines, and iii) inform the conservation management of remaining allied populations.

We used the Formalin-Fixed Sequencing (FFS) protocol of O'Connell et al. (2021) and the Anchored Hybrid Enrichment (AHE) sequencing protocol of Lemmon et al. (2012) to generate mitochondrial and nuclear sequence data for two 50-year-old fluid-preserved specimens of *Desmognathus fuscus carri* from the type locality. Sequence capture was successful but limited, yielding enough data to confidently assign the population to the "A" lineage of *D. auriculatus* (Beamer and Lamb 2008, 2020), confirming their synonymy but suggesting some degree of genetic distinctiveness. Thus, the decline of peninsular populations is likely part of a linked phenomenon affecting *D. auriculatus* A throughout its range, and not a separate instance of extinction of a distinct taxon. Additional surveys and perhaps alternative techniques such as environmental DNA should be employed to search for potential remnant populations.

Materials & Methods

Sampling & Sequencing

Wilfred T. Neill described *Desmognathus fuscus carri* from a holotype (ERA-WTN 14188, now presumed to be in the Florida Museum of Natural History; Darrel R. Frost, *pers. comm.*) collected at "Silver Glen Springs, in the Ocala National Forest, Marion County, Florida" on 12 October 1950, and 47 paratypes collected from 4 nearby peninsular localities (Neill 1951). However, no specimens with data matching the holotype or the paratypes ERA-WTN 14167–87 and 14189–93 are catalogued in the FLMNH; Neill destroyed many of his specimens before depositing them (Paul C. Moler, *pers. comm.*). The extant accessioned paratypes are UF 2962–3, 3063, and 17446–63 (formerly ERA-WTN 14197–214). He also assigned 7 other known populations from peninsular Florida to this taxon (Fig. 1; Table 1).

On 25 January 1971, the noted plethodontid biologist Richard D. Highton (see Kuchta 2019) collected 36 specimens from the type locality, now accessioned in the U.S. National Museum of Natural History as USNM 468081–468115 & 490016. Exact preservation details were not recorded for these specimens, but the general approach of Highton at that time consisted of ~24 hours of fixation in 10% unbuffered formalin, ~24 hours in flowing water, and subsequent preservation in 70% EtOH (AHW *pers. obs.*). In August 2019, one of us (AHW) extracted liver tissue from two of these specimens (468094 & 468095), and another (KAO) extracted DNA following O'Connell et al. (2021). These yielded concentrations of 20 and 52 ng/µl (in a volume of 35µl) for a total of 700ng and 1.8µg, respectively, quantified using the high sensitivity kit on a Qubit. While the DNA was somewhat sheared and did not form high molecular-weight bands on an agarose gel, fragments corresponding to ~300–500bp were visible in florescence (Fig. 2), and we therefore proceeded with AHE sequencing.

Data were generated using the Anchored Hybrid Enrichment (AHE) approach (Lemmon et al. 2012) as described in Hime et al. (2020) using the "*Desmognathus* version 2.0" probe set from Pyron et al. (2020). Sequencing was performed in the Translational Science Laboratory in the College of Medicine at Florida State University using PE150 Illumina HiSeq2000 lanes. We obtained 1,636,114 raw reads for USNM 468094 and 2,043,106 for 468095 (SRA PRJNA743148). We first trimmed adapters and low-quality bases using trimmomatic v0.39 (Bolger et al. 2014). We then filtered for off-target reads against the default references (human, mouse, rat, *Drosophila,* worm, yeast, *Arabidopsis, E. coli,* rRNA, mitochondria, PhiX, Lambda, vectors, and adapters) in FastQ Screen v0.14.1 (Wingett and Andrews 2018).

Bioinformatic Analyses

For mitochondrial analysis, we mapped trimmed reads to the mitochondrial *ND2*/tRNA/*COI* fragment of *Desmognathus auriculatus* A from the closest known, extant population to Silver Glen Springs, DAB349 from Olustee Creek, Baker Co., Florida (MH403587). We used the Geneious mapper in Geneious Prime 2020.1.2 (Biomatters Ltd.) with a minimum mapping quality of 30, retaining all mapped reads that did not induce frame-shifted indels in protein-coding regions. Given the manageable number of

mapped reads, we assessed each one individually, checking the identity of unusually divergent reads using BLAST against the full database of mitochondrial data from known *Desmognathus* lineages (Kozak et al. 2005; Beamer and Lamb 2020, etc.). We added the consensus sequences to the 161-individual alignment from our previous analysis (Pyron et al. 2020), along with six additional individuals from four populations from recent studies (Means et al. 2017; Beamer and Lamb 2020) and seventh individual from a fifth sub-population sampled in our recent fieldwork (Table 1).

For the AHE loci, we first mapped cleaned reads to the consensus sequence of each of the 381 AHE loci from our previous analysis (Pyron et al. 2020) to calculate locus-specific coverage statistics. For phylogenetic inference (see below), we then mapped the reads directly to the consensus of the full 381-locus alignment for DAB349 with a custom sensitivity comprising a minimum mapping quality of 30, gaps disallowed, a maximum of 5% mismatches, and maximum ambiguity of 4. The ungapped consensus sequence of the mapped reads for each fluid-preserved specimen was then added back to the full alignment using the '—add' and '—keeplength' options in mafft v7.475 (Katoh and Standley 2013). Finally, we manually trimmed this 163-taxon alignment to the stretches of loci for which both fluid-preserved specimens were represented with at least 10x coverage for at least 50bp using a custom R script.

Phylogenetic Inference

Our aim in this preliminary analysis is to assess the phylogenetic placement of the Silver Glen Springs population in the context of existing mitochondrial and nuclear frameworks (Beamer and Lamb 2020; Pyron et al. 2020). Our sampling of both populations and loci is too sparse to allow fine-scale population-genetic inference or estimation of phylogenetic networks showing hybridization between species, though both would be desirable in future studies due to their conservation relevance (Ewart et al. 2019). We are also cautious regarding the potential for high rates of historical-sample-related base composition error confounding such estimates (see Hykin et al. 2015; Oh et al. 2015; O'Connell et al. 2021). Thus, we do not phase alleles, model admixture, or estimate networks. Rather, we use the consensus sequences for the two individuals and estimate a bifurcating topology to place them in an ecodrainage-based mitochondrial lineage and a mito-nuclear candidate species corresponding to our previous studies.

First, we estimated a mitochondrial phylogeny for the 163-individual *ND2*/tRNA/*COI* alignment using IQ-TREE v2.1.3 (Minh et al. 2020) with optimal models and merged partitions (Chernomor et al. 2016) selected using ModelFinder (Kalyaanamoorthy et al. 2017) and support estimated using 1000 ultrafast bootstraps (Hoang et al. 2017) and the SHL-aLRT branch statistic (Guindon et al. 2010). As per the IQ-TREE recommendations, we interpret UFBoot > 95 and SHL>80 as "strong" support. Second, we estimated a concatenated phylogeny using partitioned models under the optimal merging strategy for the combined loci, also with UFBoot and SHL-aLRT support. We did not estimate a multi-locus species tree due the lack of full-length loci to accurately resolve incomplete lineage sorting, the close resemblance of previous concatenated and species-tree estimates for this dataset, and the limited nature of the question at hand

regarding the terminal lineage-level placement of the two fluid-preserved specimens, which should not be significantly altered by coalescent-based multi-locus species-tree analysis.

Results

Mitochondria

For the mitochondrial assemblies, bycatch of non-AHE reads was relatively low as originally intended for AHE library preparations (Lemmon et al. 2012). However, sufficient data were still recovered; for USNM 468094, 51 reads mapped with 1–11x coverage for 1,437bp of the *ND2*/tRNA/*COI* fragment, and 20 reads with 1–7x coverage totaling 988bp for 468095. Three reads were excluded from each alignment; for 468094, 1 unpaired read BLAST-ed to *Desmognathus valentinei* and a paired couplet to the *D. conanti* Gamma clade*;* while for 468095, 1 unpaired read matched *D. ocoee D* and a paired couplet to *D. marmoratus* E. For USNM 468094, only 831bp of ND2 formed a sufficiently long fragment for Genbank accessioning (see Table 1), while the consensus of mapped reads for USNM 468095 did not form contiguous stretches greater than 250bp, so none were uploaded. However, we analyzed the full alignment of all fragments for both. The estimated phylogeny (Fig. 3) is similar to our previous analysis (Pyron et al. 2020), yielding 100% bootstrap and SHL support for a clade containing the two historical specimens and the 9 recent *D. auriculatus* A specimens. The two fluid-preserved specimens form a clade that is sister to all more northerly samples from extant populations, potentially indicating a distinct genetic unit in the extirpated peninsular populations.

AHE Loci

Contamination was present but relatively low in both samples, with 7% of trimmed USNM 468094 reads mapping to common references and 10% for 468095. The most frequent contaminant was human, matching 4.5% and 7.7% of reads respectively. Non-zero amounts of non-exclusive matches up to ~3% were seen from all other sources (except for *E. coli,* PhiX, and Lambda) in both samples. After trimming and filtering, 1,107,198 reads remained for 468094 (68% of the raw reads), and 1,475,305 for 468095 (72%). However, initial assemblies yielded relatively low on-target mapping, <10% for both individuals, with an apparently high rate of mismatched reads from unknown sources yielding highly divergent consensus sequences. This may be expected generally as a consequence of the massive genomes found in *Desmognathus* and other salamanders (Sclavi and Herrick 2019)

To improve assembly efficiency and reduce the probability of off-target mapping, we repeated the FastQ Screen analysis using the consensus sequence of DAB349 for the 381 AHE loci as the reference genome to isolate on-target reads. This yielded 63,634 reads for 468094 and 119,923 for 468095. Of these, 57,492 mapped to 305 of the 381 loci with coverage ranging from 1–1,967x for 468094, and 109,265 mapped to 304 loci with 1–2,433x coverage for 468095. After trimming for quality and coverage, the final merged set of orthologous loci contained 163 individuals with sequence data from up to 73 AHE locus fragments ranging from 3–286bp, totaling up to 7,651bp, and was 95.5% complete. Three of the locus fragments

were very short and contained no parsimony-informative sites; these were combined into a single partition.

The estimated phylogeny is overall relatively similar to our previous concatenated AHE estimate (Pyron et al. 2020), even given its significantly reduced length at ~1.4% of the original. The two fluid-preserved specimens form a clade with the two recent *Desmognathus auriculatus* A specimens supported at 100% by both measures (Fig. 4). In contrast to the mitochondrial results, USNM 468094 is more closely related to the two extant populations than to 468095. Notably, the *D. auriculatus* A lineage occupies an earlier-branching position relative to the other *D. "auriculatus"* and *"fuscus"* lineages than in our previous analysis (Pyron et al. 2020). Additionally, *D. valentinei* forms the outgroup to this clade, rather than the *D. "conanti"* species-group as in that tree. Interestingly, *D. valentinei* was described from Gulf Coastal Plain populations previously assigned to *Desmognathus "auriculatus"* (see Means et al. 2017). Whether this pattern is real and suggestive of more complex historical evolutionary dynamics in the group, or simply a consequence of the dramatically reduced dataset is unclear but deserves further scrutiny in the future.

Similar to the mitochondrial results but more exaggerated, the terminal branch lengths for the two historical samples are noticeably longer than those of the extant *Desmognathus auriculatus* A or most other samples. However, this might have arisen due to the incompleteness of the gene fragments, sequencing error, or mis-mapped reads, as we note a qualitatively larger number of ambiguous (possibly heterozygous) or divergent base calls in those sequences. Visual examination of the alignment reveals the sporadic presence of both private alleles and SNPs shared with lineages other than *auriculatus* A, which are nonetheless supported by our stringent filtering, mapping, and coverage thresholds. These may result from a combination of legitimate genetic diversity, assembly error, contamination from closely related species, and DNA damage. Congeneric contaminants should produce excessive heterozygous SNP calls, while DNA damage generally yields an excess of false homozygous SNPs (Ewart et al. 2019; O'Connell et al. 2021).

Untangling these factors will require additional study, and our ability to diagnose this damage is limited without phased assemblies, as ambiguities representing heterozygotes were only called in limited circumstances given the strictness of our read mapping. However, we can describe initial patterns in the fluid-preserved *Desmognathus auriculatus* A compared to the recent samples. Within the 7,651bp AHE consensus sequences of the four *D. auriculatus* A specimens, there are 125 variable sites. Of these, 28 are SNPs unique to USNM 468094 and 51 to 468095, compared to 10 for DAB349 and 6 for DAB1391. In the former two samples, 5/28 (18%) and 3/51 (6%) are heterozygotes; the counts are 6/10 (60%) and 1/6 (17%) for the latter two. Across all 125 variable sites, the counts are 5 (4%), 4 (2%), 22 (18%), and 16 (13%), respectively. Thus, while the difference in heterozygosity may be due to differences in assembly parameters, the overall pattern is one of an increased frequency of unique homozygous SNPs in the fluid-preserved specimens from the same population relative to the recent specimens from different populations. We interpret this result as at least partially artifactual and consistent with DNA damage.

Discussion

The availability of historical museum specimens to genomic inquiry opens exciting new applications in the field of conservation genomics (see Nakahama 2021). Corroborating the identity of recently extinct populations and assessing their genetic distinctiveness may prove crucial for the conservation management of extant populations (Moodley et al. 2017). Similarly, historical genomic DNA can be used to assess the taxonomic identity of historical specimens and the nomenclatural validity of putatively extinct taxa (Kirchman et al. 2010; McGuire et al. 2018; Kehlmaier et al. 2020). However, few if any studies have combined these to study what we term Alexandrian extinctions; enigmatic declines or extinctions of historically named taxa whose identity is of immediate conservation relevance (Roycroft et al. 2021). We provide a test case for *Desmognathus fuscus carri*, a synonym of *D. "auriculatus"* associated with populations that disappeared from peninsular Florida in the late 1960s or early 1970s (Means 2015). We obtained high-quality mitochondrial and nuclear data for two 50-year-old formalin-fixed, ethanol preserved specimens from the type locality, confirming that they are indeed closely related to other extant populations of *D. auriculatus* A (see Rossman 1959).

It can be difficult to separate DNA damage from legitimate sequence divergence (Do and Dobrovic 2015; Hykin et al. 2015). While our sample size is small and coverage limits our ability to test all hypotheses fully, there is an increased prevalence of homozygous SNPs unique to each of the fluid-preserved specimens captured on the same day in the same population relative to the two recent specimens from widely separated populations in Florida and Georgia. Thus, we suspect at least some of the genetic divergence in the historical samples is artifactual (Ewart et al. 2019; O'Connell et al. 2021). In contrast, the peninsular populations are nonetheless supported as distinct by multiple SNPs in both the mitochondrial and nuclear data, shared by both historical specimens, called with high coverage and accuracy.

Accordingly, our preliminary phylogenetic results suggest some degree of genetic differentiation for the extirpated population in peninsular Florida (Figs. 3, 4). Most historical peninsular records (Fig. 1; Table 1; see Neill 1951) occurred in a distinct river drainage (St. Johns) and Level IV Ecoregion (75c Central Florida Ridges and Uplands/75d Eastern Florida Flatwoods) from the more northerly extant populations (Means et al. 2017; Beamer and Lamb 2020). Other populations to the west from the Alafia and Wacassassa Rivers near Tampa (Fig. 1) occurred in 75a Gulf Coast Flatwoods and 75b Southwest Florida Flatwoods. These are Gulf drainages, the former of which extends to the extant panhandle sites. This suggests the potential for one or more genetically distinct ecogeographic population segments in the peninsula, which is a well-known nexus of phylogeographic divergence during Pleistocene glacial and sea level cycles (Soltis et al. 2006). Thus, *D. auriculatus* A may have historically contained greater genetic diversity across its range than is observed in extant populations, but portions of this diversity have been erased due to enigmatic range-wide declines (see Munshi-South et al. 2013).

This result was also hinted at in our previous phylogenetic network analyses (Pyron et al. 2020), which suggested a sister-group relationship between *Desmognathus fuscus* C and *D. auriculatus* A. This group was implicated in an ancestral hybridization event with *carolinensis*, and we noted that untangling this pattern was complicated by the lack of samples from extinct populations. Our reduced AHE analysis here also finds an earlier-diverging position of *D. auriculatus* A and a closer relationship to *D. valentinei* than

our previous concatenated and species-tree estimates. Populations of *D. valentinei* were previously classified as *D. "auriculatus"* (Means et al. 2017). Thus, phylogenetic network analysis at the species level and gene-flow modeling at the population level would be highly desirable including more peninsular and coastal plain samples. We did not attempt this here due to limited signal of our AHE capture data, but future attempts to sequence other fluid-preserved specimens may yield sufficient data for such investigations.

We note that the enigmatic decline of *Desmognathus auriculatus* A also involved another geographically distinct segment in ravine streams of the western panhandle of Florida (Means and Travis 2007). These populations have also never been sampled genetically, and are only presumed to represent *D. auriculatus* A (Means et al. 2017). Both the ravine-dwelling NW Florida and many peninsular populations are notable for inhabiting free-flowing, sandy-bottomed, clear-water streams, unlike the swamp-associated populations in northern Florida and southern Georgia (Means 1974). This may suggest that the panhandle segment was indeed also *D. auriculatus* A, and that stream-dwelling *D. auriculatus* A suffered disproportionately from the unknown forces of decline compared to swamp-dwelling populations. Both swamp- and stream-dwelling populations previously assigned to *Desmognathus "auriculatus"* in the extreme western part of their range in Texas and Louisiana also apparently declined around the same time, though all recent genetic samples from this area have been referred to *D. conanti* E (Hibbitts et al. 2015; Beamer and Lamb 2020).

Similarly, ravine-dwelling populations of *Desmognathus conanti* C declined in the Florida panhandle by up to 68% at some sites (Means and Travis 2007), but this lineage is still abundant and widespread across the region (Beamer and Lamb 2008). Astonishingly, another species exhibited no change in abundance at some of these panhandle sites (e.g., *Eurycea cirrigera*), while a fourth (*Pseudotriton ruber*) showed a complex pattern of both increases and decreases depending on which other species had previously been present and their former abundance. This illustrates the complex interplay of abundance and species composition across sites for amphibians experiencing (presumably) anthropogenic disturbance (Nowakowski et al. 2018; Pyron 2018).

In a conservation framework, these results are of particular relevance in unifying the geographic context of enigmatic declines in *D. auriculatus* A. While previous studies have assumed that this species included the extinct peninsular populations (Dodd 1998), this was based only on early morphological assessments (Rossman 1959). Given the named status of *Desmognathus fuscus carri* representing an Alexandrian extinction, such a conclusion was far from certain. We can now conclude that whatever the causes of these declines, they operated across both a wide geographic area and a significant span of genetic diversity, though extirpation was apparently limited to this single species-level lineage. Additional studies are now ongoing to quantify the remaining gene pool of *D. auriculatus* A and attempt to isolate the presently enigmatic nature and causes of these declines and extirpations.

A variety of problems besiege our ability to understand the world's biodiversity, including rapid biodiversity loss including enigmatic extinctions in groups such as amphibians (Stuart et al. 2004), and

the general difficulty of retrieving sufficient genetic resources from many of the most important fluid-preserved specimens in natural history collections (Hykin et al. 2015). While successes have been found across time periods, taxa, and preservation methods (O'Connell et al. 2021; Straube et al. 2021), various unknown factors still inhibit broad-scale extraction of genomic data and yield idiopathic failures (Ruane and Austin 2017; McGuire et al. 2018). Nevertheless, methods continue to improve and future laboratory protocols may offer significant advancements in the recovery of mitochondrial and nuclear data from a wide variety of specimens (Hahn et al. 2021). This offers significant potential for understanding not only historical biodiversity patterns, but also present-day conservation concerns.

Conclusion

Named taxa that have gone extinct prior to modern genetic analysis (here termed Alexandrian extinctions) represent a particularly crucial segment of focus for conservation genomics. If they are indeed valid taxa, then their inclusion in phylogenetic and population-genetic analysis is necessary to inform our knowledge of the spatial and trait-based drivers of extinction. If they are not valid, they may still reshape our understanding of the geographic context of declines and offer insight for the conservation management of remaining populations of extant conspecifics. We demonstrate this with the example of *Desmognathus fuscus carri*, a putative synonym of *D. "auriculatus"* from peninsular Florida which has apparently gone extinct, while a few remaining populations of *D. auriculatus* A persist in northern Florida and southern Georgia. Formalin-fixed sequencing of two historical specimens from the type locality of *D. f. carri* yielded sufficient mitochondrial and genomic data to confirm synonymy with *D. auriculatus* A. These data also suggest that these peninsular populations may have formed a distinct genetic population segment. Thus, the enigmatic decline of *D. auriculatus* A is now known to be geographically unified across the peninsular and continental populations, but the as-yet unknown causes acted across a wide cross-section of genetic diversity. Advances in sequencing technology hold the promise of unlocking further genomic resources from historical museum collections, potentially offering further resolution of similar Alexandrian extinctions in the future.

Declarations

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Conflicts of interest/Competing interests None

Availability of data and material SRA/Genbank accessions given in text

Code availability N/A

Authors' contributions RAP, DAB, and KAO conceived the study, AHW and KAO performed lab work, CRH performed fieldwork and contributed background literature, EML and ARL performed sequencing and bioinformatics, all authors contributed to writing.

Ethics approval GW IACUC A426

Consent to participate N/A

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Tables

Table 1. Site localities for extant and historical (extirpated) populations of *Desmognathus auriculatus* A from (Dodd 1998; Means et al. 2017; Beamer and Lamb 2020) and *D. fuscus carri* (Neill 1951). Historical lat/long are approximate.

Specimen	Taxon	Locality	Accession	Lat	Long	Site
DAB348	*D. auriculatus* A	GA: Bryan, Otter Hole Branch	MH403590	32.08	-81.66	Modern
DAB349	*D. auriculatus* A	FL: Baker, Olustee Creek	MH403587	30.19	-82.43	Modern
DAB1385	*D. auriculatus* A	GA: Clinch, Suwannoochee Creek	MH403586	31.04	-82.88	Modern
JYL269	*D. auriculatus* A	FL: Wakulla, Bradwell Bay	KY658977	30.14	-84.57	Modern
JYL270	*D. auriculatus* A	FL: Wakulla, Bradwell Bay	KY658978	30.14	-84.57	Modern
DAB1391	*D. auriculatus* A	GA: Bryan, Otter Hole Branch	MH403588	32.08	-81.66	Modern
DAB2580	*D. auriculatus* A	GA: Wayne, Little Penholoway Creek	MH403589	31.50	-81.91	Modern
RAP0596	*D. auriculatus* A	FL: Wakulla, Bradwell Bay	MZ491222	30.14	-84.61	Modern
BTL239	*D. auriculatus* A	FL: Wakulla, Monkey Creek	KR732333 KX764610	30.16	-84.52	Modern
See Dodd, 1998	*D. auriculatus* A	FL: Alachua, Devil's Millhopper	-	29.71	-82.39	Historical
USNM 468094	*D. fuscus carri*	FL: Lake/Marion, Silver Glen Springs	MZ491223	29.25	-81.64	Type
FLMNH 2962	*D. fuscus carri*	FL: Polk, 6 miles south of Lakeland	-	27.95	-81.98	Paratype
FLMNH 3063	*D. fuscus carri*	FL: Hillsborough, Lithia Woods Creek, near Lithia Springs	-	27.87	-82.23	Paratype
ERA-WTN14189	*D. fuscus carri*	FL: Lake, Alexander Springs	-	29.08	-81.58	Paratype
ERA-WTN14191	*D. fuscus carri*	FL: Marion, Juniper Springs	-	29.18	-81.71	Paratype
See Neill,	*D. fuscus*	FL: Marion, Rainbow	-	29.10	-82.44	Historical

1951	*carri*	Springs				
See Neill, 1951	*D. fuscus carri*	FL: Marion, Half-mile Creek	-	29.22	-82.05	Historical
See Neill, 1951	*D. fuscus carri*	FL: Marion/Putnam, Orange Springs and Orange Creek	-	29.50	-81.95	Historical
See Neill, 1951	*D. fuscus carri*	FL: Putnam, Ravine Gardens at Palatka	-	29.64	-81.65	Historical
See Neill, 1951	*D. fuscus carri*	FL: Levy, Fannin (Fanning) Springs	-	29.59	-82.93	Historical
See Neill, 1951	*D. fuscus carri*	FL: Orange, Rock Springs run	-	28.80	-81.45	Historical
See Neill, 1951	*D. fuscus carri*	FL: Orange, Wekiwa Springs	-	28.73	-81.48	Historical

Figures

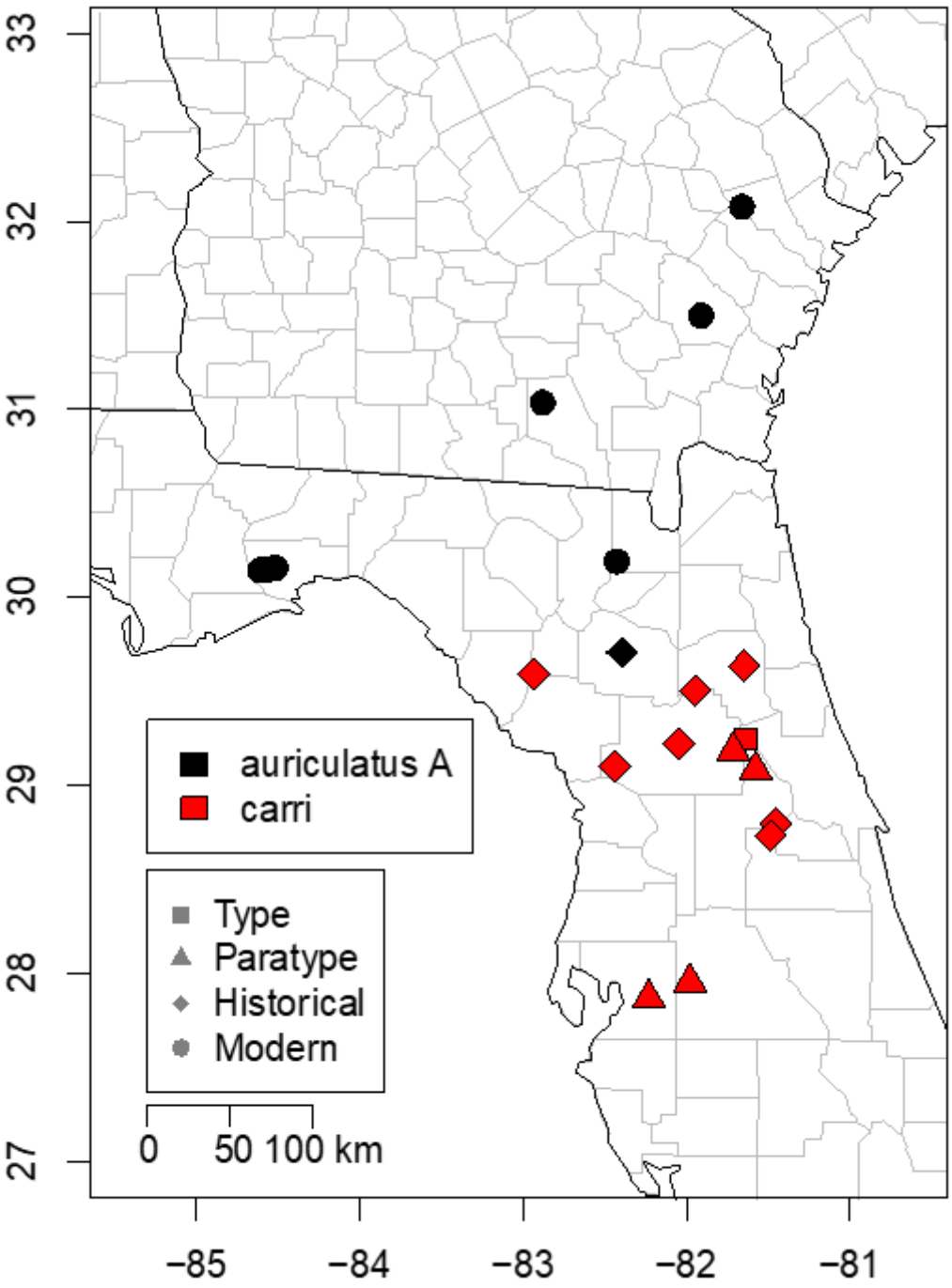

Figure 1

Map of recent extant populations of Desmognathus auriculatus A plotted as black circles (Means et al. 2017; Beamer and Lamb 2020); the notably extinct peninsular population of D. auriculatus A at Devil's Millhopper in Alachua Co., Florida, as a black diamond (Dodd 1998); and the sampled type locality (red square), 4 paratype localities (red triangles), and 7 referred populations (red diamonds) of D. fuscus carri from Neill (1951). Data in Table 1.

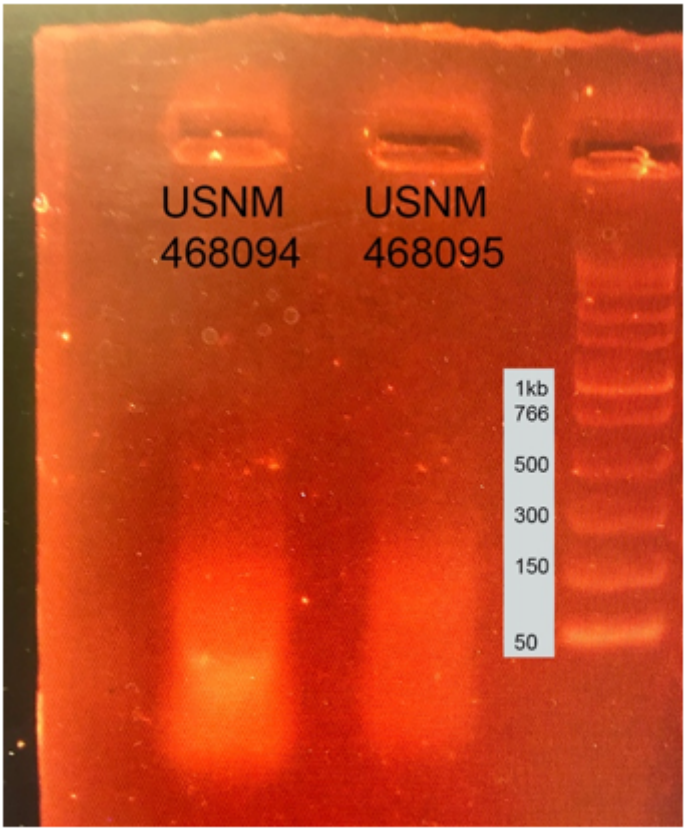

Figure 2

Gel image for FFS extraction of USNM 468094¬–5, showing extensive degradation (shearing) of genomic DNA. Most fragments are <50bp, but faint smears can be seen from ~150–500bp in both lanes. In general, successful AHE loci were represented only by single 150-300bp fragments that were covered by one read-pairs, though these were often captured to high depth, with coverage up to 2,433x.

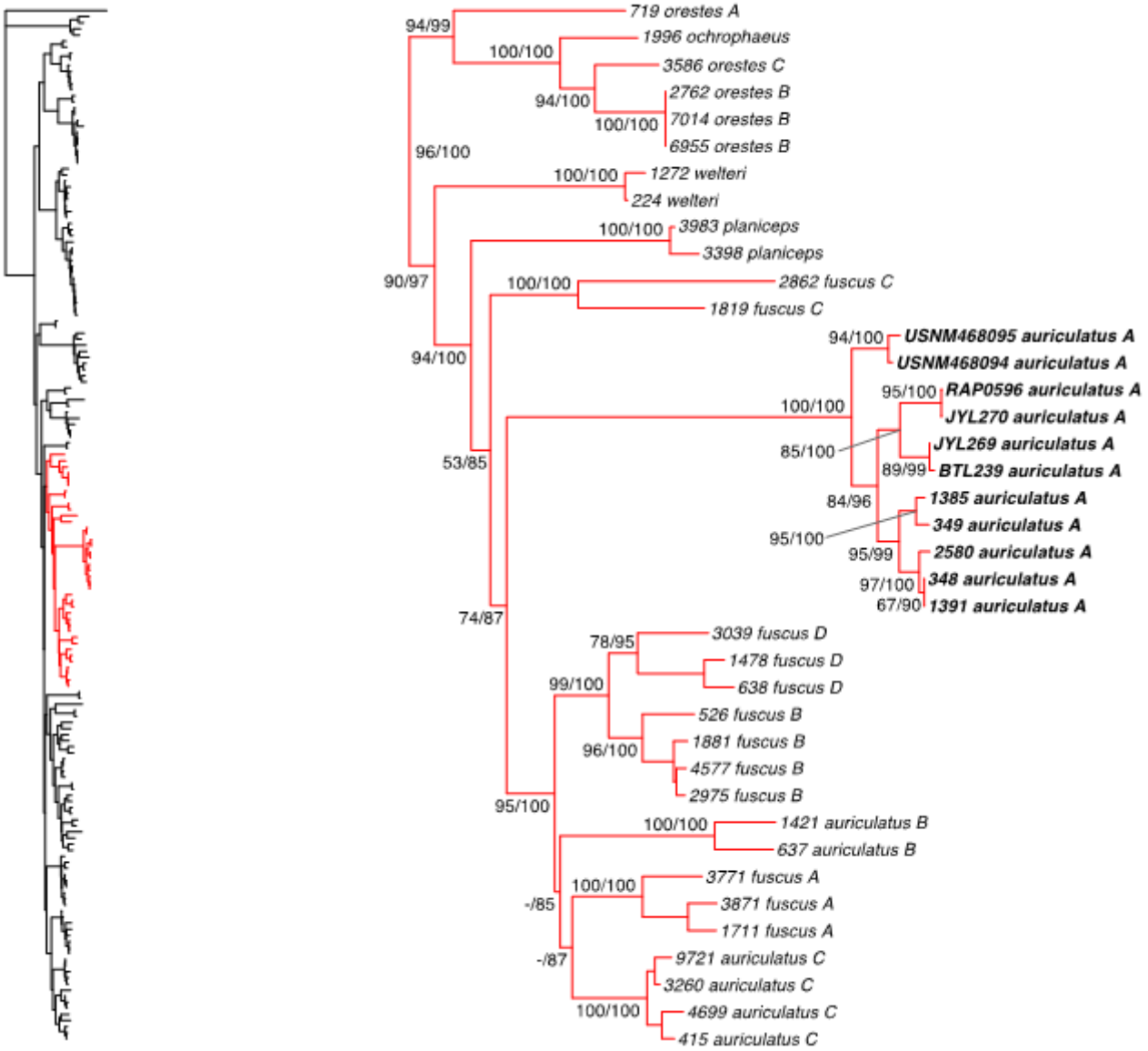

Figure 3

Concatenated ML phylogeny of the ND2/tRNA/COI fragment (1,993bp) for 169 individuals, highlighting the clade containing Desmognathus auriculatus A and the two fluid-preserved USNM samples. Specimen numbers without codes are ‘DAB’ (David A. Beamer field series). Support values >50 are shown at or above the clade level (except for D. auriculatus A); UFBoot >95 (left) and SHL >80 (right) are “strong.”

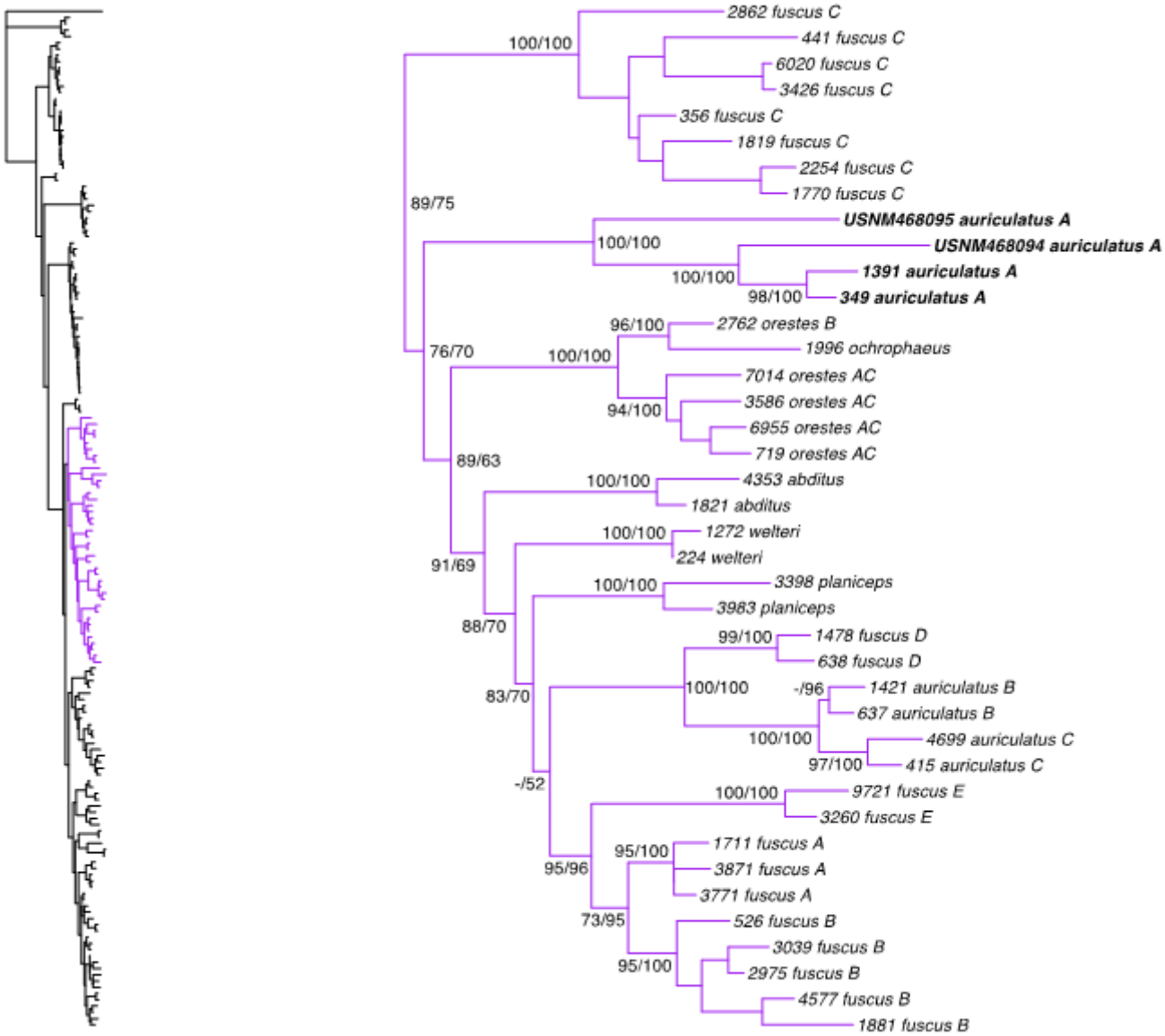

Figure 4

Concatenated ML phylogeny of 73 AHE locus fragments (7,650bp) for 163 individuals, highlighting the clade containing the two fluid-preserved USNM specimens. All other specimen numbers are ‘DAB’ (David A. Beamer field series). Support values >50 are shown at or above the clade level (except for Desmognathus auriculatus A); UFBoot >95 (left) and SHL >80 (right) are “strong.”