Conservation genetics of two critically imperiled freshwater mussels

by

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Abstract

North America is a hotspot of freshwater mussel diversity. However, over the last century many anthropogenic stressors have led to substantial declines of freshwater mussels of the families Unionidae and Margaritiferidae in North America. Conserving the remaining mussel fauna is a priority, as they play an integral role in freshwater ecosystems. Genetic information, particularly genomic technologies, can provide crucial information and guide conservation strategies. In chapter 1, I use genomic data to explore the genetic diversity and demographic history of a federally endangered freshwater mussel species, *Arcidens wheeleri* (Unionidae: Anodontini). In chapter 2, I generated population genomic data from *Alasmidonta varicosa,* an imperiled freshwater mussel, from across its range and from captively reared cohorts. No population genetic study has been conducted on either, and these two chapters can both be used to inform and improve conservation efforts for freshwater mussels.

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Chapter 1: Population Genomics of the Endangered Freshwater Mussel, *Arcidens wheeleri* (Unionoidea: Unionidae: Anodontini), in the Little River, Arkansas, USA

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INTRODUCTION

Surface freshwater habitats make up only 0.1% of the world's water, yet freshwater ecosystems support *c*. 6%, or 1.8 million, of all described species (Dudgeon *et al.*, 2006). Freshwater habitats have relatively high species richness and remarkable diversity given their small range on Earth (Román-Palacios, Moraga-López & Wiens, 2022). Anthropogenic stressors such as habitat degradation, pollution, flow regulation, water extraction, fisheries exploitation, and the introduction of invasive species increasingly imperil freshwater ecosystems and the species they hold (Strayer *et al.*, 2010). Furthermore, declines in biodiversity are far greater in freshwater ecosystems than in the most distressed terrestrial ecosystems (Reid *et al.*, 2019). Making matters worse, freshwater ecosystems are understudied and rates of aquatic biodiversity loss are currently lacking in many regions (Sala *et al.*, 2000), meaning the biodiversity crisis in freshwater ecosystems may be even worse than currently understood.

The southeastern United States (U.S.) is a global freshwater biodiversity hotspot that is becoming increasingly imperiled as a result of anthropogenic stressors (Lydeard & Mayden 1995; Johnson *et al.*, 2013; Elkins *et al.,* 2019). The U.S. is home to the richest freshwater mussel fauna in the world with nearly 300 species in 55 genera in the families Margaritiferidae and Unionidae (Graf & Cummings, 2021). Both the southeastern U.S. and the Interior Highlands

of Arkansas, Missouri, Kansas and Oklahoma are freshwater mussel hotspots (Harris *et al.,* 2009; Haag, 2010). As in other freshwater ecoregions, numerous mussel species from the Interior Highlands are imperiled. There are currently over 80 native freshwater mussels that occur in Arkansas, and the Arkansas Wildlife Action Plan (2015) lists 45 native freshwater mussels as state species of greatest conservation need. Of these, 18 are federally threatened, endangered, or proposed for listing under the Endangered Species Act (Hopper *et al.* 2023).

The freshwater mussel *Arcidens wheeleri* (Ortmann & Walker, 1912), or Ouachita Rock Pocketbook, is one such mussel that is listed as federally endangered (Fig. 1, Supplementary Material Figs S1, S2). In the Arkansas Wildlife Action Plan "Calculated Priority Scores for Mussel Species" section, *A. wheeleri* has the third highest priority ranking (Arkansas Wildlife Action Plan, 2015). Ortmann & Walker (1912) described *A. wheeleri* from "Old River, Arkadelphia, Arkansas", which is an oxbow lake of the Ouachita River (Posey, Harris & Harp, 1996). Observations of the species in the Ouachita River have been rare (USFWS, 2004), and the species is likely extirpated from its type locality (Posey *et al*. 1996; Harris *et al*. 1997; USFWS 2004). The two remaining populations are found in the Kiamichi River in Oklahoma and the Little River in Oklahoma and Arkansas (USFWS, 2004). Past reports indicated that the Kiamichi River population was recently the most abundant population, with population size estimates of less than 1,800 individuals (USFWS, 2004). However, Galbraith, Spooner & Vaughn (2005) and Galbraith, Spooner & Vaughn (2008) found no evidence of *A. wheeleri* at any of the ten sites they surveyed in the Kiamichi River, including six sites that were known to hold *A. wheeleri* in the 1990s. Both studies concluded that *A. wheeleri* had declined dramatically over a 10-year period in the Kiamichi River. The only location with recent reports of *A. wheeleri* gravid females is the Little River in Arkansas (Tackett *et al.*, 2022).

The recent decline of *A. wheeleri* has been attributed to impoundment, channelization and water quality degradation (Tackett *et al.*, 2022). In the Little River in Arkansas, the completion of Millwood Dam in 1966 resulted in loss and degradation of *A. wheeleri* habitat (USFWS, 2004). Historic collections in the Little River include White Cliffs, Little River County, and the border of Little River and Sevier Counties (Beacham *et al.*, 2001). In 1987, the range of *A. wheeleri* in the Little River was reported to be as small as an 8 km stretch in Sevier and Little River Counties, Arkansas (Ecosearch, 1987). Davidson (2017) reported that *A. wheeleri* occurred at only 4 of 15 locations sampled in the Little River in Arkansas, and only six *A. wheeleri* were collected despite sampling over 10,000 mussels from 360 quadrats at these sites. Thus, conservation efforts are essential to prevent future declines of *A. wheeleri* in the Little River.

Originally placed in the monotypic genus *Arkansia* (Ortman & B. Walker, 1912) when first described (Ortmann & Walker, 1912), molecular phylogenetic analyses determined that *A. wheeleri* is sister to *Arcidens confragosus* (Say, 1829), which resulted in synonymization of *Arkansia* with *Arcidens* (Inoue *et al.,* 2014). *Arcidens wheeleri* is medium sized compared to other unionids, with a maximum documented shell width of 60 mm and length of 112 mm (USFWS, 2004). The shell exterior can range in shades from chestnut brown to black and often has a silky texture (Beacham *et al.*, 2001; Fig. 1, Supplementary Material Figures S1, S2). The habitat of *A. wheeleri* is often characterized by pools, backwaters and side channels of rivers and large creeks that have sluggish currents with gravel or sandy substrates (Tackett *et al.*, 2022), but in the Little River in Arkansas it is often found in main channel mussel beds associated with gravel/cobble bars (Davidson, 2017; K. Moles, unpub.). *Arcidens wheeleri* typically occurs within large mussel beds containing a diverse group of other mussel species (USFWS, 2004). That is, as many as 11 to 24 other mussel species have been documented in the same bed as *A.*

wheeleri (Beacham *et al.*, 2001; Davidson, 2017), most often with *Quadrula quadrula* (Rafinesque, 1820), *Megalonaias nervosa* (Rafinesque, 1820), and *Ellipsaria lineolata* (Rafinesque, 1820) (USFWS, 2004). Life history data are almost entirely lacking for *A. wheeleri*, and available genetic data are limited to two mitochondrial genes and one nuclear gene that have limited utility for understanding population genetics and historical demography for *A. wheeleri* (Inoue *et al.,* 2014). A better understanding of *A. wheeleri* is needed so management efforts can be informed by species-specific data, rather than broad, and possibly inapplicable, generalizations that are made from studies on other mussels.

In the face of limited information and inherent difficulties associated with long-term field studies of critically endangered species like *A. wheeleri*, genomic tools are well-suited to provide valuable information about *A. wheeleri* biology and its historical demography (Hohenlohe, Funk & Rajora, 2021). Furthermore, genomic data are critical for informing potential captive propagation efforts and ensuring that reintroduction efforts are effective (Strayer *et al.*, 2019; Geist *et al.*, 2021). For example, genomic data recently revealed unexpected levels of genetic diversity within *Epioblasma brevidens* (Lea, 1831) in the lower Tennessee River drainage, resulting in a new perspective on what populations would be most ideal as broodstock for captive propagation (Gladstone *et al.*, 2022). Recent genomics research has been also used to guide the propagation program for *Margaritifera hembeli* (Conrad, 1838) (Garrison, Johnson & Whelan, 2021; Sikes, 2020). To date, most conservation genetics research on mussels has used a limited number of microsatellites or mitochondrial genes (Geist & Kuehn, 2005; Paterson *et al.*, 2015), but genome-scale analyses can provide better resolution for inferring population structure, facilitating more accurate effective population size estimates, and allow for fine-scale demographic modeling (Garrison *et al.*, 2021; Hohenlohe *et al.*, 2021; Gladstone *et al.*, 2022;).

Using genome-scale data, we assessed the genetic diversity and historical demography of *A. wheeleri* in the Little River of central Arkansas. We focused on the Little River in Arkansas because during the planning stages of this study, initial communication with biologists at the Oklahoma Department of Wildlife Conservation casted doubt on our ability to sample even a few individuals from the Kiamichi River given recent population declines (C. Tackett, personal communication; Galbraith *et al.*,2005, 2008). A follow-up conversation with the USFWS species recovery lead for *A. wheeleri* also indicated that sampling in the Kiamichi River for population genomics would be impractical and inadvisable, given the amount of effort that would be required (D. Martinez, personal communication). We hypothesized that a single genetic population of *A. wheeleri* persists in the Little River and that the population has undergone a severe demographic decline. We used a single enzyme restriction-site associated DNA-sequencing (RADseq) approach to generate a genome-scale dataset of thousands of single nucleotide polymorphisms (SNPs). Data generated here will enhance the understanding of *A. wheeleri* and guide recovery efforts.

MATERIAL AND METHODS

Sample collection

We sampled 12 individuals from three sites in the Little River in Arkansas (Fig. 2). One individual was collected from the upstream most site, *c.* 19 km from two individuals collected at the middle collection site (both sites are upstream of Millwood reservoir). Nine individuals were collected downstream of Millwood Reservoir, *c.* 54 km away from the middle site. Although we

only sampled 12 individuals, this represented a considerable sampling effort of over 220 man hours, and additional sampling was not practical. Furthermore, past studies have indicated that population genetic statistics like heterozygosity, and even accurate demographic modeling, can be determined from sample sizes similar, or lower, to those used here when thousands of loci are available (e.g. Willing *et al.* 2012; Nazarenzo *et al.* 2017; Nunziata *et al.*, 2018).

Samples were collected by hand and genetic material was obtained non-lethally by rubbing a sterile buccal swab (Isohelix) on the foot. Swabs were immediately placed in a stabilization buffer (Isohelix). Mussels were immediately returned to the substrate where they were collected from after swabbing.

Genomic data generation and assembly

DNA was extracted with the Isohelix Xtreme DNA isolation kit. Each extraction was quantified with a Qubit Fluorometer and visualized with a 1% agarose gel and electrophoresis to ensure integrity of high molecular weight DNA. Samples were normalized to a concentration of 20 ng/μL and sent to Floragenex for single enzyme RADseq using the *PstI* restriction enzyme following Baird *et al.* (2008). Size selection during library preparation ranged from 300 to 500 base pairs (bp). Samples were tagged with unique barcodes, pooled together and combined with 84 samples from other studies, and sequenced on three Illumina Hiseq 4000 lanes using 150 bp paired-end chemistry.

Reads from all three sequencing lanes were combined for each individual and processed with the STACKS v. 2.3 pipeline for population genomic analyses (Rochette, Rivera-colón & Catchen, 2019). Paired-end reads were demultiplexed with the command process_radtags,

allowing for one mismatched nucleotide per barcode. After demultiplexing, RADseq data were assembled with the STACKS pipeline denovo_map. Assembly parameters were selected following Paris, Stevens & Catchen (2017), resulting in a minimum stack depth (i.e. coverage) of 5, the distance between stacks set to 1, and the distance allowed between catalog loci set to 1.

The STACKS assembly was filtered with the STACKS module populations to create final datasets. For the dataset used for most analyses, herein referred to as the "primary dataset", the following filtering parameters were used: -r set to 0.75, --min-maf set to 0.025 and --max-obs-het set to 0.5. A second dataset for analyses that assumed SNPs are unlinked was filtered like the primary dataset, except only one SNP per locus was retained. For the dataset used for effective population size estimation via the linkage disequilibrium method, we filtered the same as the single SNP per locus dataset, except we removed all SNPs outside of Hardy–Weinberg equilibrium, as calculated by *populations* ($P < 0.05$) and required each SNP to be present in all individuals. The dataset for demographic modeling was filtered similarly to the primary dataset but requiring to be present for a locus to pass filtering, retaining only a single SNP per locus, and having a minimum minor allele count of 1 because modeling with the allele frequency spectrum benefits from rare alleles being present in the dataset.

Population genomic analyses

Observed heterozygosity, expected heterozygosity, nucleotide diversity and inbreeding coefficient (F_{IS}) were calculated in populations. We used AdmixPipe 3 (Mussmann *et al.*, 2020) to convert files for use in ADMIXTURE (Alexander *et al.*, 2009) and to automate testing best-fit number of genetics clusters (K) with 20% cross validation. We also tested the bestfit number of genetic clusters with the R (R Core Team, 2023) package adegenet (Jombart T, 2008) using the

command find.clusters, as a part of DAPC analysis. The R package PopGenReport (Adamack & Gruber, 2014) was used to quantify rarefied allelic richness. To investigate co-ancestry levels among samples, we conducted analyses with fineRADstructure v. 0.3.2 (Malinsky *et al.*, 2018). We examined fine-scale genetic diversity spread between individuals through principal component analysis (PCA) using the factoextra Visualization Principal Component Analysis R package (Kassambara & Mundt, 2020). All analyses used the multiple SNPs per locus dataset, except ADMIXTURE.

Effective population size (N_e) was calculated using the linkage disequilibrium method (Waples & Do, 2008), as implemented in NeEstimator V2.1 (Do *et al.*, 2014). A critical value of 0.05 was used for N_e calculations. 95% Confidence intervals were measured via the parametric (Waples, 2006) and jackknife approaches (Jones, Ovenden & Wang, 2016) implemented in NeEstimator.

Demographic history for *A. wheeleri* was inferred using δaδi v. 2.0.5 (Gutenkunst *et al.*, 2009). δaδi estimates demographic parameters for simple to complex scenarios using the allele frequency spectrum and a diffusion-based approach (Gutenkunst *et al.*, 2009). The dataset created for demographic modeling was converted to a folded allele frequency spectrum using the python script vcf2dadi.py (https://github.com/CoBiG2/RAD_Tools.git) with an easySFS (https://github.com/isaacovercast/easySFS) projection of 12 individuals. Five single population demographic scenarios were modeled in δaδi: (1) standard neutral model, which maintains a constant population size; (2) growth model, a population size change via continuous exponential growth or decline; (3) two-epoch model, which describes an instantaneous population increase or decrease; (4) bottleneck-growth model, an instantaneous increase or decrease followed by exponential growth or decline; and (5) three-epoch model, which includes two instantaneous

population changes. One hundred replicates were performed for each model and the best-fit model was identified using the Akaike's information criterion (AIC; Akaike, 1974) and Bayesian information criteria (Schwarz, 1978), which were calculated in R. Nonparametric bootstrapping was used to estimate 95% confidence intervals (95% CI) for each best-supported model parameter using 100 bootstrapped datasets.

To convert δaδi output parameters into timing and population size estimates, mutation rates and generation times are required. In the absence of specific information for *A. wheeleri*, we used a mutation rate of 5.0×10^{-9} per site/generation, which was calculated by Pogson & Zouros (1994) for the bivalve species, *Placopecten magellanicus* (Gmelin, 1791). This mutation rate was also used by Rogers *et al.* (2021) for genomic analyses on another unionid species, *Megalonaias nervosa* (Rafinesque, 1820), so we think it is an adequate estimate for *A. wheeleri*. The exact generation time of *A. wheeleri* is also unknown, but other unionid species of *Anodonta* Lamarck, 1799; *Pyganodon* Fischer & Crosse, 1894; and *Utterbackia* Baker, 1927 have a generation time of 3–5 years (Heard, 1975), so we used a generation time of 3 years for calculating timing of demographic events. We estimated the reference population size (N_{ref}) using the following equation: $\boxed{Nref = \frac{\theta}{4 \times \mu \times BP}}$ (formula from the ∂a∂I manual; Gutenkunst *et al.*, 2009). Theta was estimated by ∂a∂i, μ was the mutation rate, and 4,062,424 bp was used, which was the total number of sites across assembled loci in the ∂a∂i dataset. Population size estimates before and after demographic events of each model were calculated by multiplying the estimated ∂a∂i parameter ratio for each population size by N_{ref}. Timing of demographic events for each of the five models was calculated using ∂a∂i parameter estimates, multiplied by two times the calculated reference population size, and then multiplied by a generation time of 3 years (formula from the ∂a∂I manual; Gutenkunst *et al.*, 2009).

RESULTS

The 12 sequenced individuals had an average of 9,386,329 raw paired-end reads (range from 2,647,975–15,731,380 reads). Filtering implemented in populations resulted in 59,155 loci for the primary one SNP per locus dataset and 159,102 SNPs in primary dataset with multiple SNPs per locus. The final dataset for calculating N_e had 2,730 loci and SNPs. The δaδi dataset had 7,579 loci with 6,079 SNPs.

Analyses with ADMIXTURE and adegenet indicated that only one genetic cluster, or population, was present in the dataset, so DAPC and ADMIXTURE plots are not reported because both are uninformative at K = 1. Observed heterozygosity was considerably lower than expected heterozygosity, with $H_0 = 0.14$ and $H_e = 0.22$. Average nucleotide diversity (π) was 0.24. The average inbreeding coefficient (F_{is}) was 0.33. Rarefied allelic richness was 1.88 (Table 1).

NeEstimator reported an effective population size of 32.4 individuals (parametric 95% CI 30.8 - 34.1; jackknife 95% CI: 7.9 - infinite). We note that the estimation of "infinite" with the jackknife CI should not be inferred as a potentially large contemporary N_e, but rather a sampling error (see Marandel *et al.*, 2018). Given the rarity of *Arcidens wheeleri* in the Little River, sampling additional individuals for a more precise confidence intervals of N_e was not possible. The rarity of *A. wheeleri* also corroborates the low N_e point estimate.

As with DAPC and ADMIXTURE, no sub-population structure was inferred with fineRADstructure . The fineRADstructure results showed extremely high co-ancestry values, indicating that all sampled *A. wheeleri* individuals are closely related (Fig. 3). However, PCA

showed genetic diversity spread among the three collection sites, indicating some, albeit limited, genetic variation and subpopulation structure across the landscape (Fig. 4).

According to AIC and BIC, the best-fitting ∂a∂i demographic model was the three-epoch model (Fig. 5A, Tables 2, 3), with the bottleneck-growth model being the second bestfit model (Fig. 5B, Tables 2, 3). The three-epoch model suggested that *A. wheeleri* in the Little River went through an initial large expansion *c.* 47,000 years ago, followed by a period of constant population size and then a drastic population bottleneck *c.* 1,100 years ago (Fig. 5A, Table 4). The second bestfit model was the bottleneck-growth model which also suggests a large instantaneous population expansion during the Late Pleistocene followed by a continuous exponential decline, resulting in a population decrease of 99% and a contemporary effective population size of *c.* 1,200 individuals (Fig. 4B, Table 4). The remaining demographic models were poorly supported (ΔAIC and ΔBIC ≥ 5; Table 2), indicating that *A. wheeleri* in the Little River has neither maintained its population size over time or undergone population growth since the Pleistocene.

DISCUSSION

Population genomic analyses of *Arcidens wheeleri* from the Little River confirm survey-based observations that the species has undergone severe decline in the Little River. The decline appears exacerbated by high inbreeding levels and a low effective population size that will likely result in future loss of genetic diversity without conservation interventions (Table 1). Demographic analyses inferred two well-supported scenarios for *A. wheeleri* in the Little River. The first is an instantaneous expansion during the Late Pleistocene followed by a steady population size for thousands of years and then a severe population reduction. The exact cause of

the inferred instantaneous expansion is unclear, but inferred expansions from the reference, or ancestral, population size may be an artifact of the modelling process; similar expansions at the first demographic event are commonly seen with allele frequency spectrum demographic modeling (e.g. Noskova *et al.*, 2020; Xiong *et al.*, 2020; Gladstone *et al.*, 2022;). Nevertheless, the two bestfit models consistently infer population size decreases sometime in the Pleistocene. The worst-fitting models are those with a constant population size and population expansion (Table 2), further indicating that population genomic data are indicative of population decline even if the exact pattern of decline cannot be teased apart at this time. Population declines may have resulted from cyclical periods of warming and cooling brought on by numerous glaciation events during the Late Pliocene and the Pleistocene (Richmond & Fullerton, 1986; Pielou, 1991). A population contraction that never recovered, combined with the relatively small range of *A. wheeleri*, likely resulted in a loss of evolutionary potential (Forester *et al.*, 2022). This could explain why *A. wheeleri* is rare than its congener and sympatric mussels.

Archeological data corroborate the best-fitting demographic models. For instance, archaeological excavations of Caddoan Mississippian mounds below the Little River and Red River confluence in Arkansas recovered numerous shell artifacts and fragments from several species of *Quadrula* Rafinesque, 1820; *Amblema* Rafinesque, 1820; and *Lampsilis* Rafinesque, 1820 dating from 800 to 1400 CE (Webb, 1959; Durham & Kizzia, 1964; McKinnon, 2013). These excavations did not recover any shells of *A. wheeleri*, despite the previously identified positive association with *Quadrula* and other common mussel species (USFWS, 2004). More recent survey records, as captured by museum records, also support rarity of *A. wheeleri* prior to major anthropogenic influences like the construction of Millwood dam. For example, according to Pfieffer, Dubose & Keogh (2024), among 45 natural history museums in the U.S., there are

only 49 georeferenced occurrences of *A. wheeleri*, of which only 24 were from before 1960. Comparisons to the number of museum records of its sister species *A. confragosus* (1,058) and sympatric species like *E. lineolata* (1,716) further corroborate demographic modeling and contemporary N_e calculations that portray *A. wheeleri* as a species that has experienced long-term rarity. Such long-term rarity, probably combined with low evolutionary potential, likely explains why *A. wheeleri* is one of the rarest mussel species west of the Mississippi River.

Genetic patterns seen in *A. wheeleri* provide empirical data that mussel declines inferred by survey work can result in a loss of genetic diversity and increased inbreeding. This contrasts with what genetics research revealed for *Epioblasma brevidens*, where one population was difficult to find during survey work in the last 15+ years but harboured relatively high amounts of genetic diversity (Gladstone *et al.*, 2022). Several other characteristics of genomic diversity in *A. wheeleri* from the Little River also deserve close inspection. Despite low heterozygosity, nucleotide diversity is higher than seen in several apparently more stable molluscs like the mussel *E. brevidens* (Gladstone *et al.,* 2022) and several freshwater gastropods (Whelan *et al.*, 2019; Redak *et al.*, 2021). Heterozygosity values are lost faster than nucleotide diversity values over time, and the *A. wheeleri* summary statistics likely explain a relatively recent and drastic bottleneck that is not picked up by demographic model;ing (Amos *et al.*, 2010) but can be inferred from high F_{IS} values and low N_e value inferred with the linkage disequilibrium method.

Previously documented habitat fragmentation across the range of *A. wheeleri* (Tackett *et al.*, 2022) seems particularly problematic since the only likely avenue for increased genetic diversity in *A. wheeleri* from the Little River is migration, given high levels of current inbreeding (Liu, Wu & Chen, 2019). Unfortunately, options for management of *A. wheeleri* that follow best practices appear limited (Patterson *et al.*, 2018). Many recovery plans for freshwater mussels

include translocation and/or propagation and reintroduction, with the goals of increasing the amount of genetic variation within a population or increasing redundancy by re-establishing the species at an extirpated location. However, without proper planning, such efforts can waste resources and cause more harm than good (Strayer, 2019). One significant concern is not being able to maintain genetic diversity or capture promising individuals to rear genetically diverse cohorts, which can reduce evolutionary fitness and increase inbreeding even further (Willoughby *et al*., 2017). With proper planning these concerns could be avoided. In the case of *A. wheeleri*, translocation from the Kiamichi River could potentially increase genetic diversity, but it could also have the unintended effect of swamping out natural alleles. However, *A. wheeleri* in the Kiamichi River is exceedingly rare (Galbraith *et al.*, 2005, 2008), and risks to the Kiamichi River population from removing any number of remaining individuals for translocation may not outweigh the benefits.

Ideally, genetic data should be generated for the Kiamichi River population to determine if the genetic background is so different that outbreeding depression could be an issue if translocation occurs. Outbreeding depression has not been empirically demonstrated in freshwater mussels, but it has posed a threat to population viability in some species of marine bivalves (Lannan, 1980; Gaffney *et al.*, 1993). Broadly, introducing freshwater mussel individuals with a different genomic background, particularly thousands of captively reared individuals placed at a location with a much smaller census size, could have harmful effects to natural genetic adaptations (Jones, Hallerman & Neves, 2006). Of course, captive propagation is also not possible at this time, as a suitable fish host is unknown. Past host fish trial work resulted in juveniles recovered from potential fish hosts surviving no more than 1 month (Barnhart, 2018). Attempts at *in vitro* metamorphosis have also been unsuccessful (Barnhart, 2018). If a

suitable host fish can be identified, the data generated here will be an important baseline for evaluating whether propagated cohorts maintain as much genetic diversity as seen in the Little River, which is the most likely source of gravid females that could be used for propagation (Tackett *et al.*, 2022).

We hope this study will spur additional research and increased active management of *A. wheeleri*. Understanding the genetic diversity of the Little River populations is important for conservation assessment and planning. The data are also critical as a baseline for future population monitoring and evaluation of conservation efforts. Future decreases in genetic diversity would be indicative of a species in free fall. *Arcidens wheeleri* in the Little River may not survive another bottleneck event given strong evidence of already high inbreeding and a loss of genetic diversity. We advocate for immediate habitat protection and restoration to stop the decline of *A. wheeleri*, which are the only current active management options that would follow best practices given the absence of captive propagation protocols. Increased host-fish studies and development of propagation protocols should be high priorities so captive reintroductions can be attempted while genetic diversity still exists within *A. wheeleri*.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY

Sequence data has been uploaded to NCBI SRA (PRJNA1106917). Processed datasets in various file formats and certain program input and output files are available on Figshare (DOI: 10.6084/m9.figshare.23808777). Code related to the execution of pipelines used are available online at Github (https://github.com/miacadcock/wheeleri-dataset).

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Table 1. Population summary statistics for *Arcidens wheeleri* including sample size (N), allelic richness (AR), observed heterozygosity (Ho), expected heterozygosity (He), nucleotide diversity (π), inbreeding coefficient (F_{is}) and effective population size inferred with the linkage disequilibrium method (N_e).

N	AR (SE)	Ho (SE)	He (SE)	π (SE)	Fis (SE)	Ne (parametric 95% CI)
12	1.88 (0.12)	0.1371 (0.0005)	0.2245 (0.0006)	0.2369 (0.0006)	0.3264 (0.0046)	32.4 (30-34.1)

Table 2. Results of the ∂a∂i demographic analysis including the highest log-likelihood values and model fit information.

Model	*k*	Max est log-likelihood	AIC	ΔAIC	AICw_i	BIC	ΔBIC	BICw_i
SNM	0	-714.81	3292.41	3175	0.000	3292.41	3173	0.000
Growth	2	-30.29	143.52	26	0.000	144.49	25	0.000
Two-epoch	2	-25.83	122.97	6	0.036	123.94	5	0.053
Bottleneck-growth	3	-24.45	118.62	1	0.319	120.07	1	0.366
Three-epoch	4	-23.71	117.21	0	0.645	119.15	0	0.581

Table 3. Demographic parameter estimates for the two best-fitting models inferred by ∂a∂i for *Arcidens wheeleri* (see Figure 5 for model schematics).

	Three-epoch model
nuB^1	65.16 (95% CI: 25.55-129.31)
nuF^2	0.66 (95% CI: 0.05-1.19)
TB^3	1.31 (95% CI: 1.13-1.56)
TF^4	0.033 (95% CI: 0.000-0.063)
θ^5	485.419

	Bottleneck-growth model
nuB^1	134.15 (95% CI: 115.72-168.29)
nuF^2	5.20 (95% CI: 4.61-5.89)
T^6	1.50 (95% CI: 1.26-1.79)
θ^5	461.439

[1] Ratio of the population size at second demographic event in three-epoch model and first demographic event in bottleneck-growth model to $N_{ref.}$
[2] Ratio of the contemporary population size to $N_{ref.}$
[3] Length of time between first and second demographic event, units = $2 \times N_{ref} \times$ GenerationTime.
[4] Length of time from present to first demographic event, units = $2 \times N_{ref} \times$ GenerationTime.
[5] $\theta = 4 \times N_{ref} \times$ MutationRate $\times$ NumberOfSites
[6] Length of time from present to first demographic event, units = $2 \times N_{ref} \times$ GenerationTime.

Table 4. Demographic population sizes and timing estimates for the two best-fitting models inferred by ∂a∂i for *Arcidens wheeleri* (see Figure 5 for model schematics).

Three-epoch model	
Reference population size	5,974
Population size at demographic event 2	389,298 (95% CI: 152,635-772,498)
Contemporary population size	3,913 (95% CI: 299-7,109)
Timing of demographic event 2 (years)	46,816 (95% CI: 40,503-55,917)
Timing of demographic event 1 (years)	1,183 (95% CI: 0-2,259)
Bottleneck-growth model	
Reference population size	5,679
Population size at demographic event 1	761,891 (95% CI: 657,174-955,719)
Contemporary population size	29,555 (95% CI: 26,180-33,449)
Timing of demographic event 1 (years)	50,978 (95% CI: 42,933-60,992)

Fig. 1. Photograph of *Arcidens wheeleri* specimen found in the Little River, AR. Photo credit: K. Moles.

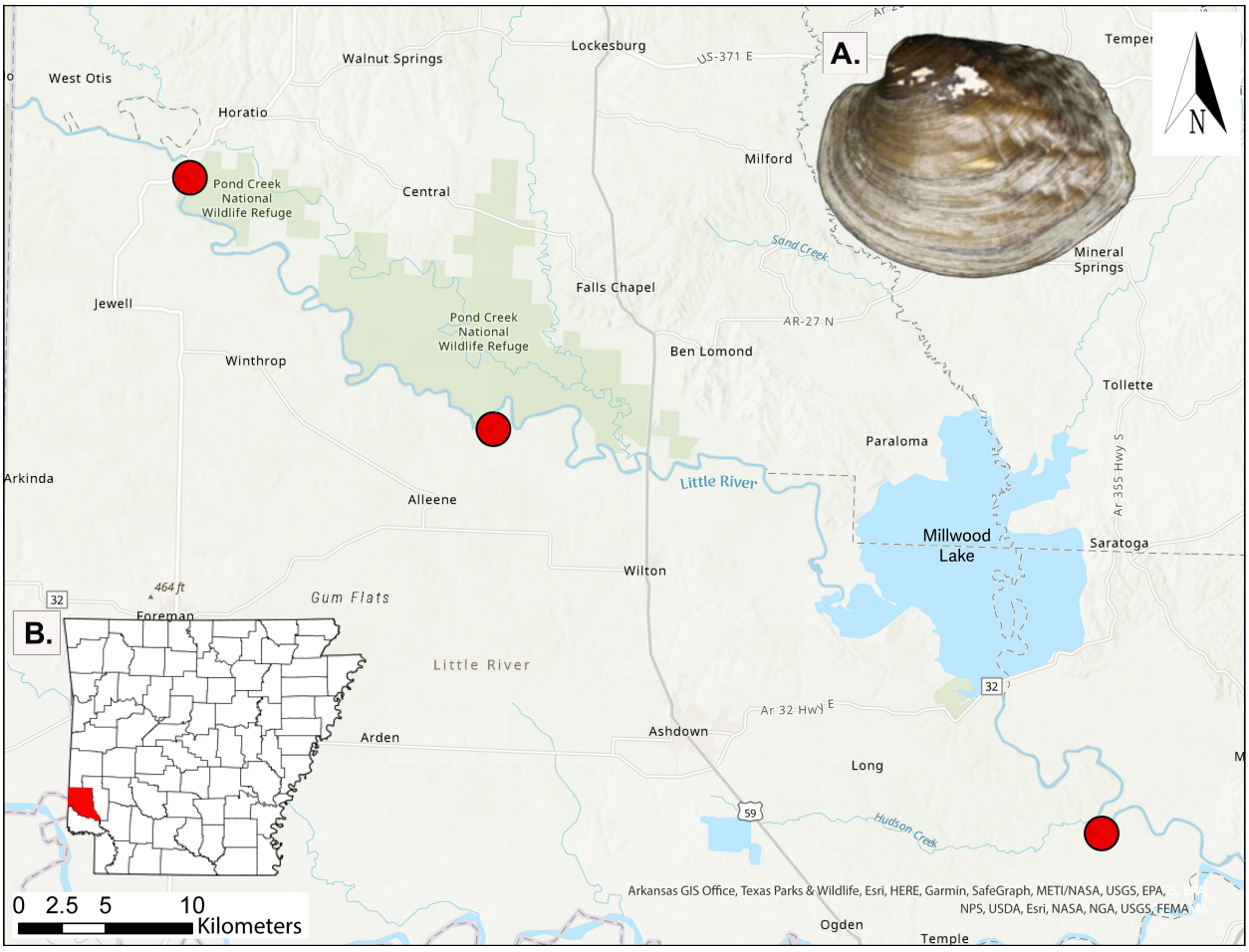

Fig. 2. Map of sample sites. **A.** *Arcidens wheeleri*. Photo credit: The University of Michigan Museum of Zoology, Division of Mollusks. **B.** Arkansas with highlighted county indicating where samples were collected.

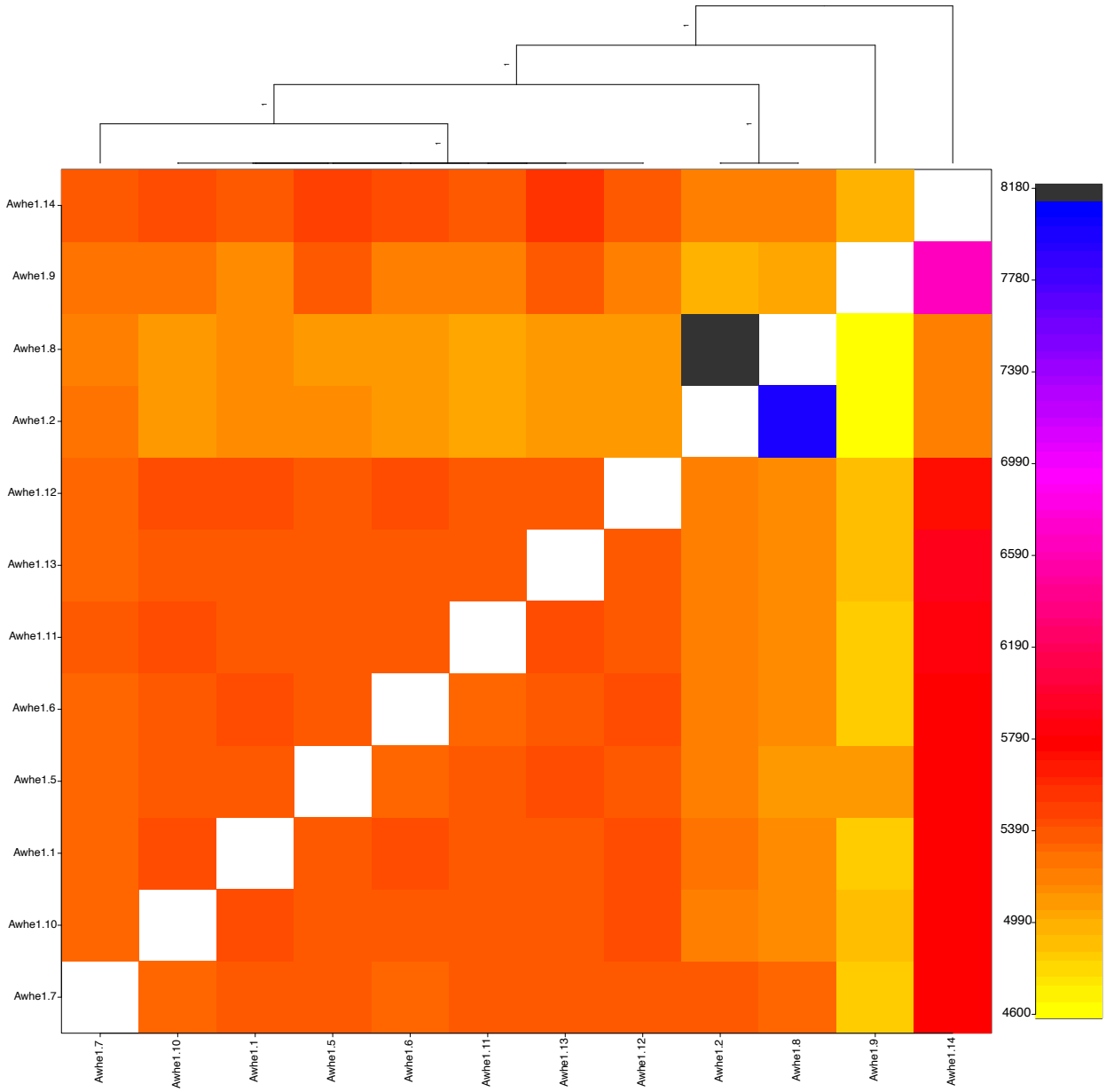

Fig. 3. Plot of co-ancestry values inferred by fineRADstructure and a simple tree displaying relationships among individuals.

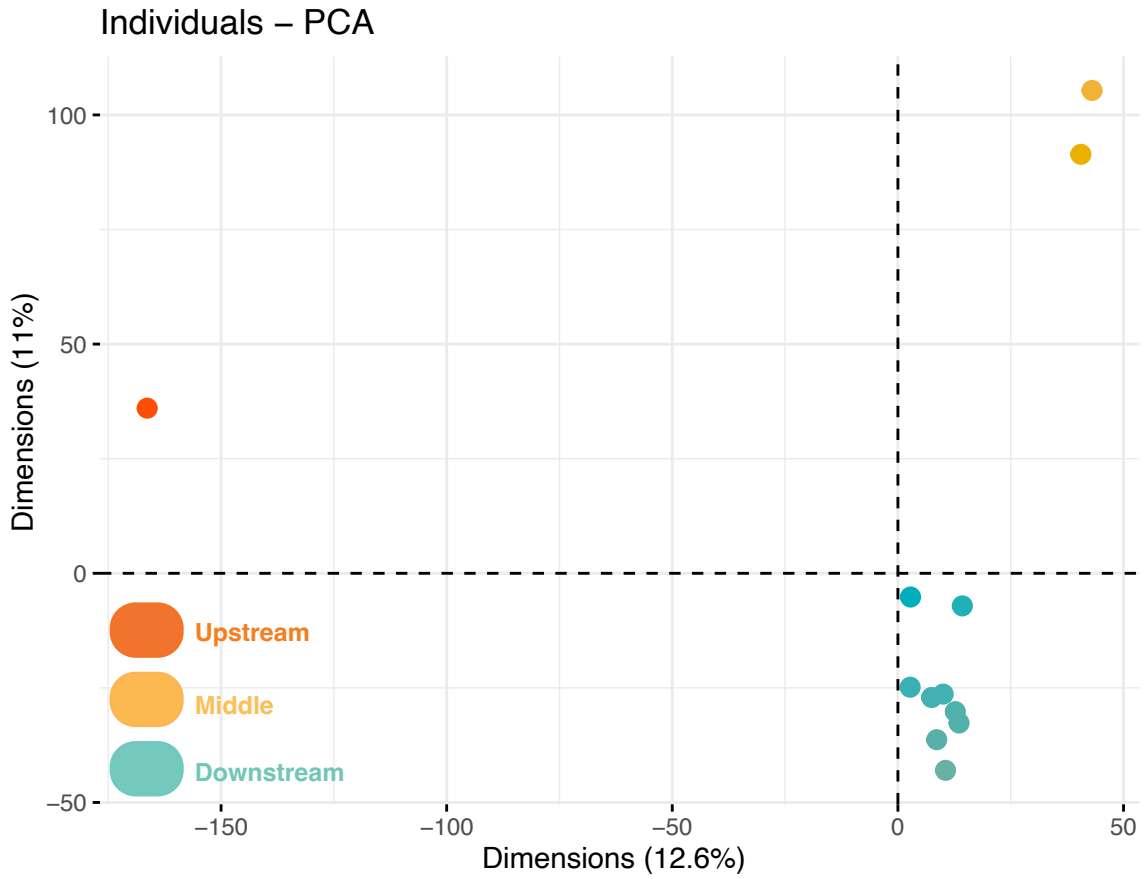

Fig. 4. Results of PCA, showing the genetic spread of individuals coloured by collection site.

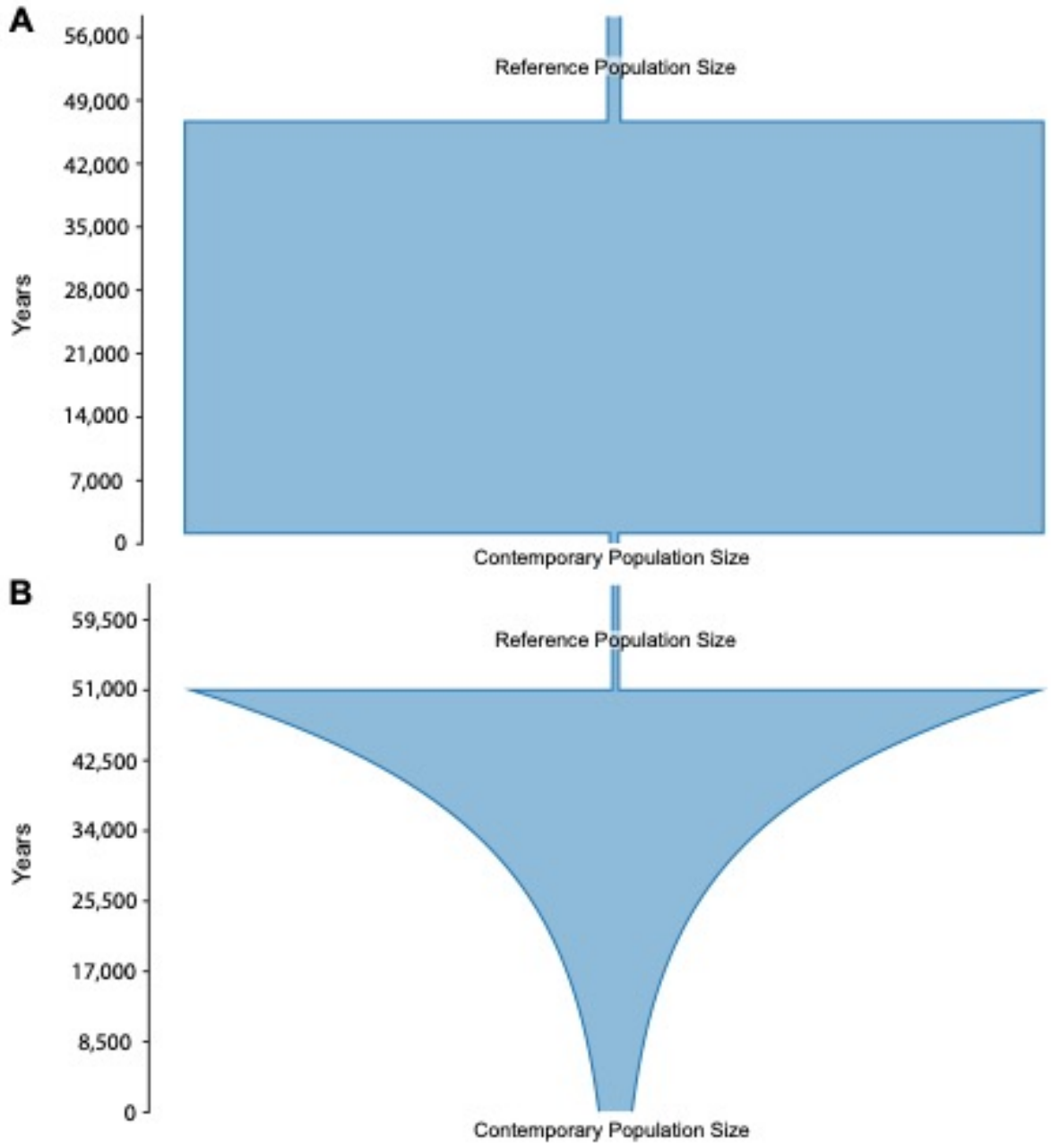

Fig. 5. Best-fit demographic models. Visualization of the three-epoch model (**A**) and the bottleneck-growth model (**B**) drawn with estimated parameters inferred by ∂a∂i for *Arcidens wheeleri*. The width is proportional to the population size.

SUPPLEMENTARY MATERIAL

S1. Photograph of *Arcidens wheeleri* specimens found in the Little River, AR. Photo credit: K. Moles.

S2. Photograph of *Arcidens wheeleri* specimens found in the Little River, AR. Photo credit: K. Moles.

Chapter 2: Population Genetics of *Alasmidonta varicosa* (Brook Floater) Throughout its Range

Authors: Mia C. Colley, Shannon Julian, Meredith L. Bartron, Brian T. Watson, Nathan V. Whelan

Introduction

Biodiversity is facing numerous threats worldwide, which must be understood so appropriate tools can be used to conserve vulnerable species (Hohenlohe et al. 2021). However, critical information for wildlife conservation is lacking for most imperiled species, including population size and connectivity, the potential for populations to adapt to environmental change, and the presence or extent of hybridization and inbreeding (Shafer et al. 2015). Genetic information, particularly with genomic technologies, can provide such information and guide conservation strategies (Hohenlohe et al. 2021).

North America has one of the most diverse freshwater mussel (Unionida) faunas in the world (Graf and Cummings 2021). Currently, over 300 species of freshwater mussels are recognized in North America, but 127 of those are considered extinct, possibly extinct, critically endangered, or vulnerable (Aldridge et al. 2023). The decline of freshwater mussels has detrimental effects to ecosystem function as freshwater mussels cycle nutrients by filter feeding and burrowing(Vaughn 2018). Mussels are also a source of food and modify habitat for many other organisms (Haag and Williams 2014). The causes of freshwater mussel declines include habitat loss and degradation, population fragmentation, introduction of invasive species, and climate change (Strayer et al. 2004). Freshwater mussels are also sensitive to declines in

freshwater fish populations as almost all mussels have an obligate, parasitic life stage with a host fish (Strayer 2008, Modesto et al. 2018).

Alasmidonta varicosa (Lamarck, 1819), or Brook Floater (Fig. 1), is a small and thin-shelled mussel with a moderate life span and moderate age of reproduction. The species has low fecundity, reported as below 8,000 glochidia for four *A.varicosa* mussels from a population in New Hampshire (Wicklow et al. 2017), which is much lower than some other freshwater mussels like *Arcidens confragosus* (Say 1829), which has high fecundity that has been estimated to range between 75,833-897,500 glochidia (Haggerty et al. 2011). Most *A. varicosa* individuals are relatively small, reaching only 7.5 cm in in length. The species can be distinguished from other mussels by its elliptical to trapezoidal shell shape and its cantaloupe orange colored foot (Fig. 1; Wicklow et al. 2017).

Alasmidonta varicosa is a host generalist species (Wicklow et al. 2017). Host use can vary across geographic regions (Douda et al. 2014), and with *A. varicosa* having such a substantial range, it has been shown to have differences in host use among populations. For example, the Margined Madtom (*Noturus insignis)* and the Tessellated Darter (*Etheostoma olmstedi*) were found to be suitable host fish in New Hampshire but not in North Carolina, whereas the Redbreast Sunfish (*Lepomis auritus*) was found to be a suitable host fish in North Carolina but not in New Hampshire (Eads et al. 2007, Wicklow et al. 2017). In another study, Skorupa et al., (2022) found that for Brook Floater populations in Massachusetts and Maine, Slimy Sculpin (*Cottus cognatus*) and Brook Trout (*Salvelinus fontinalis*) had the highest metamorphosis rate. Longnose Dace (*Rhinichthys cataractae*), Golden Shiner (*Notemigonus crysoleucas*), Pumpkinseed (*Lepomis gibbosus*), Tellow Perch (*Perca flarescens*) and Blacknose

Dace (*Rhinichthys atratulus*) have also been recorded to serve as glochidial hosts (Strayer and Jirka 1997, Schulz and Marabain 1998).

The range of *A. varicosa* spans much of the North American Atlantic slope, from the Savannah River Basin in Georgia and South Carolina, U.S.A to Nova Scotia and New Brunswick, Canada. However, its recent historical range (i.e., in the last 200 years, prior to widespread mussel declines) is not contiguous, as there are no historical records from much of Virginia, USA (Pfeiffer et al. 2024). Extirpations have further fragmented the current range of *A. varicosa*, including complete extirpations in Rhode Island and Delaware, USA (Wicklow et al. 2017), and likely extirpation from Virginia (Wicklow et al. 2017; Watson et al. unpublished data). Given widely distributed host fish, it is unlikely that the decline of this species is a result of some aspect of the host fish (Nedeau 2008). It is much more likely that habitat degradation, below *A. varicosa* tolerances, is the main driver of declines. For example, the Shenandoah River drainage has had high agricultural impacts, deforestation, and urbanization since the 1600s, which has significantly degraded freshwater habitats in Virginia (Lookingbill et al. 2009, Thady 2016). The last time a non-reintroduced population of *A. varicosa* was seen live in Virginia was in 1998 in Broad Run near Manassas (B. Watson, pers. communication). Similar extirpations have occurred elsewhere across the historical range of *A. varicosa*.

Despite fragmentation and some extirpations, in 2019, U.S. Fish and Wildlife Service (USFWS) determined that listing *A. varicosa* under the U.S. Endangered Species Act was not warranted as the species has resilient populations throughout its range (U.S. Fish and Wildlife Service 2018). The species is listed as special concern under the Canada Species at Risk Act (SARA; Department of Fisheries and Oceans Canada 2018) and threatened under the Nova Scotia Endangered Species Act (Nova Scotia Department of Natural Resources and Renewables

2022). Many persisting populations of *A. varicosa* are fragmented and most sites do not appear to hold large numbers of the species (Wicklow et al. 2017).

Given the decline of *A. varicosa*, a multi-state, international Brook Floater Working Group (BFWG) was created to coordinate research and conservation for the species. The group consists of 39 representatives from 14 U.S. state agencies, 3 federal agencies, and 3 universities. The group focuses on increasing range-wide cooperative conservation efforts and strategic planning to reduce further declines. Numerous studies that advance our understanding of *A. varicosa* have come out of the group (Sterrett et al. 2018, Roy et al. 2022, Skorupa et al. 2022, Sterrett et al. 2022, Skorupa et al. 2024a, Skorupa et al. 2024b). However, no range-wide genetics study has been performed for *A. varicosa*.

As with many other imperiled freshwater mussels, captive propagation is being pursued for *A. varicosa* as a tool for recovering the species (Jones et al. 2006). The ability to propagate large numbers of freshwater mussels has been a major advancement for conservation of these imperiled animals (Strayer et al. 2019). However, critical genetics research has often occurred after propagation efforts have started (e.g., Garrison et al. 2021, Gladstone et al. 2022) or not been done at all. Genetics research in the context of propagation and release is particularly important for broodstock choices and establishing baseline values for program evaluation. Genetic information can also be used to assess the effective number of breeders contributing to captively reared cohorts, which is of particular interest for freshwater mussel propagation as females are almost always brought into propagation facilities after mating in the wild. Thus, genetic information is the only way to determine the number of males contributing to a captive cohort. Without proper planning, including assessment of genetics data, releasing freshwater mussels into the wild can do more harm than good for mussel populations and their ecosystems

(Strayer et al. 2019). Given that propagation and release programs are already underway for *A. varicosa*, genetics data are critically needed.

Past molecular research on *Alasmidonta* has focused on phylogenetic analyses and species-level taxonomic questions. Although such research has been essential for clarifying species boundaries in *Alasmidonta*, including determining that a population that was once considered *A. varicosa* was a novel species (i.e., *A. uwharriensis*; Whelan et al. 2023), little is known about intraspecific genetic diversity of *Alasmidonta* species. Therefore, we sampled *A. varicosa* from across its range and from captively reared cohorts to generate population genomic data that can be used to inform and improve conservation efforts.

Materials and Methods

Sample collecting and sequencing

In total, 268 individuals from 22 different sites were sampled across North American Atlantic Slope drainages (Fig. 2; Table 1). At each site, we aimed for collecting at least 20 individuals, but at numerous sites we were unable to collect that many as a result of low *A. varicosa* abundance. Wild samples were collected by hand, sometimes while snorkeling. Genetic material was obtained non-lethally by rubbing a sterile buccal swab (Isohelix) on the foot. Swabs were immediately placed in stabilization buffer (Isohelix) and mussels were returned to the substrate where they were collected from. We also sampled 135 hatchery individuals from three different hatcheries. We sampled 20 individuals from the Cronin Aquatic Resource Center in Connecticut that were produced with broodstock sourced from the Wesserunsett Stream, using swabs. Ten broodstock individuals from the Chattooga River, in the Savannah River drainage, were collected and swabbed by the Orangeburg National Fish Hatchery. We also received 15

swabs from 2023 broodstock individuals and 90 tissue samples from individuals from various propagated cohorts that were raised at the Virginia Department of Natural Resources Hatchery (Table 2). We also included 16 DNA samples from a previous study, including *A. uwharriensis* as an outgroup (Whelan et al. 2023).

Dataset assembly

For buccal swabs, DNA was extracted with the Isohelix Xtreme DNA isolation kit following manufacturer's instructions. For tissue clips, DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit following manufacturer's instructions. Each extraction was quantified with a Qubit Fluorometer and visualized with a 1% agarose gel and electrophoresis to ensure integrity of high molecular weight DNA. Samples were normalized to a concentration of 20 ng/µL prior. RAD-seq library perp followed the 3RAD protocol with enzymes *XbaI, EcoRI,* and *NheI* restriction enzymes, mostly following Bayona-Vásquez (2019). Size selection during library preparation ranged from 440 to 608 base pairs (bp). Samples were tagged with unique barcodes, pooled together, and sequenced on an Illumina NovaSeq 6000 using 150 bp paired-end chemistry and a single SP flow cell lane. The full 3RAD protocol used here can be found at https://github.com/NathanWhelan/3RAD_protocols.

Raw Illumina reads were assembled with STACKS v2.3 (Rochette et al. 2019). Paired-end reads were demultiplexed with the command *process_radtags*, allowing for one mismatched nucleotide per barcode. The command *clone_filter* was used to remove PCR clones, which were identified with the random 8-N i5 Illumina index that was attached during library prep (see 3RAD protocol for more details). After demultiplexing, RADseq data were assembled with the STACKS pipeline *denovo_map*. Assembly parameters were selected following Paris et al.

(2017), resulting in a minimum stack depth (i.e., coverage) of 3, the distance between stacks set to 2, and the distance allowed between catalog loci set to 2. All other parameters were set to default for paired-end data. STACKS assembly was done separately one with only wild individuals, and one with wild and captive individuals, that were sampled from hatcheries.

The STACKS assembly was filtered with the STACKS module *populations* to create final datasets. The dataset used for most analyses, herein referred to as the "primary dataset", was filtered with the following parameters: the minimum percentage of individuals required to process a locus was set to 0.8, the minimum minor allele frequency setting was set to 0.025, and the maximum heterozygosity was set to 0.5. A second dataset for analyses that assume SNPs are unlinked was filtered like the primary dataset, except only one SNP per locus was retained. Three other smaller datasets were used for the samples from three different hatchery facilities, these datasets include the propagated individuals and the wild population from which the broodstock was sourced (Table 3); hatchery datasets were filtered as above.

Population genomic analyses

All analyses used the multiple SNPs per locus dataset, except admixture-like analyses with LEA that used the single SNP dataset. Sites with sample sizes less than 3 individuals were removed for LEA and DAPC to avoid error. Observed heterozygosity (H_o), expected heterozygosity (H_e), nucleotide diversity (Π), and inbreeding coefficients (F_{IS}) were calculated in *populations* for each collection site and captively reared cohort. The fixation index (F_{ST}), which is a measure of genetic structure, was calculated in STACKS using the command *fstats*. We assessed population structure among collection sites with an Analysis of Molecular Variance

(AMOVA;(Excoffier et al. 1992), using the *poppr.amova* command in the R package poppr (Kamvar et al. 2014); significance was tested with a 999 permutation randomization test.

For wild individuals, genetic clustering across the landscape was assessed with discriminant analysis of principal components (DAPC), which was implemented using the R package adegenent and ape (Jombart 2008, Paradis and Schliep 2018). The best-fit number of clusters (K) was assessed using Bayesian Information Criterion (BIC), and the number of retained principle components was one less than K, following Thia (2023). Genomic admixture of each wild individual was assessed with the sparse non-negative matrix factorization (*snmf*) function of the R package LEA (Frichot and François 2015); the best-fit number of genetic clusters (K) was determined by calculating the K with the lowest cross-entropy values. We examined fine-scale genetic diversity spread among propagated individuals and the broodstock populations with principal component analysis (PCA) using the *factoextra* R package (Kassambara and Mundt 2020).

Many species and higher-level taxa, display a latitudinal gradient of genetic diversity, with higher genetic diversity in lower latitudes (Rohde, 1992). However, such a pattern has rarely been tested for in freshwater mussels. We tested for a latitudinal genetic diversity gradient using observed heterozygosity, expected heterozygosity and nucleotide diversity values with linear regression functions in R, using latitude as the predictor variable for genetic diversity measures.

Phylogenetic inference

To visualize relationships among populations and assess phylogeographic patterns, we inferred a maximum likelihood phylogenetic tree. First, we trimmed the primary dataset to

include a maximum of five individuals per site; individuals with the most missing data were removed. This was done to limit required computational time for model testing and tree inference. The fasta file output by STACKS was converted to individual loci with the script Convert_Stacks_Fasta_to_Loci.py (https://github.com/dportik/Stacks_pipeline); heterozygous sites were coded with IUPAC nucleotide ambiguity codes). Loci were then concatenated with FASconCAT-G 1.0 (Kück and Longo 2014).

The best-fit nucleotide substitution model was determined with MixtureFinder (Ren et al. 2024), as implemented in IQ-TREE 2.3.2 (Minh et al. 2020). MixtureFinder infers the best-fit site-heterogeneous model by testing for the best-fit number of site-frequency mixture profiles and the best-fit nucleotide exchangeability model for each site-frequency mixture profile. It then tests for the best-fit rate-heterogeneity model. For model-testing, we allowed for a maximum of 10 mixture profiles and model-fit was determined with Bayesian information criteria (BIC). After model testing, the maximum likelihood tree was inferred with IQ-TREE using the best-fit substitution model. We performed 10 independent tree searches, and we reported the tree with the lowest log-likelihood value. 1,000 ultrafast bootstrap replicates (UFboot) were performed to assess support for relationships (Hoang et al. 2018).

Results

The 403 sequenced individuals had an average of 1,531,720 raw paired-end reads (range from 1,731-5,403,659 reads). After removing 6 individuals with high amounts of missing data, the remaining 397 individuals had an average of 1,554,335 raw paired-end reads (range from 17,639-5,403,659). Filtering implemented in *populations* resulted in 10,621 loci and 9,027 SNPs for the single SNP per locus dataset and 10,621 loci with 30,572 SNPs in primary dataset with

multiple SNPs per locus (Table 3). The captive dataset with samples from the Orangeburg National Fish Hatchery contained 11,416 loci and 15,222 SNPs. The dataset with samples from Virginia Department of Natural Resources Hatchery contained 13,790 loci and 20,039 SNPs. The Cronin Aquatic Resource Center dataset contained 15,289 loci and 31,294 SNPs.

Population genomics of wild A. varicosa

Observed heterozygosity was higher than expected heterozygosity at most sites (Table 4). Linear regression analysis showed a strong ($r^2 > 0.8594$) and significant ($p < 0.05$) correlation of genetic diversity and latitude, with an increase in diversity from southern sites to northern sites (Fig. 4). AMOVA was significant ($p = 0.001$), with 24.6% of genetic variation explained by sample location. Pairwise F_{ST} values between sites ranged from 0.0139–0.3589, with lower values being between geographically proximate locations.

Analyses with LEA indicated that data were best explained by 8 genetic clusters, K=8. Shared ancestry inferred with LEA was associated with geographical distribution (Fig. 2). Multiple sites had individuals with admixed ancestry, indicating at least some gene flow among genetic clusters (Fig. 2). DAPC analysis indicated that a K of 7 best fit the data. DAPC clusters were similar to that of LEA analyses (Fig. 3).

Population genomics of A. varicosa hatchery individuals

Individuals used for broodstock at the Orangeburg National Fish Hatchery had slightly higher observed heterozygosity than expected heterozygosity, whereas the wild individuals from the Chattooga River that were used for broodstock had equal observed and expected heterozygosity values (Table 5). Orangeburg National Fish Hatchery produced individuals had a

lower F_{IS} value compared to the wild individuals (Table 5). The hatchery produced individuals from the Virginia Department of Natural Resources Hatchery had nearly identical genetic diversity values as the wild individuals from the Cacapon River, except that F_{IS} values were slightly lower for the wild population (Table 5). Hatchery individuals from the Cronin Aquatic Resource Center also had equal values for observed and expected heterozygosity is the same as expected, while the broodstock site in the Wesserunsett Stream had slightly lower observed heterozygosity than expected heterozygosity (Table 5). The hatchery produced individuals from the Cronin Aquatic Resource Center also had a lower F_{IS} value than their corresponding broodstock population (Table 5). PCA analyses of offspring propagated at all three hatcheries show more genetic diversity among hatchery individuals than among wild individuals, indicating some, albeit limited, genetic variation compared to the wild individuals from which they were sourced (Fig. 5).

Phylogenetics

Relationships among *A. varicosa* individuals inferred with maximum likelihood phylogenetic inference were consistent with clustering patterns inferred with LEA and DAPC (Figures. 2, 3, 6). The sister clade to all other *A. varicosa* was from central North Carolina (Fig. 6). The tree has a pectinate, or ladder-like, topology, with samples from the southern portion of the *A. varicosa* range comprising clades that split towards the base of the tree (Fig. 6). Most relationships were strongly supported (UFboot > 99), especially for deeper relationships. As expected for a within-species analysis, branch lengths within the clade of *A. varicosa* were short, especially compared to the branch length between *A. varicosa* and *A. uwharriensis*.

Discussion

Landscape genetic analyses revealed unexpected patterns of genetic diversity across the range of *A. varicosa*. For example, *A. varicosa* has significantly higher genetic diversity at higher latitudes and at the northern and western edge of its range (Fig. 4), which conflicts with the common, and almost universal, characteristic of species having greater genetic diversity at the center of their ranges (Brussard 1984). This is especially notable given phylogenetic relationships that suggest *A. varicosa* originated in central North Carolina, near its southern range extent, and then diversified via migration north (Fig. 6). Typically, species lose genetic diversity as they expand their range through repeated bottlenecks that occur with colonization events (Shirk et al., 2014; Bock et al., 2016). However, this does not appear to have happened in *A. varicosa*, or it at least does not drive current genetic diversity patterns across the landscape. Furthermore, anthropogenic effects do not seem to be the cause of landscape genetic distributions as we have no evidence that *A. varicosa* habitat degradation has followed a latitudinal gradient. More research will be needed to understand the processes driving the patterns revealed here and to understand if similar patterns are present in other freshwater mussels or if *A. varicosa* is unique.

Despite extirpations across the range of *A.varicosa* and currently fragmented populations, almost all sampled sites have genetic diversity that is similar or higher to other freshwater mussels for which SNP data are available (e.g., Farrington et al. 2020, Garrison et al. 2021, Kim and Roe 2021, Smith et al. 2021, Gladstone et al. 2022, Perea et al. 2022). The one exception we are aware of is that some populations of *Obovaria olivaria* were previously reported to have higher observed heterozygosity values than seen in *A. varicosa* (Bucholz et al. 2022). Such high genetic diversity measures, coupled with low inbreeding coefficients, casts doubt on the past

claims that *A. varicosa* populations are in decline, at least for populations sampled here. Notably, our analyses show similar, or higher, levels of genetic diversity in captively-reared individuals compared to the sites from which broodstock were sourced (Table 5). Thus, current hatchery protocols appear sufficient for reestablishing populations that have genetic diversity that is comparable to, or higher than, naturally occurring populations.

Landscape genetic diversity and biogeography of A. varicosa

Genetic drift, gene flow, and natural selection dictate the genetic characteristics of populations, and they are strongly influenced by the spatial distributions of populations (Eckert et al. 2008). Furthermore, the "abundant center" model predicts that any given species will have a greater population size, and therefore genetic diversity, at the center of its range, with populations becoming progressively less diverse the farther a population is from the range center (Brussard 1984, Sagarin and Gaines 2002, Eckert et al. 2008). Many empirical studies have found support for the abundant center model (Wulff 1950, Udvardy 1969, Naumov 1972, Kendeigh and Kendeigh 1974, Brown and Gibson 1983, Maurer 2009, Rapoport 2013, Cox et al. 2016), so much so that it has been referred to as the 'general rule' of biogeography (Hengeveld and Haeck 1982, Hochberg and Ives 1999, Sagarin and Gaines 2002). However, *A. varicosa* does not follow the abundant center model. Instead, peripheral populations in Canada have the highest levels genetic diversity, and sites near the center of the range have intermediate levels of genetic diversity.

The causes of the latitudinal gradient in genetic diversity of *A. varicosa* are unclear. Moreover, genetic diversity is typically higher near the tropics (Brown, 2014), so the landscape genetics of *A. varicosa* further defies common biogeographic patterns. We are also unable to

explain the large, historical distributional gap in Virginia. The profound gap in populations in Virginia could help explain the lower diversity in the southernmost sites, as the gap isolates populations and restricts gene flow. Furthermore, during repeated glaciation cycles, *A. varicosa* may have maintained genetic diversity in northern populations by whole-population downstream expansions to glacial refugia that would now be under the Atlantic Ocean, followed by upstream migrations. Such migrations could have been facilitated by the use of relatively vagile fish host fish like Brook Trout in northern populations, whereas southern population apparently use less vagile fish (Eads et al., 2007), possibly meaning that moving to glacial refugia was associated with historical bottlenecks that influence contemporary genetic diversity of southern populations.

Unlike the latitudinal genetic diversity gradient, genetic structure seen in *A. varicosa* follows a predictable pattern. Genetic clusters follow a clear geographic pattern, with populations grouping based on geographic proximity and sometimes by drainage. Genetic structure of *A. varicosa* is best explained by considering river connectivity during the last glacial maximum. For example, individuals from the Cacapon River, Potomac River, and Darling Creek in Pennsylvania seem disconnected based on the river paths of today, but all three rivers flow into the Chesapeake Bay and were likely connected during the Pleistocene. The grouping of individuals from the Kouchibouguacis River, Petitcodiac River, and Little River are also likely the result of river connectivity during the last glacial maximum. Although some redundancy in genomic composition exists across the range of *A. varicosa*, populations in the East Branch Pleasant River, Wesserunsett Stream, and Nissitissit River have unique ancestry profiles (Fig. 2).

Genetic analyses indicate stable populations

A major motivating factor for this study was to generate data to improve *A. varicosa* conservation because the species is widely seen as being in decline. However, landscape and population genetic patterns for *A. varicosa* are drastically different from other critically imperiled freshwater mussels. For instance, almost all *A. varicosa* populations have higher observed heterozygosity than seen in federally threatened *Margaritifera hembeli* and federally endangered *Arcidens wheeleri* (Garrison et al. 2021, Adcock et al. 2024). Most *A. varicosa* populations have higher observed heterozygosity than seen in federally endangered *Epioblasma brevidens* (Gladstone et al. 2022). In cases where *A. varicosa* had lower genetic than the aforementioned species, observed and expected heterozygosity values were nearly identical, which is not the case for any population of *M. hembeli*, *A. wheeleri*, or *E. brevidens* (Garrison et al. 2021, Gladstone et al. 2022, Adcock et al. 2024). In other words, past population genomic studies on federally listed mussels have revealed genetic evidence of bottlenecks, whereas no such evidence exists in *A. varicosa*.

Even in rivers with exceptional water quality and mussel habitat, not every species will be naturally abundant. *Alasmidonta varicosa* has previously been reported to be in relatively low abundances at many known occurrence sites, but past research has been equivocal as to whether this is a result of recent declines or some other process (Wicklow et al. 2017). In contrast, we found a striking lack of genetic evidence for population declines, bottlenecks, or inbreeding (Table 4). Therefore, genomic information indicates that *A. varicosa* may simply be a species that will never be among the most abundant mussel species in any given river. This has important implications for how managers should think about both population status and recovery objectives.

Conservation implications and future management guidelines

The absence of a genetic signature of population bottlenecks or inbreeding for sampled populations calls into question the need for active management of existing populations like augmentation with hatchery individuals. Instead, our data indicate that sampled populations are healthy and not at risk of losing genetic diversity without a catastrophic event like habitat destruction. Thus, we argue that hatchery and other conservation efforts should focus on 1) preserving existing populations via habitat protections and 2) reestablishment of extirpated populations. If reintroductions are pursued, we recommend doing so in the context of landscape genetic structure naturally present in *A. varicosa*. We suggest managing *A. varicosa* as 9 distinct management units, splitting out East Branch of the Pleasant River as an additional cluster from the number of clusters revealed by LEA because the population has clearly unique genomic ancestry (Fig. 2). Broodstock populations for reintroductions should follow geographic patterns, combing consideration of geographic proximity of planned reintroduction sites to potential broodstock sources and locations in river networks. We think doing so is particularly important given past reports of differential host fish use across the range of *A. varicosa*; ensuring that the host fish used for hatchery transformation is present at reintroductions site should help mitigate risks with establishing populations that cannot use sympatric fish as hosts. Both differential host fish use and natural landscape genetic structure mean northern populations should not be used as broodstock for southern reintroductions even though the northern populations have greater genetic diversity.

Perhaps the most difficult result to explain is the higher genetic diversity seen in captively-reared individuals, at all three hatcheries, compared to the wild sites from which they were sourced (Fig. 5). This pattern is apparent in the PCA plots for broodstock sites and

hatchery-reared individuals, indicating that hatchery managers have been able to increase and/or maintain genetic integrity. We are unsure the exact cause of the higher genetic diversity and it warrants further study. Bramwell et al. (2024) found a similar pattern in farmed *Mytilus* bivalves, but was also unable to explain the cause of higher genetic diversity in hatchery produced individuals. As hatchery production efforts continue for *A. varicosa*, we think that future genetic monitoring will be essential to 1) explain the aforementioned genetic diversity pattern and 2) examine if reintroduced populations maintain the genetic diversity seen among hatchery individuals or if selection after reintroduction decreases genetic diversity to levels that are consistent with broodstock sources.

Conclusions

Our findings show that some common biographic and landscape genetic patterns do not apply to *A. varicosa*. Future studies should determine if *A. varicosa* is unique among Unionidae or if freshwater mussels, more broadly, have life history strategies, possibly associated with parasitism, that make them a counter example to common biogeographic processes. This study also provides vital information for *A. varicosa* that should be used to inform international conservation efforts. Broadly, this study, including the large BFWG partnership that made range-wide sampling possible, should be used as a framework for studying landscape genetics and biogeography of species with large ranges but high conservation concern. Most sampled sites have high genetic diversity, and no site had inbreeding levels that suggest genetic diversity is being lost. This is indicative of healthy *A. varicosa* populations, and it is positive finding for an imperiled species. Nevertheless, we advocate for habitat protection, continued population monitoring, and consideration of incorporating the 9 management units identified here into

formal conservation plans. Propagation efforts should continue with currently used protocols as offspring have comparable or large amounts of genetic diversity as the broodstock sources, which is important for establishing reintroduced populations with adequate evolutionary potential.

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Table 1. Collection information for *Alasmidonta varicosa*

Species	Location	Number of Samples	Latitude	Longitude
Alasmidonta varicosa	Cacapon River, West Virginia	31	39.6165	-78.2811
Alasmidonta varicosa	East Branch Pleasant River, Maine	20	45.4690	-68.9854
Alasmidonta varicosa	West Branch Farmington River, Massachusetts	20	42.1950	-73.0911
Alasmidonta varicosa	Wesserunsett Stream, Cornville Maine	20	44.9112	-69.6713
Alasmidonta varicosa	Petitcodiac River, NB, Canada	15	45.9977	-65.0913
Alasmidonta varicosa	Little River, NB, Canada	15	46.0199	-65.0211
Alasmidonta varicosa	Truro, NS, Canada	20	45.3691	-63.3365
Alasmidonta varicosa	Nississit River, Massachusetts	24	42.6974	-71.6063
Alasmidonta varicosa	Ware River, Massachusetts	3	42.2411	-72.2661
Alasmidonta varicosa	Propagation - Connecticut River	20		
Alasmidonta varicosa	West River, Vermont	12	43.0479	-72.6959
Alasmidonta varicosa	Potomac River, Maryland	15	39.6030	-77.9091
Alasmidonta varicosa	Bachelor Brook, Massachusetts	6	42.2794	-72.5617
Alasmidonta varicosa	Kouchibouguacis River, Canada	6	46.7116	65.0601
Alasmidonta varicosa	Chattooga River Trail, Georgia	19	34.8151	-83.3067
Alasmidonta varicosa	Darling Run Pine Creek Rail Trail, Pennsylvania	20	41.7401	-77.4297
Alasmidonta varicosa	Orangeburg National Fish Hatchery	10		
Alasmidonta varicosa	Virginia Department of Wildlife Resources Hatchery	105		
Alasmidonta varicosa	Turkey Creek, North Carolina	4	35.2873	-82.6960
Alasmidonta varicosa	Mulberry Creek, North Carolina	5	35.9545	-81.6253
Alasmidonta varicosa	Deep River, North Carolina	5	35.4779	-79.5197
Alasmidonta varicosa	Rock River, North Carolina	1	35.6545	-79.2394
Alasmidonta varicosa	Wilson's Creek, North Carolina	2	35.8871	-81.7166
Alasmidonta varicosa	Mitchell River, North Carolina	1	36.3958	-80.8396
Alasmidonta uwharrensis	Hannah's Creek, North Carolina	1	35.5846	-79.9434
Alasmidonta uwharrensis	Tom's Creek, North Carolina	1	35.6399	-79.9792
Alasmidonta uwharrensis	Densons Creek, North Carolina	5	35.3864	-78.8679
Alasmidonta uwharrensis	Dumas Creek, North Carolina	2	35.3935	-79.8972
Alasmidonta uwharrensis	Barnes Creek, North Carolina	7	35.4798	-79.9527

Table 2. Sample information from the Virginia Department of Natural Resources Hatchery

Sample Name	Sample Information
AVVA-1	propogated, batch 2020
AVVA-2	propogated, batch 2021
AVVA-3	propogated, batch 2020 -01
AVVA-4	propogated, batch 2020 - 01
AVVA-5	propogated, batch 2020 - 01
AVVA-6	propogated, batch 2021 - 02
AVVA-7	propogated, batch 2021 - 02
AVVA-8	propogated, batch 2021 - 03
AVVA-9	propogated, batch 2021 - 03
AVVA-10	propogated, batch 2022-01
AVVA-11	propogated, batch 2022-01
AVVA-12	propogated, batch 2022-01
AVVA-13	propogated, batch 2022-01
AVVA-14	propogated, batch 2022-01
AVVA-15	propogated, batch 2022-01
AVVA-16	propogated, batch 2022-01
AVVA-17	propogated, batch 2022-02
AVVA-18	propogated, batch 2022-02
AVVA-19	propogated, batch 2022-02
AVVA-20	propogated, batch 2022-02
AVVA-21	propogated, batch 2022-02
AVVA-22	propogated, batch 2022-02
AVVA-23	propogated, batch 2022-03
AVVA-24	propogated, batch 2022-03
AVVA-25	propogated, batch 2022-03
AVVA-26	propogated, batch 2022-03
AVVA-27	propogated, batch 2022-03
AVVA-28	propogated, batch 2022-03
AVVA-29	propogated, batch 2022-03
AVVA-30	batch 1, 2023 cohort
AVVA-31	batch 1, 2023 cohort
AVVA-32	batch 1, 2023 cohort
AVVA-33	batch 1, 2023 cohort
AVVA-34	batch 1, 2023 cohort
AVVA-35	batch 1, 2023 cohort
AVVA-36	batch 1, 2023 cohort
AVVA-37	batch 2, 2023 cohort

AVVA-38	batch 2, 2023 cohort
AVVA-39	batch 2, 2023 cohort
AVVA-40	batch 2, 2023 cohort
AVVA-41	batch 2, 2023 cohort
AVVA-42	batch 2, 2023 cohort
AVVA-43	batch 3, 2023 cohort
AVVA-44	batch 3, 2023 cohort
AVVA-45	batch 3, 2023 cohort
AVVA-46	batch 3, 2023 cohort
AVVA-47	batch 3, 2023 cohort
AVVA-48	batch 3, 2023 cohort
AVVA-49	batch 3, 2023 cohort
AVVA-50	2023 broodstock swabs
AVVA-51	2023 broodstock swabs
AVVA-52	2023 broodstock swabs
AVVA-53	2023 broodstock swabs
AVVA-54	2023 broodstock swabs
AVVA-55	2023 broodstock swabs
AVVA-56	2023 broodstock swabs
AVVA-57	2023 broodstock swabs
AVVA-58	2023 broodstock swabs
AVVA-59	2023 broodstock swabs
AVVA-60	2023 broodstock swabs
AVVA-61	2023 broodstock swabs
AVVA-62	2023 broodstock swabs
AVVA-63	2023 broodstock swabs
AVVA-64	2023 broodstock swabs
AVVA-65	broodstock from 2021
AVVA-66	2021 cohort batch 1
AVVA-67	2021 cohort batch 1
AVVA-68	2021 cohort batch 1
AVVA-69	2021 cohort batch 1
AVVA-70	2021 cohort batch 1
AVVA-71	2021 cohort batch 1
AVVA-72	2021 cohort batch 1
AVVA-73	2021 cohort batch 2
AVVA-74	2021 cohort batch 2
AVVA-75	2021 cohort batch 2
AVVA-76	2021 cohort batch 2
AVVA-77	2021 cohort batch 2

AVVA-78	2021 cohort batch 2
AVVA-79	2021 cohort batch 3
AVVA-80	2021 cohort batch 3
AVVA-81	2021 cohort batch 3
AVVA-82	2021 cohort batch 3
AVVA-83	2021 cohort batch 3
AVVA-84	2021 cohort batch 3
AVVA-85	2021 cohort batch 3
AVVA-86	2020 cohort batch 1
AVVA-87	2020 cohort batch 1
AVVA-88	2020 cohort batch 1
AVVA-89	2020 cohort batch 1
AVVA-90	2020 cohort batch 1
AVVA-91	2020 cohort batch 1
AVVA-92	2020 cohort batch 1-3
AVVA-93	2020 cohort batch 1-3
AVVA-94	2020 cohort batch 1-3
AVVA-95	2020 cohort batch 1-3
AVVA-96	2020 cohort batch 1-3
AVVA-97	2020 cohort batch 1-3
AVVA-98	2020 cohort batch 1-3
AVVA-99	2020 cohort batch 1-3
AVVA-100	2020 cohort batch 1-3
AVVA-101	2020 cohort batch 1-3
AVVA-102	2020 cohort batch 1-3
AVVA-103	2020 cohort batch 1-3
AVVA-104	2020 cohort batch 1-3
AVVA-105	2020 cohort batch 1-3

Table 3. Dataset information

Dataset	Collection Sites	Number of Individuals	Number of Loci	Number of SNPs
Primary	24	376	10621	30,572
Single	21	286	10621	9,027
OrangeburgHatchery	2	23	11416	15,222
VirginiaHatchery	2	136	13790	20,039
ConneticutHatchery	2	38	15289	31,294

Table 4. Population summary statistics for *A. varicosa*. Number of individuals (N), observed heterozygosity (H_o), expected heterozygosity (H_e), nucleotide diversity (π), and inbreeding coefficient (F_{IS}).

Collection Site	N	H_o (SE)	H_e (SE)	Π (SE)	F_{IS} (SE)
Kouchibouguacis River	5	0.1903 (0.0014)	0.1761 (0.0011)	0.2004 (0.0013)	0.0214 (0.0051)
Chattooga River	11	0.1061 (0.0011)	0.1076 (0.0010)	0.1143 (0.0011)	0.0193 (0.0122)
Darling Run Pine Creek Rail Trail	20	0.1873 (0.0011)	0.1929 (0.0011)	0.1987 (0.0011)	0.0322 (0.0147)
Ware River	3	0.1834 (0.0017)	0.1511 (0.0012)	0.1971 (0.0016)	0.0231 (0.0042)
Turkey Creek	3	0.1090 (0.0014)	0.0931 (0.0010)	0.1146 (0.0012)	0.0098 (0.0028)
Mulberry Creek	5	0.1115 (0.0012)	0.1097 (0.0010)	0.1248 (0.0012)	0.0277 (0.0059)
Deep River	4	0.1054 (0.0013)	0.0970 (0.0010)	0.1185 (0.0013)	0.0243 (0.0056)
Cacapon River	30	0.1695 (0.0011)	0.1743 (0.0011)	0.1776 (0.0011)	0.0230 (0.0160)
Potomac River	12	0.1715 (0.0012)	0.1708 (0.0011)	0.1787 (0.0012)	0.0197 (0.0068)
West River	10	0.2013 (0.0012)	0.2028 (0.0011)	0.2153 (0.0012)	0.0327 (0.0072)
Bachelor Brook	5	0.1969 (0.0015)	0.1839 (0.0011)	0.2099 (0.0013)	0.0264 (0.0045)
East Branch Pleasant River	20	0.2153 (0.0011)	0.2263 (0.0010)	0.2324 (0.0011)	0.0499 (0.0080)
West Branch Farmington River	18	0.1895 (0.0011)	0.1953 (0.0011)	0.2012 (0.0011)	0.0312 (0.0080)
Little River	15	0.2216 (0.0011)	0.2292 (0.0010)	0.2376 (0.0011)	0.0453 (0.0061)
Nississit River	24	0.2192 (0.0011)	0.2267 (0.0010)	0.2320 (0.0011)	0.0363 (0.0149)
Petitcodiac River	15	0.2219 (0.0011)	0.2297 (0.0010)	0.2380 (0.0011)	0.0448 (0.0061)
Truro River	19	0.1976 (0.0011)	0.2043 (0.0011)	0.2104 (0.0011)	0.0371 (0.0120)
Wesserunsett Stream	18	0.2175 (0.0011)	0.2251 (0.0010)	0.2318 (0.0011)	0.0406 (0.0065)

Table 5. Population summary statistics for A. varicosa between wild populations and hatchery individuals including; number of individuals (N), observed heterozygosity (H_o), expected heterozygosity (H_e), nucleotide diversity (π), and inbreeding coefficient (F_{IS}).

Collection Site	N	H_o (SE)	H_e (SE)	π (SE)	F_{IS} (SE)
Orangeburg	10	0.1080 (0.0012)	0.1015 (0.001)	0.1073 (0.0011)	0.0029 (0.0083)
Chattooga	11	0.1061 (0.0011)	0.1076 (0.001)	0.1143 (0.0011)	0.0193 (0.0122)
Virginia Hatchery	105	0.1699 (0.0012)	0.1720 (0.0011)	0.1769 (0.0011)	0.0196 (0.0084)
Cacapon River	30	0.1695 (0.0011)	0.1743 (0.0011)	0.1776 (0.0011)	0.023 (0.016)
PropagationConneticut	20	0.2179 (0.0012)	0.2143 (0.0011)	0.2203 (0.0011)	0.0066 (0.0139)
Wesserunsett	18	0.2175 (0.0011)	0.2251 (0.001)	0.2318 (0.0011)	0.0406 (0.0065)

Fig. 1. *Alasmidonta varicosa* specimens

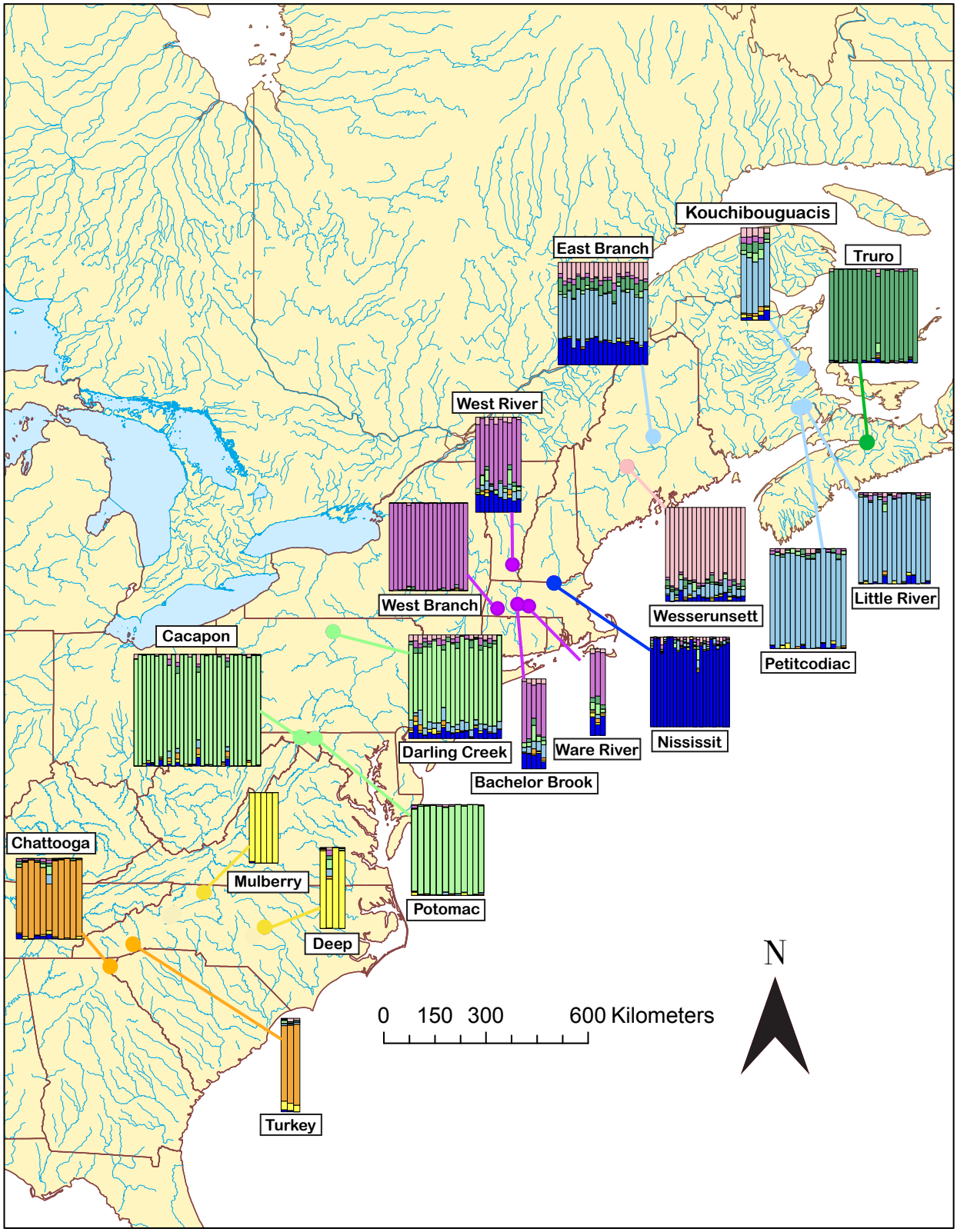


Fig. 2. Map of collection sites and individual admixture for all wild individuals by site, as inferred by LEA analysis

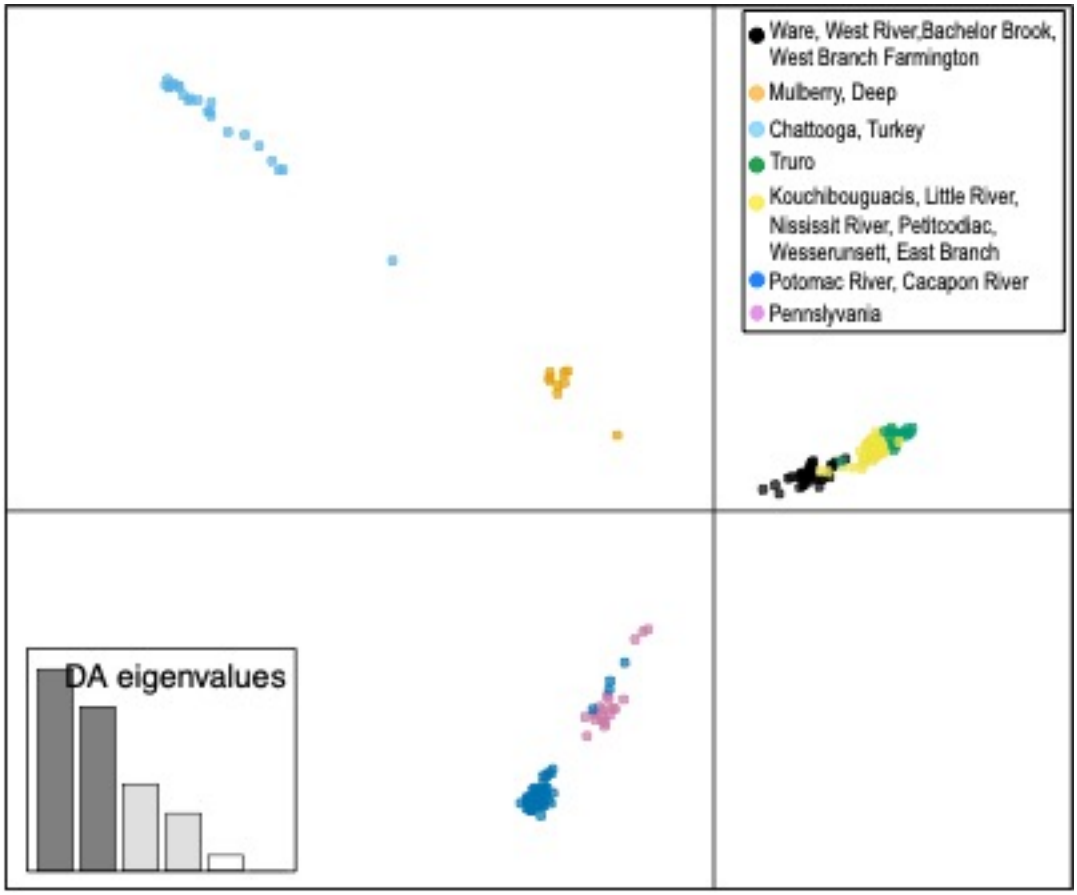


Fig. 3. Discriminant analysis of principal components output showing clustering of sites

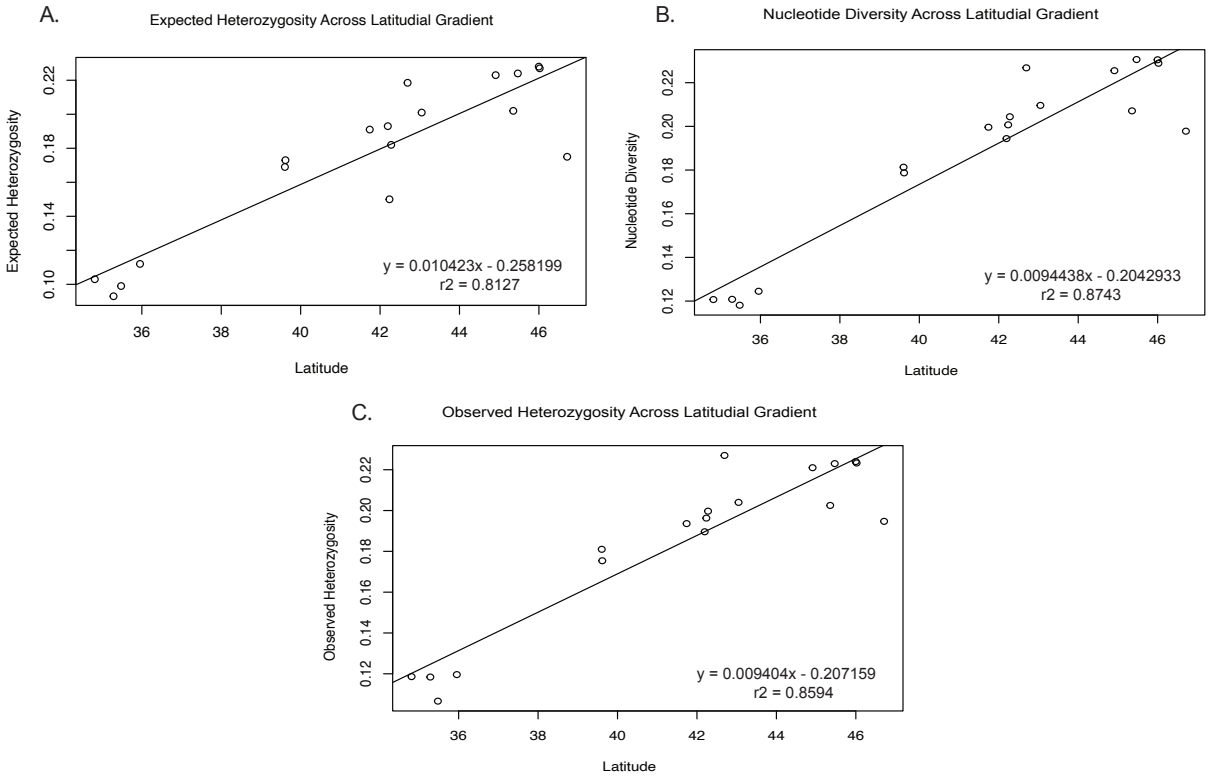


Fig. 4. Genetic diversity across latitudinal gradient

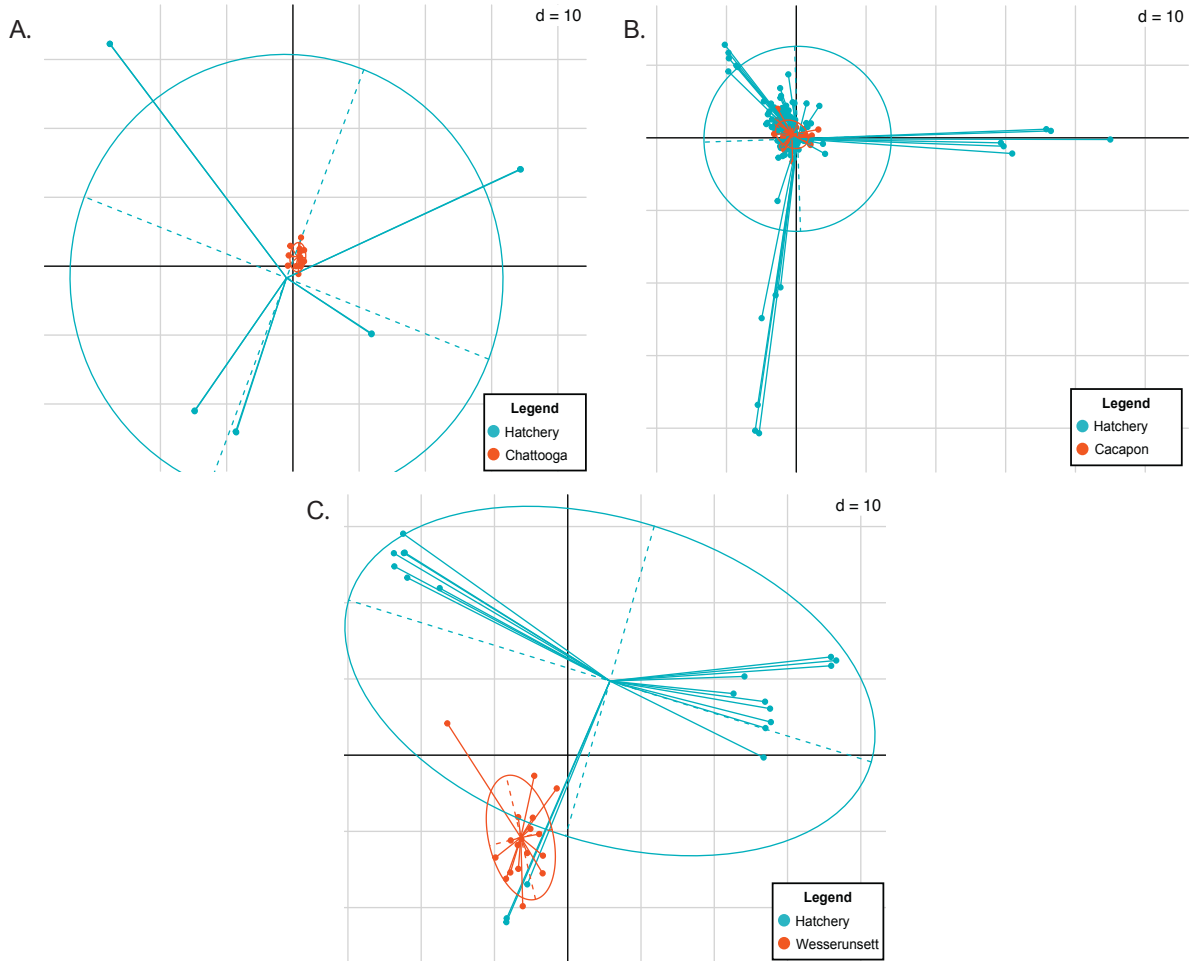
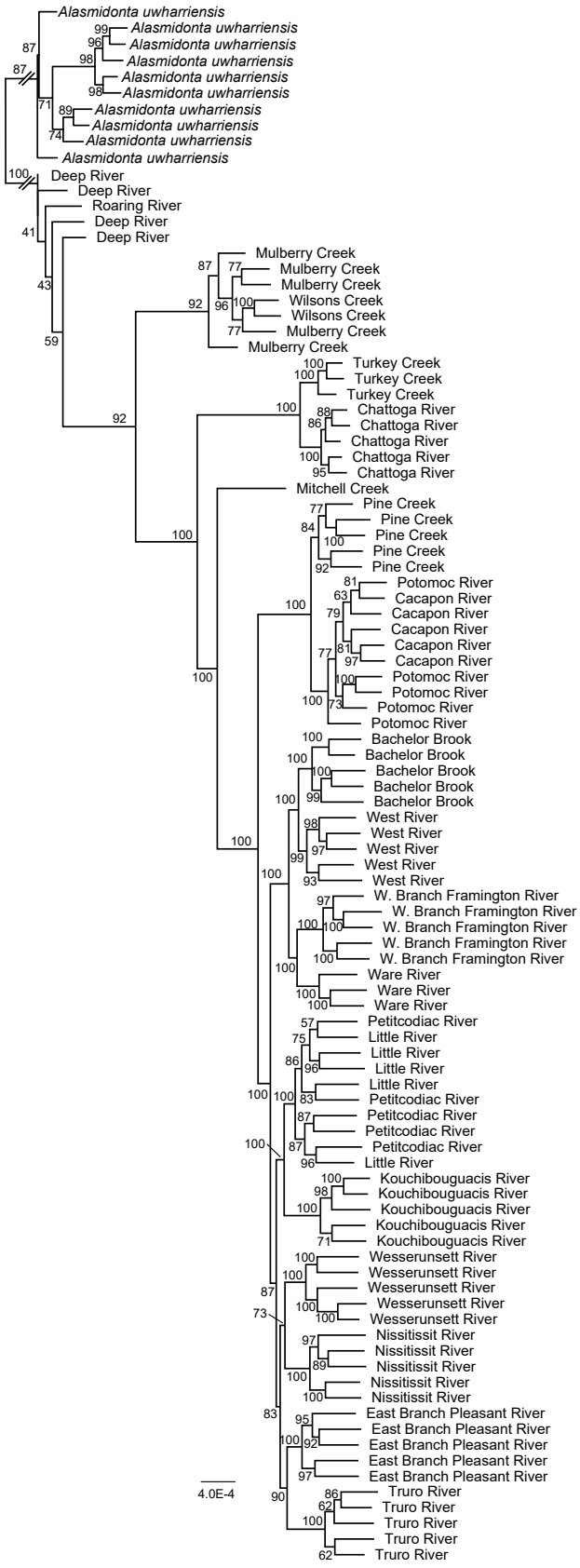


Fig. 5. Principal component analysis (PCA) showing fine-scale genetic diversity spread among propagated individuals and their broodstock populations


Alasmidonta uwharriensis
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Deep River
Deep River
Roaring River
Deep River
Deep River
Mulberry Creek
Mulberry Creek
Mulberry Creek
Wilsons Creek
Wilsons Creek
Mulberry Creek
Mulberry Creek
Turkey Creek
Turkey Creek
Turkey Creek
Chattoga River
Chattoga River
Chattoga River
Chattoga River
Chattoga River
Mitchell Creek
Pine Creek
Pine Creek
Pine Creek
Pine Creek
Pine Creek
Potomoc River
Cacapon River
Cacapon River
Cacapon River
Cacapon River
Cacapon River
Potomoc River
Potomoc River
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Bachelor Brook
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Bachelor Brook
Bachelor Brook
West River
West River
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W. Branch Framington River
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Wesserunsett River
Wesserunsett River
Wesserunsett River
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East Branch Pleasant River
East Branch Pleasant River
Truro River
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Truro River
4.0E-4

Fig. 6. Maximum likelihood phylogenetic tree with *Alasmidonta uwharrensis* as an outgroup. Tips with sample location names are *A. varicosa* individuals.