Guiding Species Recovery through Assessment of Spatial and Temporal Population Genetic Structure of Two Critically Endangered Freshwater Mussel Species (Bivalvia: Unionidae)

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Abstract

The Cumberlandian Combshell (*Epioblasma brevidens*) and Oyster Mussel (*E. capsaeformis*) are critically endangered freshwater mussel species native to the Tennessee and Cumberland River drainages, major tributaries of the Ohio River in the eastern United States. The Clinch River in northeastern Tennessee (TN) and southwestern Virginia (VA) harbors the only remaining stronghold population for either species, containing tens of thousands of individuals per species; however, a few smaller populations are still extant in other rivers. We collected and analyzed genetic data to assist with population restoration and recovery planning for both species. We used an 888 base-pair sequence of the mitochondrial NADH dehydrogenase 1 (*ND1*) gene and ten nuclear DNA microsatellite loci to assess patterns of genetic differentiation and diversity in populations at small and large spatial scales, and at a 9-year (2004 to 2013) temporal scale, which showed how quickly these populations can diverge from each other in a short time period. Intraspecific mitochondrial DNA (mtDNA) and microsatellite DNA variation was higher in *E. capsaeformis* than in *E. brevidens*. These two species have maintained quite different levels of genetic diversity within their Clinch River stronghold and in their smaller peripheral populations in the Big South Fork Cumberland and Nolichucky rivers, TN. For instance, with only three mtDNA haplotypes detected overall across populations, *E. brevidens'* capacity for maintaining genetic diversity appears to be less than that of *E. capsaeformis*, which had 18 haplotypes. At the relatively small spatial scales (15-30 kilometers) investigated in the Clinch River, demes of both species exhibited minimal genetic differentiation in either the 2004 or 2013 sampling periods, typically <0.02 based on F_{ST} and <0.1 based on Jost's D. Our genetic data suggest that mussels at the numerous shoals in a 32-kilometer section of the Clinch River comprise a single, large population of each respective species with very high gene-flow among individual demes. However, we also observed a high level of genetic differentiation among demes at the 9-year temporal scale, with differentiation metrics for *E. brevidens* (D = 0.47 and F_{ST} = 0.12) and *E. capsaeformis* (D = 0.31 and F_{ST} = 0.05) proving higher than the within-year values. This result strongly suggests that genetic drift is playing an important role in allele frequency change over time in these populations. At the spatial and temporal scales investigated in this study, various demographic, life history, and environmental factors are influencing maintenance of genetic variation and need to be considered during conservation planning for each species.

Introduction

Habitat fragmentation and restricted gene flow often lead to a loss of genetic diversity and inbreeding within fragmented populations (Frankham et al. 2010). For imperiled species, this may impact individual fitness and increase the probability of extinction of a species. Combined analysis of spatial and temporal variation during the investigation of population genetic structure enhances our ability to understand opposing forces of gene flow and genetic drift, while weighing the impacts of population fragmentation and facilitating gene flow through human-mediated conservation practices. In this study, we investigated the spatial and temporal genetic structure of the Cumberlandian Combshell *Epioblasma brevidens* (Lea, 1831) and Oyster Mussel *E. capsaeformis* (Lea, 1834), two critically endangered freshwater mussel species (U.S. Fish and Wildlife Service 2004) native to the Tennessee and Cumberland River drainages, major tributaries of the Ohio River in the eastern United States (U.S.) (Figure 1). The abundance and distribution of both species have declined by over 90% in the last 100 years, with complete extirpation of populations occurring in dozens of streams throughout each species' range (U.S. Fish and Wildlife Service [USFWS] 2004). These declines are the result of large-scale habitat loss and fragmentation caused by dam construction and operation and pollution of aquatic ecosystems (Neves et al. 1997). Collectively, these drivers of decline have caused widespread population loss and extinction of dozens of species, making freshwater mussels the most imperiled group of animals in the U.S. (Williams et al. 2017). Today, only a handful of streams in these drainages contain extant populations of *E. brevidens* and *E. capsaeformis*. A stronghold for both species is the Clinch River in northeastern Tennessee (TN) and southwestern Virginia (VA) of the eastern U.S., which contains tens of thousands of individuals of *E. brevidens* and hundreds of thousands of individuals of *E. capsaeformis*, representing the largest remaining populations of either species (Jones et al. 2014; Ahlstedt et al. 2016; Jones et al. 2018; Lane et al. 2021). Small native populations not exceeding a few thousand individuals of *E. brevidens* remain in the Powell River in eastern TN and southwestern VA, in Bear Creek in western Alabama (AL) and eastern Mississippi (MS), and in the upper Cumberland River system in the Big South Fork Cumberland River and Buck Creek in north-central TN and south-central Kentucky (KY); the species is being reintroduced to the Duck River and the upper Nolichucky River, TN, and the upper Clinch River, VA (Figure 1). The only remaining native population of *E. capsaeformis* outside of the Clinch River occurs in the lower 12 kilometers

of the Nolichucky River, but the species is being reintroduced to the upper Clinch River, VA, Powell River, TN and VA, upper Nolichucky River, TN, and Paint Rock River, AL (Figure 1). The robustness of the Clinch River populations provides opportunities to reintroduce and restore populations to these and perhaps other rivers throughout each species' native range. Hence, current management efforts are focusing on the release of translocated and hatchery-reared mussels to augment and reintroduce populations to achieve species recovery (USFWS, 2004).

The larvae (glochidia) of *E. brevidens* and *E. capsaeformis* parasitize darters, small (60-120 mm total length) benthic-dwelling percid fishes in the genera *Etheostoma* and *Percina* to metamorphose to the juvenile stage, drop off the host, and thereby to disperse to new habitats (Yeager and Saylor 1995). The mantle of females of both species is modified to mimic small aquatic insect prey (see Figure 2) to lure the host fish into close contact, so that the mussel can capture its host in a manner similar to how a Venus flytrap flower captures insects (Jones et al. 2006). The mantle lures of *E. brevidens* and *E. capsaeformis* exhibit complex morphological variation, likely mimicking caddisfly larvae (Trichoptera), stonefly larvae (Plecoptera), eggs of fish and crayfish and other aquatic species, i.e, a suite of prey-item mimics that they present to attract and capture their host fish (Figure 2). This morphological variation is poorly understood and has not been extensively documented photographically, may represent important phenotypic variation among populations, and should be inventoried and conserved as part of the morphological and adaptive heritage of each species. For example, the mantle-lure color of *E. capsaeformis* is predominately white in the Nolichucky River, TN but predominately blue in the Clinch River, TN (Figure 2); however, both color types occur in each population, but at much lower frequencies (personal observation of the authors). Once the female mussel captures the host fish, her larvae are released from the two outer gills to attach and parasitize the host (Jones et al. 2020). However, due to the presence of dozens of dams fragmenting habitat in the Tennessee and Cumberland River basins, natural re-colonization of previously occupied habitats by dispersal of parasitized host fishes is impossible. Thus, migration assisted by humans will be needed to circumvent these dispersal barriers and thereby allow re-colonization of habitat and maintenance of gene-flow and high genetic diversity.

Outside of the Clinch River, remaining populations are small and comprised of both native and restored populations across each species' range. As these stronghold and small populations fluctuate in size over time due to natural stochasticity, and in some cases, from augmentations of hatchery-reared mussels, we would expect that their genetic composition and diversity would also change over time. Development of effective management strategies for maintaining species-level genetic diversity will require knowing which populations have low and high genetic variation, and which populations are highly genetically diverged from the others and why that divergence has occurred. Has divergence arisen because populations harbor high amounts of unique genetic variation, or is it because they have been bottlenecked due to a range of factors such as habitat fragmentation and loss, losing most of their original genetic variation? Hence, the purpose of this study was to utilize both mitochondrial and nuclear DNA markers to assess spatial and temporal genetic variation of *E. brevidens* and *E. capsaeformis* populations. Genetic data, especially measures of genetic diversity and population structure, are needed to inform population restoration and recovery planning for both species.

Materials And Methods

Tissue Collection and Preparation

Mantle tissue or tissue swab samples of *E. brevidens* and *E. capsaeformis* were obtained in 2004 and 2013 from live mussels collected at various stream locations in the upper Tennessee and Cumberland river basins; see Table 1 for location and sample size information. In 2004, a small piece of mantle tissue (20–30 mg) was collected non-lethally from live mussels from each population (Naimo et al. 1998; Jones et al. 2015). Tissues were preserved in 95% ethanol and stored at -20 °C prior to DNA extraction. Total genomic DNA was isolated from ~20 mg of fresh mantle tissue using the Purgene DNA Extraction kit (Gentra Systems) following the manufacturer's instructions. The concentration of DNA was determined using a Hoefer TKO 1000 fluorometer to provide a standardized quantity for use in the polymerase chain reaction (PCR). In 2013, the DNA samples were collected non-lethally by gently opening each mussel and vigorously swabbing the foot with a buccal swab (Isohelix kit DDK-50). The DNA was isolated and extracted using the Isohelix DNA isolation kit, and its concentration and purity were assessed by using a μLite PC spectrophotometer (Biodrop, Cambridge, UK).

DNA Sequence Analyses

The DNA sequence from the first subunit of NADH dehydrogenase (*ND1*), a protein-encoding, mitochondrial gene, was amplified by polymerase chain reaction (PCR) in a PTC-200 Thermal Cycler (MJ Research) using primers and conditions reported in Serb et al. (2003). Primer sequences for *ND1* were: forward: 5'-TGGCAGAAAAGTGCATCAGATTAAAGC-3'; and reverse: 5'-CCTGCTTGGAAGGCAAGTGTACT-3'. The PCR reaction mixture for *ND1* consisted of 100 ng of genomic DNA, 1x PCR buffer, 4.0 mM $MgCl_2$, 0.4 mM dNTPs, 1.0 mM each primer, and 1.5 U Ampli*Taq* Gold DNA polymerase (Thermo Fisher Scientific), with ddH_2O added to a total volume of 25 mL. The thermal cycling profile consisted of an initial 95°C for 8 min; followed by 35 cycles of: 94°C for 40 sec, 50°C for 60 sec and 72°C for 90 sec; with a final extension step at 72°C for 2 min; and a final hold at 4°C. All PCR products were sequenced with a Big Dye Terminator Cycle Sequencing kit (Thermo Fisher) with Ampli*Taq* DNA polymerase. Cycle sequence reactions were purified using a Qiagen DNA Purification kit before electrophoresis and sequencing using an ABI 3100 automated DNA sequencer (Applied Biosystems, Inc. [ABI], Foster City, CA) at the Virginia Bioinformatics Institute.

The DNA sequences were aligned and edited using the alignment algorithm in the program SEQUENCHER (version 3.0, Gene Codes Corporation, Ann Arbor, MI). Standard indices of intraspecific mtDNA genetic diversity were assessed for populations of each species, including: (1) haplotype diversity (*h*), i.e., the probability that two randomly chosen mtDNA sequences were different in a sample; (2) number of polymorphic or segregating sites (*s*); (3) mean number of nucleotide differences among sequences (*k*); and (4) nucleotide diversity (p), i.e., the probability that two randomly chosen homologous nucleotides were different in the sample (see Nei and Kumar 2000 for equations). The mtDNA diversity was assessed for all populations. including those at three sites or demes in the Clinch River, TN [Wallen Bend (WB), Frost Ford (FF), Swan Island (SI)] and for the global sampled population (i.e., data combined for all three demes within each species), in the Clinch River's largest tributary, the Powell River, and in the Big South Fork Cumberland River (Table 1). The *ND1* DNA sequences from WB, FF, and SI in 2004 were obtained from Jones et al. (2015). Parameter estimates and associated variances were calculated using DNAsp 6.12.03X64 software (Rozas *et al.*, 2018). Mean uncorrected genetic distance (D_{xy}) between populations was calculated in DnaSP, and genetic differentiation (F_{ST}) between populations was calculated in the program ARLEQUIN, version 3.0 (Excoffier et al. 2005).

DNA microsatellites Analyses

Microsatellite loci and primers were developed and characterized using DNA of *E. capsaeformis* (Jones *et al.*, 2004) and *L. abrupta* (Eackles & King, 2002) and were screened in all sampled individuals using a subset of the loci available in these two primer sets (Table 2). The DNA microsatellite genotypes from individuals collected at WB, FF, SI in 2004 were obtained from Jones et al. (2015). PCR amplification protocols followed Eackles & King (2002) and consisted of 100 ng of genomic DNA, 1x PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 mM each primer, and 1.0 U Ampli*Taq* DNA polymerase (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA) in a total volume of 20 mL. PCR thermal cycling conditions were: 94 °C for 2 min; followed by 35 cycles of 94 °C for 40 sec, 58 °C for 40 sec, and 72 °C for 1 min; followed by a final extension at 72 °C for 1 min; and a hold at 4 °C (Eackles & King, 2002).

Amplification products containing microsatellite loci were examined for size polymorphism using an ABI 3100 automated sequencer and GENOTYPER (ABI) software to determine allele size. Results were stored as GENESCAN files; GENEMAPPER software (ABI) was used to visualize allele size and score the results. Microsatellite data sets were tested for genotyping errors caused by null alleles, stuttering, and short allele dominance using a Monte Carlo simulation of expected allele size differences using MICROCHECKER (Van Oosterhout et al. 2004). Populations were screened for linkage disequilibrium (LD) between all pairs of loci and for deviations from Hardy-Weinberg equilibrium (HWE) at each locus in ARLEQUIN. Significance of LD pairwise tests was determined using the likelihood-ratio test with 10,000 permutations (Slatkin and Excoffier 1996), and HWE using the exact test with a Markov chain of 1,000,000 steps and 100,000 dememorisation steps (Guo and Thompson 1992). We used a sequential Bonferroni correction to account for Type I errors associated with all multiple pair-wise comparisons (Rice 1989).

Genetic variability across microsatellite loci for populations of *E. brevidens* and *E. capsaeformis* was quantified in terms of percentage of polymorphic loci, observed heterozygosity, expected heterozygosity, number of alleles per locus, total number of alleles, number of private alleles, and allele frequencies per locus. Evidence for a bottleneck in each population at each locus was tested using the Garza-Williams index (*M*-ratio) as implemented in ARLEQUIN, which is the ratio of the number of alleles to those possible within the observed range in allele size; values below 0.7 suggest the occurrence of a bottleneck (Garza and Williamson

2001). We used program BOTTLENECK, which tests for an excess of heterozygosity as a consequence of a genetic bottleneck to test for a recent bottleneck in each population (Cornuet and Luikart 1996; Piry et al. 1999). The Wilcoxon's sign-rank test was used to test for significant excess of heterozygosity in population samples across loci, which is interpreted as evidence of a recent bottleneck. This is a one-tailed test and is considered the most powerful of all similar tests available in program Bottleneck when the number of polymorphic loci tested is low (<20) (Cornuet and Luikart 1996). The Wilcoxon's sign-rank test was conducted using the two-phase mutation model (TPM), which is considered the best mutation model for DNA microsatellites. The TPM was implemented with the variance set to 12 and the proportion of stepwise mutations set to 90 (Piry et al. 1999; Garza and Williamson 2001). Evidence for inbreeding within each population was tested for using F_{IS}, which measures the extent of departure from HWE due to inbreeding within a subpopulation(s) and can range from -1.0 (all heterozygotes) to +1.0 (all homozygotes) (Wright 1978).

Population differentiation was quantified using the F_{ST} metric in ARLEQUIN, which ranges in value from 0 (no differentiation) to 1 (complete differentiation) (Wright 1978; Balloux & Lugon-Moulin 2002). However, because F_{ST} will approach zero when gene diversity is high, D, — an estimator of actual differentiation developed by Jost (2008) — was estimated using GenAlEx v.6.5 (Peakall and Smouse 2006). This metric also ranges from 0 to 1, but provides a more accurate estimate of differentiation due to genetic drift and gene flow at highly polymorphic loci. The program POPTREE2 (Takezaki et al. 2010) was used to construct phylogenetic trees from our DNA microsatellite allele frequency data by using the neighbor-joining (NJ) method (Saitou and Nei 1987) and Nei's standard genetic distance (D_{ST}) (Nei 1972), utilizing a total of 10,000 bootstrap replications.

We used program STRUCTURE version 2.3.4 (Pritchard et al. 2000) to infer the most likely number of genetically distinct, multilocus genotypic clusters (~populations) and to assign individual genotypes to such populations. An admixture ancestry model with correlated allele frequencies was used to determine population structure of *E. brevidens* and *E. capsaeformis* individuals. No priors or population information were used in the analysis. The best-supported number of populations (K) was assessed by calculating the mean natural logarithm of the probability of K [mean Ln$P(K)$] (Pritchard and Wen 2003) across iterations from K = 2-9 and with ten independent runs for each K. The largest mean Ln$P(K)$ is considered the optimal value of K. All runs consisted of a burn-in of 10,000 steps, followed by 100,000 iterations. Finally, runs generating the highest likelihood estimates were imported to the program STRUCTURE PLOT at http://btismysore.in/strplot/index.php (Ramasamy et al. 2014) to create a visualization of population structure.

Contemporary effective population sizes (N_e) were estimated for each population using the linkage disequilibrium (LD) method of Hill (1981). The method is known to be downwardly biased, but the program LDNe corrects the bias and was used to estimate N_e (Waples 2006; Waples and Do 2007).

Results

Genetic variation of mitochondrial DNA sequences

Intraspecific mitochondrial DNA sequence variation at the *ND1* gene generally was higher in *E. capsaeformis* than in *E. brevidens* (Table 3). The number of polymorphic nucleotides was higher in *E. capsaeformis* (n = 22) than in *E. brevidens* (n = 4), and a total of 18 haplotypes were observed among all *E. capsaeformis* populations, compared to just three haplotypes observed among all *E. brevidens* populations (Figure 3, Panels A and B; Table 4). Further, none of the three *E. brevidens* haplotypes were unique to the study samples; they were shared among the Big South Fork Cumberland River, Clinch River and Powell River populations, and shared between the 2004 and 2013 sampling periods. However, the Big South Fork Cumberland River sample only contained one haplotype (*Ebrev2*), and it was the same haplotype observed in the Clinch and Powell river samples (Table 4). In contrast, for *E. capsaeformis*, eleven of the thirteen haplotypes observed in the Clinch River population and five of the seven haplotypes observed in the Nolichucky River population were unique. Two haplotypes (*Ecap1* and *Ecap5*) were shared between the two populations, but at different proportions in the respective samples. Several haplotypes were unique to the three demes in the Clinch River (WB, FF, SI) and between the 2004 and 2013 sampling periods (Table 4).

To summarize the genetic diversity metrics for both species, global haplotype diversity for *E. brevidens* was h = 0.59, but ranged from a low of h = 0 in the Big South Fork Cumberland River sample to a high of h = 0.66 in the Clinch River sample at Frost Ford in 2004 (Table 3). Global nucleotide diversity was p = 0.0018, with p in population samples ranging from 0 to 0.0017. Mean k was 1.57 nucleotide differences among sequences, ranging from 0 to 1.82. Global haplotype diversity for *E. capsaeformis* was h = 0.68, but ranged from a low of h = 0.49 in the Clinch River sample at WB in 2013 to a high of h = 0.90 in the Nolichucky River sample in 2013 (Table 3). Global nucleotide diversity was p = 0.0024, with p in population samples ranging from 0.0017 to 0.0040. Mean k was 2.09 nucleotide differences among sequences, ranging from 1.54 to 3.59.

Mitochondrial DNA genetic distance and differentiation among populations

For *E. brevidens*, uncorrected sequence divergence (D_{xy}) values among populations were highest when involving comparisons with the Big South Fork Cumberland (range = 0.00243 − 0.00308) and the Powell River (range = 0.00217 − 0.00263), whereas D_{xy} values were lowest among Clinch River demes (WB, FF, SI) and sampling years (2004, 2013) (Table 5). Haplotype differentiation (F_{ST}) among populations ranged from zero (with numerous negative values indicative of negligible differentiation) to a high of 0.75 between the Wallen Bend (2004) and Big South Fork Cumberland (2004) samples (Table 5). Similarly, pairwise F_{ST} comparisons involving the Big South Fork Cumberland (range = 0.59 − 0.75) and the Powell River (range = 0.27 − 0.47) and other populations were the highest and significantly different, whereas F_{ST} among demes and years in the Clinch River were lower and non-significant.

For *E. capsaeformis*, uncorrected sequence divergence (D_{xy}) values among populations were highest between the Nolichucky River to the Clinch River demes, typically averaging 0.011 among pairwise comparisons, whereas D_{xy} values were lowest among the Clinch River demes (WB, FF, SI) and sampling years (2004, 2013) (Table 5). Similarly, pairwise F_{ST} comparisons involving the Nolichucky River (range = 0.23 − 0.39) with other populations were the highest and significantly different, whereas F_{ST} among demes and years in the Clinch River were lower and mostly non-significant. However, pairwise comparisons involving WB (2013) and the other Clinch River demes were higher and significantly different.

Genetic variation of nuclear DNA microsatellites

Genetic diversity at microsatellite loci generally was lower in *E. brevidens* than in *E. capsaeformis* (Table 6). Mean expected heterozygosity in *E. brevidens* ranged from a low of H_e = 0.68 in the Powell River in 2013 to a high of H_e = 0.79 at Frost Ford and Swan Island in the Clinch River in 2013, whereas mean expected heterozygosity in *E. capsaeformis* ranged from a low of H_e = 0.79 in the Nolichucky River in 2013 to a high of H_e = 0.85-0.86 at nearly all sites in the Clinch River in 2004 and 2013. Similarly, allelic diversity (mean number of alleles per locus) in *E. brevidens* ranged from a low of A = 3.9 in the Powell River in 2013 to a high of A = 9.8 at Frost Ford in 2013, whereas allelic diversity in *E. capsaeformis* ranged from a low of A = 11.3 at Wallen Bend in 2013 to a high of A = 12.8 at Frost Ford in 2004 and Swan Island in 2013. Allele frequencies at each microsatellite locus for each population are reported in the Appendix.

After the threshold for the significance of *p*-values for the respective locus pairs was Bonferroni corrected ($p < 0.001$; a = 0.05), none of the *E. brevidens* populations in either the 2004 or 2013 samples contained loci with significant deviations from linkage equilibrium. Significant deviations ($p < 0.001$; a = 0.05) from Hardy-Weinberg equilibrium were observed at *Ecap01*, *Ecap06*, *Ecap08*, and *Ecap09*, but the loci in disequilibria were distributed randomly among 1-3 loci per population. Further, no evidence was found for genotyping errors or large-allele drop-out, although increased homozygosity at some loci suggested the possible segregation of null alleles, or inbreeding due to small population size and hermaphroditic reproduction (van der Schalie 1970). Each population contained a small number of private alleles not detected in other sampled populations, with 1-6 private alleles observed per population; however, the Big South Fork Cumberland River population contained a high number of 19 private alleles (Table 6; see Appendix).

After the threshold for significance of *p*-values for the respective locus pairs was Bonferroni corrected ($p < 0.001$; a = 0.05), none of the *E. capsaeformis* populations in either the 2004 or 2013 samples contained loci with significant deviations from linkage equilibrium. Significant deviations ($p < 0.001$; a = 0.05) from Hardy-Weinberg equilibrium were observed at *Ecap01*,

Ecap02,Ecap04, Ecap06, Ecap08, and *Ecap09, Lab206* and *Lab213*, but the loci in disequilibria were distributed randomly among a small number of loci per population. Further, no evidence was found for genotyping errors or large-allele drop-out, although increased homozygosity at some loci suggested the possible segregation of null alleles, or inbreeding due to small population size and hermaphroditic reproduction (van der Schalie 1970). Each population contained a small number of private alleles not detected at other sampled populations, with 2-6 private alleles observed per population; with the Nolichucky River population containing the highest number of private alleles, 7 (Table 6).

Genetic evidence for bottlenecks and inbreeding in populations

For *E. brevidens*, mean observed *M*-ratio values per population (Table 6) ranged from 0.27 in the Powell River to 0.60 at Frost Ford (2013) in the Clinch River population, suggesting the effects of past bottlenecks at certain loci. Results of the Wilcoxon sign-rank tests conducted in program BOTTLENECK were not significant for most populations, except for the Clinch River at Frost Ford in 2004 ($p = 0.001$) and 2013 ($p = 0.001$) (Table 6). Mean F_{IS} values per population ranged from 0.05 at Swan Island in the Clinch River to 0.27 in the Big South Fork Cumberland River, indicating a low to moderate level of inbreeding in each population (Table 6).

For *E. capsaeformis*, mean observed *M*-ratio values per population (Table 5) ranged from 0.54 in the Nolichucky River to 0.61 at Swan Island (2013) in the Clinch River, suggesting the effects of past bottlenecks at certain loci. Results of the Wilcoxon sign-rank tests conducted in program BOTTLENECK were not significant for any of the tested populations (Table 6). Mean F_{IS} values per population ranged from 0.02 at Swan Island (2004) to 0.23 at Wallen Bend (2013) in the Clinch River, indicating a low level of inbreeding in each population (Table 6).

Genetic divergence among populations at nuclear DNA microsatellites

For *E. brevidens*, spatial and temporal allele frequency divergence among populations and sampling years 2004 and 2013 was moderate to high based on *D* and F_{ST} metrics (Figure 4, Panels A and B). At a broader spatial scale (Panel A), divergence values among the three populations ranged from a high of $D = 0.81$ and $F_{ST} = 0.23$ between the Big South Fork Cumberland River and Powell River population samples, to a low of $D = 0.35$ and $F_{ST} = 0.10$ between the Big South Fork Cumberland River and the 2004 Clinch River population samples. However, all of the among-population divergence levels presented in Panel A as quantified by *D* are considered high, while the F_{ST} values are moderate to high. At a finer spatial scale (Panel B), divergence levels among the three demes in the Clinch River were much lower. In 2004, values ranged from a high of $D = 0.021$ and $F_{ST} = 0.008$ between Frost Ford and Swan Island, to zero or negligible levels of $D = 0.0$ and $F_{ST} = 0.0001$ between Frost Ford and Wallen Bend; similarly in 2013, values ranged from a high of $D = 0.09$ and $F_{ST} = 0.03$ between Frost Ford and Swan Island, to a low of $D = 0.009$ and $F_{ST} = 0.004$ between Wallen Bend and Swan Island. All of the between-deme *D* and F_{ST} values in either 2004 or 2013 among Clinch River samples are considered low. However, temporally (Panel B), when comparing divergence levels of the same demes between years 2004 and 2013, divergence levels were much higher, averaging $D \approx 0.6$ and $F_{ST} \approx 0.15$. These divergence values are considered high and an order of magnitude higher than among-deme, within-year comparisons. When genotype data from the three demes are combined per year and the 2004 and 2013 sampling periods compared, then $D = 0.47$ and $F_{ST} = 0.12$ (Panel A). The NJ tree (Figure 5; Panel A) shows strong separation with high bootstrap support (99%) between the 2004 and 2013 Clinch River data sets, but with minimal separation and low bootstrap support among demes within each respective year; further, the Big South Fork Cumberland River population is embedded within the 2004 Clinch River demes and the Powell River population is embedded with the 2013 Clinch River demes.

For *E. capsaeformis*, spatial and temporal allele frequency divergence among populations and sampling years 2004 and 2013 was low to moderate based on *D* and F_{ST} metrics (Figure 4, Panels C and D). At a broader spatial scale (Panel C), divergence levels between the Nolichucky River sample and the 2004 and 2013 Clinch River samples were $D = 0.37$ and $F_{ST} = 0.08$ and $D = 0.31$ and $F_{ST} = 0.06$, respectively. At a finer spatial scale (Panel D), divergence levels among the three demes of *E. capsaeformis* in the Clinch River were much lower. In 2004, values ranged from a high of $D = 0.079$ and $F_{ST} = 0.015$ between the Wallen Bend and Swan Island demes, to a slightly lower level of $D = 0.17$ and $F_{ST} = 0.004$ between the Frost Ford and Swan Island demes;

similarly in 2013, values ranged from a high of D = 0.039 and F_{ST} = 0.008 between the Frost Ford and Swan Island demes, to a low of D = 0.005 and F_{ST} = 0.004 between the Frost Ford and Wallen Bend demes. All of the between-deme D and F_{ST} values in either 2004 or 2013 in the Clinch River are considered low. However, at a temporal scale, when comparing the divergence of the same demes between years 2004 and 2013 (Panel D), the divergence values were much higher, typically at $D \approx 0.31$ and $F_{ST} \approx$ 0.05. These divergence levels are considered low to moderate, but are much higher than among-deme within-year comparisons. When genotype data from the three demes are combined per year and the 2004 and 2013 sampling periods compared, then D = 0.31 and F_{ST} = 0.05 (Panel C). The NJ tree (Figure 5; Panel B) shows strong separation with high bootstrap support (99%) between the 2004 and 2013 Clinch River data sets, but with minimal separation and low bootstrap support among demes within each respective year; further, the Nolichucky River population is embedded with the 2013 Clinch River demes.

Testing for population genetic structure using assignment tests

For *E. brevidens*, results of the assignment test analysis conducted in program STRUCTURE yielded the best-supported K value at K = 4 biologically informative clusters, which respectively included Big South Fork Cumberland River, Clinch River 2004, Clinch River 2013, and Powell River populations and sample years (Figure 6, left top panel). None of the three demes (Wallen Bend, Frost Ford, Swan Island) in the Clinch River in either the 2004 or 2013 sample periods clustered as a separate population. The best supported values of K = 4 yielded an average Ln$P(K)$ value of -7107.4 (SD = 3.1) (Figure 6, left bottom panel).

For *E. capsaeformis*, results of the assignment test analysis conducted in program STRUCTURE yielded the best-supported K value at K = 3 biologically informative clusters, which respectively included the Clinch River 2004, Clinch River 2013, and Nolichucky River populations and sample years (Figure 4, right top panel). None of the three demes (Wallen Bend, Frost Ford, Swan Island) in the Clinch River in either the 2004 or 2013 sample periods clustered as a separate population. The best supported K=3 value yielded an average Ln$P(K)$ value of -9954.1 (SD = 1.7) (Figure 6, right bottom panel).

Effective population size N_e

For *E. brevidens*, estimates of N_e ranged from a high of 222 (CI = 49 − infinity) individuals at Wallen Bend in 2004 to a low of 105 (52 − infinity) individuals at Frost Ford in 2013 in the Clinch River (Figure 7). Estimated N_e at Wallen Bend in 2013 was -132 (effectively 0), and was not considered an accurate estimate; therefore, no value is reported in Figure 7. Estimated N_e in 2004 was 96 (CI = 29 − infinity) individuals in the Big South Fork Cumberland River. Generally, the per-year and per-site estimates of N_e ranged between 100-200 individuals. However, when genotype data from all three sites in the Clinch River were combined, N_e in 2004 was 612 (CI = 175 − infinity) and N_e in 2013 was 619 (CI = 235 − infinity) (Figure 7). In 2004, estimates of N_e were available from all three sites, and when these separate estimates are totaled together, N_e equals 584, a value that is very similar to an estimate of 612 individuals from the pooled data.

For *E. capsaeformis*, estimates of N_e ranged from a high of 2,917 (CI = 129 − infinity) individuals at Frost Ford in 2004 to a low of 294 (95 − infinity) individuals at Swan Island in 2004 in the Clinch River, and N_e in 2013 was 224 (CI = 113 − infinity) individuals in the Nolichucky River (Figure 7). With the exception of Frost Ford in 2004, the per-year and per-site estimates of N_e ranged between about 200-750 individuals; however, when genotype data from all three sites in the Clinch River were combined, the estimated N_e in 2004 was 11,430 (CI = 391 − infinity) and in 2013 it was 18,084 (CI = 468 − infinity) (Figure 7). In 2004 and 2013, estimates of N_e were available from all three sites in the Clinch River, and when these separate estimates are totaled together, N_e equaled 3,561 and 1,433 individuals, respectively, values that are much lower than for the pooled data.

Discussion

In this study, we assessed patterns of genetic diversity and differentiation in populations of *Epioblasma brevidens* and *E. capsaeformis* at both small and large spatial scales; for the first time with freshwater mussels, we assessed diversity and differentiation at a temporal scale, showing how quickly these populations can diverge from each other over a relatively short time period of just 9 years or approximately one generation for either species. These two species have maintained quite different levels of genetic diversity within their Clinch River stronghold, and also in much smaller peripheral populations in the Big South

Fork Cumberland and Nolichucky rivers. For instance, with only three mtDNA haplotypes detected overall across populations, *E. brevidens'* capacity for maintaining genetic diversity appears to be less than that of *E. capsaeformis*. Hence, the spatial and temporal scales, demographic, life history, and environmental factors influencing maintenance of genetic variation for these two species require more in-depth discussion.

At the relatively small spatial scales (15-30 kilometers) investigated within the Clinch River, the three demes exhibited minimal genetic differentiation for both species. While some statistically significant differences were observed between demes in 2004 and 2013, differentiation levels were very low, typically <0.02 based on F_{ST} and <0.1 based on Jost's *D* for both species. The river is free-flowing in Hancock County, TN, and therefore no barriers exist among demes that would prevent dispersal of host fishes, downstream dispersal of adults or downstream drift of sperm during spawning. Thus, our genetic data suggests that at the dozen or so major shoal areas in this reach of the river (RKM 276.8-309.0), which collectively contain tens of thousands of individuals of *E. brevidens* and *E. capsaeformis* (Jones et al. 2018; Lane et al. 2021), would best be described as containing a single large functional population of each respective species, with very high gene-flow among the individual demes.

However, at a larger spatial scale, a much different pattern of genetic diversity and differentiation emerges. For *E. brevidens*, the Big South Fork Cumberland River population appears devoid of mtDNA diversity at the *ND1* gene, and as a whole range-wide, the species appears to be low in variation at the mitochondrial genome, especially given that the Clinch River population is its last stronghold and mtDNA variation is low even there. This result is expected, given the anthropogenic fragmentation caused by construction of dams, impounded back-waters, and cold-water temperature zones below hypolimnetic-releasing reservoirs. The *ND1* haplotypes sampled in the Big South Fork Cumberland and Powell rivers were identical to those in the Clinch River, suggesting that mtDNA diversity is low and similar across the species' range in the Cumberland and Tennessee river valleys. However, at the mtDNA *cytochrome-b* gene, a 1-bp fixed difference was observed between samples in the Big South Fork Cumberland and Clinch rivers (Johnson et al. 2006), indicating that some unique variation does exist even in the small remaining populations. Thus, assessing genetic diversity of the Buck Creek, KY and Bear Creek, AL and MS populations of *E. brevidens* would help test this interpretation. Genetic differentiation at nuclear DNA microsatellites among the Big South Fork Cumberland, Clinch and Powell river populations was very high based on *D* and moderate to high based on F_{ST}, which is not surprising given that the remaining populations are small and bottlenecked and separated by hundreds of river miles and numerous barriers, including large hydro-power dams. While observed mtDNA variation was not unique among *E. brevidens* populations, the Big South Fork Cumberland River population contained numerous private microsatellite alleles, suggesting that this population has unique or novel genetic variation when compared to the Clinch River population for example, which is expected given the geographic distance (1,200 river kilometers) separating the two populations.

For *E. capsaeformis*, the Nolichucky River population, while small, still retains relatively high mtDNA haplotype diversity. Our mtDNA sample size was small (*N* = 13) but a total of seven haplotypes, five of which were unique, were observed in this small peripheral population. Given our small sample size, but high observed haplotype diversity, it is likely that additional haplotypes might be discovered in this population with additional sampling. However, with eighteen total haplotypes observed—thirteen unique to the Clinch River and five unique to the Nolichucky River—global haplotype diversity is much higher in *E. capsaeformis* than in *E. brevidens*. Hence, two interpretations merit discussion here. First, the Nolichucky River may have supported a much larger population of *E. capsaeformis* in the not-too-distant past than previously thought. There are more than a dozen major island complexes located throughout a ~74 kilometer reach (RKM 19.6–93.8) of the river, each with significant shoal habitat for mussels (T. Lane, unpublished data). Nearly all of these sites are larger (>20,000 m^2) than the largest sites in the Clinch River, TN for example. Hence, the amount of physical habitat that is available in the river is more than enough to support a large population. Second, the capacity of *E. capsaeformis* to maintain genetic diversity seems to exceed that of *E. brevidens*. In the Clinch River, TN, where both species co-occur at all sites, *E. capsaeformis* genetic diversity is higher, where presumably both species have been exposed to the same environmental and historical conditions. Genetic differentiation at nuclear DNA microsatellites between the Clinch and Nolichucky river populations of *E. capsaeformis* was high based on *D* and moderate to low based on F_{ST}, although these metrics were still much higher than the among-deme comparisons in the Clinch River, which again were very low. To reiterate, this increase in differentiation at a larger spatial scale is not surprising given that these two populations also are separated by hundreds of river miles and numerous large hydro-power dams.

Demographic and life-history factors, such as changes in population size, longevity, and contrasting fish host life-histories, offer the best explanations for why genetic diversity was greater in *E. capsaeformis* than in *E. brevidens*. The census population size of *E. capsaeformis* is much larger than that of *E. brevidens* in the TN section of the Clinch River (Figure 8). From 2004-2014, populations of both species were censused annually at Swan Island, Frost Ford, and Wallen Bend, which showed that across these three sites, combined mean total population size over the 10-year period was ~12,000 individuals of *E. brevidens* versus 285,000 individuals of *E. capsaeformis* (Jones and Neves 2011; Jones et al. 2014; Jones et al. 2018; Lane et al. 2021 in review). Further, fluctuations in population size were much greater for *E. capsaeformis* relative to *E. brevidens*, with population size increasing five-fold for *E. capsaeformis* from 2007-2009 for example, then decreasing significantly over the following years, whereas *E. brevidens* exhibited a much more stable population trend with only modest, non-significant fluctuations in size over this same period (Figure 8). Hence, the *E. capsaeformis* population seems to have a greater inherent capacity to grow quickly and maintain a larger population size, allowing it to maintain more genetic variation, or stated differently, *E. brevidens* which has a much smaller population size making it more susceptible to losing genetic variation through random genetic drift. However, *E. capsaeformis* is a relatively short-lived species (maximum age of at least 12 years), and thus despite any advantages it may have in population growth rate and size under seemingly favorable conditions, *E. brevidens* is a longer-lived species (maximum age at least 28 years) and is better at persisting under unfavorable conditions (Jones and Neves 2011). For example, small recruiting populations of *E. brevidens* have persisted in the Big South Fork Cumberland and Powell rivers despite decades of impacts from coal mining, whereas *E. capsaeformis* was extirpated decades ago from both rivers (Johnson et al. 2012; Ahlstedt et al. 2016; Zipper et al. 2016).

Likely one of the biggest drivers of differences in population size between these two mussel species is the relative abundance and specialization in use of their primary fish hosts. In the Clinch River, *E. brevidens* uses two large, relatively mobile darters (Percidae), the blotchside logperch (*Percina burtoni*) and common logperch (*P. caprodes*), as its primary hosts (Yeager and Saylor 1995). Both fish species are typically 120-130 mm in total length, considered rare to uncommon in the river, and have a sturdy cartilaginous snout that it uses to flip small stones to hunt for aquatic insect prey items (Jenkins and Burkhead 1993). Importantly, the snout and skull are sufficiently strong to withstand capture and holding during infestation of glochidia by females of *E. brevidens* (Barnhart et al. 2008). In contrast, *E. capsaeformis* uses smaller, presumably less mobile darters in the genus *Etheostoma*, such as Redline Darter (*E. rufilineatum*) and Bluebreast Darter (*E. camurum*) as primary hosts (Yeager and Saylor 1995). These fish species are typically 50-60 mm in total length, and are considered common to abundant in the river (Jenkins and Burkhead 1993). Females of *E. capsaeformis* utilize a cushioned mantle-pad to cradle these small darters to minimize harm to them when they are being infested with glochidia (Jones et al. 2006; Barnhart et al. 2008; Jones et al. 2020). The fish hosts of *E. brevidens* likely number in the dozens per site, whereas the fish hosts of *E. capsaeformis* likely number in the thousands per site, which allows this species to have much greater contact with its host fish and ultimately the capacity for reproduction to build up large local populations.

Perhaps the most interesting finding in our study was the high level of genetic differentiation observed among the demes in the Clinch River, TN between the 2004 and 2013 sample periods. Our within-year data clearly show that differentiation was very low and thus high gene-flow existed among demes, indicating that the mussels inhabiting the numerous habitat patches in this section of river are collectively acting together as a single large population. This low within-year differentiation also speak to the adequacy of our sample sizes for the demes. The 2004 and 2013 STRUCTURE analyses for each species showed that individuals from each deme shared essentially the same overall genetic ancestry and thus comprised single, large populations, respectively (Figure 6). However, at a 9-yr. temporal scale, genetic differentiation increased greatly for both species, with differentiation values between 2004 and 2013 for *E. brevidens* (D = 0.47 and F_{ST} = 0.12, Figure 4 Panel A) and *E. capsaeformis* (D = 0.31 and F_{ST} = 0.05, Figure 4 Panel B) increasing greatly relative to the within-year values. This result strongly suggests that random genetic drift is playing a big role in driving allele frequency change over time in these two populations; further, the numerous individual demes within this reach of river are not drifting apart from each other, but rather they are staying inter-connected through high gene-flow, but drifting genetically together in unison over time as a single, large population. In addition, within-year differentiation was slightly less among demes for *E. brevidens* compared to *E. capsaeformis*, suggesting higher gene-flow, perhaps due to its more mobile fish hosts; further, differentiation between years was much greater (nearly double) among demes for *E. brevidens* than for *E. capsaeformis*, suggesting that the smaller population size of this species is increasing the rate

of random genetic drift. In contrast, corresponding increases in mtDNAbased genetic differentiation between 2004 and 2013 for *E. brevidens* (F_{ST} = 0.012) and *E. capsaeformis* (F_{ST} = 0.008) within the Clinch River were not observed, but rather differentiation was elevated geographically between the Clinch River populations and the small peripheral populations of each species (Table 5). The Clinch River population (all three sites combined) of *E. brevidens* changed very little with regard to haplotype frequency over the period from 2004-2013 (Figure 3, Panel A). However, the Clinch River population of *E. capsaeformis* showed noticeable changes in haplotype frequencies over the same time period, with some previously observed haplotypes absent in 2013 and several new haplotypes now frequently observed relative to the overall samples (Figure 3. Panel B).

Our estimates of N_e in 2004 and 2013 were convergent with the census size (N_c) estimates made by Jones et al. (2014, 2018) in two important ways. First, values of N_e and N_c at all three demes were lower for *E. brevidens* than for *E. capsaeformis*, and second, estimates of population size were much less variable for *E. brevidens* relative to *E. capsaeformis* (Figures 7 and 8). Since our data showed minimal differentiation and high gene-flow among demes for both species, we combined the microsatellite DNA genotype data from all three demes to estimate N_e (Figure 5). By increasing the sample size, estimates of N_e are expected to improve, i.e., to become more accurate and precise (Waples and Gagiotti 2006). Hence, the combined data indicate that N_e was very stable over time and much lower for *E. brevidens*, whereas for *E. capsaeformis* N_e was larger and more variable, and increased substantially from 2004 and 2013. Again, this pattern of lower N_c but stable N_e for *E. brevidens* likely is driven by the species' greater longevity and its less-common host fishes, whereas for *E. capsaeformis* both N_c and N_e are larger but more variable, and likely tied to the species' short lifespan and its abundant host fishes. Interestingly though, the N_e/N_c ratios were quite similar, essentially 10% in 2004 and 7% in 2013 for both species (Figure 5). While our N_e confidence intervals are large, we believe these estimated ratios should best be viewed as upper-end values, as the total N_c used to calculate them were derived by adding the N_c values only from Wallen Bend, Frost Ford, and Swan Island. For example, Jones and Neves (2011) estimated N_c at all demes from 2004-2008 in RKM 276.8-309.0 and reported that total N_c was 46,436 individuals for *E. brevidens* and 862,426 individuals for *E. capsaeformis*. Using these census values to estimate N_e/N_c ratios for 2004, for example, would be 1.3% for both species; when considered together with estimates derived from just the three sites, each species' respective N_e/N_c ratio ranges from 1-10% in the Clinch River, TN. An understanding of this ratio is important because it gives insight into the genetic and demographic viability of a population. Other invertebrate species (e.g., Pacific oysters) that broadcast-spawn have very low N_e/N_c ratios (<1%), meaning that they need large census sizes to support a breeding population capable of maintaining genetic variation over time (Hedgecock and Pudovkin 2011). Further, understanding the N_e/N_c ratio allows estimation of N_c from N_e when census data are unavailable. For instance, applying a N_e/N_c range of 1-10% to the Nolichucky River N_e of 224 individuals of *E. capsaeformis* gives the N_c estimate of 2,240-22,400 individuals, and to the Big South Fork Cumberland River N_e of 95 individuals of *E. brevidens* gives the N_c estimate of 950-9,500 individuals. Even though neither population has been quantitatively surveyed, these rough estimates of N_c are likely to be useful to conservation planners, especially when they consider the long-term adaptive potential of focal populations.

Due to various anthropogenic changes to stream hydrology and pollutant stressors over the last 100 years, the range and abundance of *E. brevidens* and *E. capsaeformis* have contracted by >90%, causing both species to become critically endangered (USFWS 2004). Outside of their last stronghold in the Clinch River, remaining populations are small (hundreds to perhaps a few thousand individuals) and completely cut off from each other demographically by numerous dams, with no contemporary gene-flow occurring among them. For *E. brevidens*, small native populations remain in the Powell River, TN and VA, Bear Creek, AL and MS, Big South Fork Cumberland River, TN and KY, and Buck Creek, KY. Four additional populations located in the Duck River, TN, Elk River, AL, upper Nolichucky River, TN and upper Clinch River, VA have been restored by releasing hatchery-reared progeny and translocating wild collected adults, all of which were derived from broodstock collected in the Clinch River, TN (Hubbs 2020). Additional genetic sampling is needed for the Bear Creek, Powell River and Buck Creek populations of *E. brevidens* but individuals are rare in these populations making sampling difficult. Few or no genetic samples or data yet exist for these populations, which would inform understanding of the remaining genetic diversity of the species. For *E. capsaeformis*, the lower Nolichucky River houses the only extant native population outside of the Clinch River; similarly, five additional populations have been restored to the Elk and Paint Rock rivers, AL, upper Nolichucky River, TN, Powell River, TN and upper Clinch River, VA; all broodstock came from the Clinch River, TN (Hubbs 2020). Strategies to maintain and enhance the genetic representation of the native population

of *E. capsaeformis* in the lower Nolichucky River should be given high priority. This small native population contains unique genetic variation for the species, and managers need to be careful that it is not lost due to further population decline or from genetic swamping from overstocking with Clinch River animals. Because the Clinch and Nolichucky River populations are strongly differentiated at nuclear and mitochondrial DNA markers, additional investigation of these populations using nuclear single nucleotide polymorphism (SNP) markers may help elucidate the phylogenetic depth of this differentiation, and further inform conservation planning for the species. Currently, the restoration site containing Clinch River individuals and the native Nolichucky River population are separated by more than 15 river kilometers, but in time mixing may occur. Hence, for now, they can be managed separately to maximize genetic representation. With so much genetic diversity still maintained in the Nolichucky River population, managers should consider using the native population as a source for broodstock alongside the Clinch River stock to further diversify augmentation of upstream and other recipient populations. Finally, consideration should be given to assessing the genetic diversity of restored populations of each species to see whether they are maintaining diversity levels similar to that in the lower Clinch River, TN populations, and to assessing growth, survival and phenotypic variation of the mantle-lure displays at the population level for both species.

Declarations

Author Contributions: Conceptualization, J.W.J. and T.L.; methodology, J.W.J. and T.L.; validation, ALL; formal analysis, J.W.J and T.L.; investigation, J.W.J., T.L., N.A.J.; resources, J.W.J., T.L., N.A.J., and E.M.H.; data curation, J.W.J.; writing—original draft preparation, J.W.J.; writing—review and editing, All; visualization, ALL; project administration, J.W.J. and E.M.H.; funding acquisition, J.W.J. and E.M.H. All authors have read and agreed to the published version of the manuscript.

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Tables

Table 1. River and site names and location data for DNA samples obtained for mussel species investigated in this study. NA = data not available.

River Name	Site location name	Year(s) Sampled	River Kilometer	River Mile	Latitude	Latitude
Big South Fork Cumberland River	Parch Corn Creek Shoal, Scott County, TN	2004	NA	NA	36°33'35.17"N	84°40'17.96"W
Clinch River	Swan Island, Hancock County, TN	2004, 2013	277.1	172.2	36°28'26.42"N	83°17'24.50"W
	Frost Ford, Hancock County, TN	2004, 2013	291.8	181.3	36°31'49.75"N	83° 9'2.77"W
	Wallen Bend, Hancock County, TN	2004, 2013	309.6	192.4	36°34'45.80"N	83° 0'14.80"W
Nolichucky River	Thomas Hurley Island, Cocke County, TN	2013	13.7	8.5	36° 8'5.81"N	83°12'9.68"W
	Beech Bottoms Island, Hamblen County, TN	2013	18.3	11.4	36° 8'35.11"N	83°10'42.41"W
	Johnson Island, Cocke County, TN	2013	21.7	13.5	36°10'3.92"N	83°11'13.40"W
	Shoal Below Solomon, Cocke County, TN	2013	30.3	18.8	36° 9'41.93"N	83°10'17.71"W
Powell River	Parkey Property, Hancock County, TN	2013	165.3	102.7	36°33'38.81"N	83°23'33.98"W

Table 2. Total sample sizes (*N*) of individuals genotyped per mitochondrial DNA sequences and DNA microsatellite loci for Cumberland combshell (*Epioblasma brevidens*) and Oyster Mussel (*Epioblasma capsaeformis*). Individuals were collected from sites in the Big South Fork (BSF) Cumberland River, Scott County, TN, Clinch River (CR) and Powell (PR) rivers, Hancock County, Tennessee and from the Nolichucky River (NR), Hamblen County, Tennessee. --- = locus not analyzed for population.

				nDNA Microsatellite Loci									mtDNA	
Species	*N*	Site	Year	*ND1*	*Ecap01*	*Ecap02*	*Ecap04*	*Ecap05*	*Ecap06*	*Ecap07*	*Ecap08*	*Ecap09*	*Lab206*	*Lab213*
E. brevidens	18	BSF	2004	17	18	18	18	18	18	18	18	18	---	---
E. brevidens	79	CR	2004	72	77	79	79	79	77	79	78	78	78	79
E. brevidens	90	CR	2013	86	90	90	90	90	90	90	90	90	90	90
E. brevidens	5	PR	2013	5	5	5	5	5	5	5	5	5	5	5
E. capsaeformis	90	CR	2004	90	90	90	90	90	90	90	90	90	89	88
E. capsaeformis	90	CR	2013	90	90	90	90	90	90	90	90	90	89	88
E. capsaeformis	39	NR	2013	14	39	39	39	39	39	39	39	39	39	39

Table 3. Summary of observed mitochondrial DNA sequence variation at 888 base-pairs of the *ND1* gene for *Epioblasma brevidens* and *Epioblasma capsaeformis* at multiple sites located in the Big South Fork (BSF) Cumberland River at Parchcorn Creek (PC) shoal, Scott County, TN, the Clinch River (CR) at three sites Wallen Bend (WB), Frost Ford (FF) and Swan Island (SI) and the Powell River (PR) at the Parkey Property (PP) all located in Hancock County, TN and the Nolichucky River (NR) located in Hamblen County, TN. Total values were obtained by pooling and analyzing data together from all three sites in the Clinch River and global values were obtained by pooling and analyzing data from all sites and years.

Species	Year	River	Site	Sample Size (N)	No. Haplotypes (unique)	Haplotype Diversity (h)	No. Segregating Sites (s)	Mean No. Nucleotide Differences (k)	Nucleotide Diversity (p)
E. brevidens	2004	BSF	PC	17	1 (0)	0	0	0	0
	2004	CR	WB	22	3 (1)	0.51	4	1.82	0.0013
			FF	28	3 (0)	0.66	4	1.66	0.0019
			SI	22	2 (0)	0.37	3	1.10	0.0012
			Total	72	3	0.54	4	1.34	0.0015
	2013	CR	WB	30	3	0.58	4	1.23	0.0014
			FF	30	3	0.59	4	1.54	0.0017
			SI	26	3	0.54	4	1.48	0.0017
			Total	86	3	0.57	4	1.42	0.0016
	2013	PR	PP	5	2(0)	0.40	3	1.20	0.0014
			Global	180	3	0.59	4	1.57	0.0018
E. capsaeformis	2004	CR	WB	30	7 (2)	0.66	10	1.65	0.0019
			FF	30	6 (1)	0.64	8	1.54	0.0017
			SI	30	5(0)	0.71	8	2.38	0.0027
			Total	90	3 (3)	0.66	12	1.82	0.0021
	2013	CR	WB	30	4 (0)	0.49	7	1.73	0.0020
			FF	30	5 (1)	0.63	7	1.63	0.0018
			SI	30	7(1)	0.67	9	1.91	0.0022
			Total	90	2 (2)	0.62	11	1.82	0.0020
E. capsaeformis	2013	NR	Total	13	7(5)	0.90	13	3.59	0.0040
			Global	193	18	0.68	22	2.09	0.0024

Table 4. Observed haplotypes and polymorphic sites for mitochondrial DNA sequences of the *ND1* (888 base-pairs) gene for *Epioblasma brevidens* (*Ebrev1-3*) and *E. capsaeformis* (*Ecap1-18*). DNA samples were obtained in 2004 and 2013 from individuals collected in the Big South Fork (BSF) Cumberland River, Scott County, TN, Clinch River at Wallen Bend (WB), Frost Ford (FF) and Swan Island (SI) and Powell River (PR), Hancock County, TN and from the Nolichucky River (NR), Hamblen County, TN.

Epioblasma brevidens:

	Polymorphic Sites and Base Pair Positions				Number of Individuals Per Haplotype Per Population								
	2	5	6	8									
	7	4	5	0		2004				2013			
Haplotype	6	8	3	7	BSF	WB	FF	SI	WB	FF	SI	PR	Total
Ebrev1	C	A	C	C	0	15	13	17	18	17	16	1	97
Ebrev2	T	.	T	T	17	4	8	5	5	9	8	4	60
Ebrev3	.	G	.	.	0	3	7	0	7	4	2	0	23
Totals					17	22	28	22	30	30	26	5	180

Table 4. Continued.

Epioblasma capsaeformis:

Table showing haplotype distribution with polymorphic sites and number of individuals per population.

	Polymorphic Sites and Base Pair Positions																						Number of Individuals Per Haplotype Per Population							
																							2004			2013				
Haplotype	2	81	129	186	263	347	386	410	425	443	551	552	561	633	635	674	716	720	737	749	760	792	WB	FF	SI	WB	FF	SI	NR	Total
Ecap1	C	G	A	G	T	T	G	G	A	T	G	A	G	G	T	T	T	C	A	C	T	T	15	14	12	21	15	15	1	93
Ecap2	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	10	12	11	3	11	9	0	56
Ecap3	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	1	0	0	0	0	0	0	1
Ecap4	.	.	.	.	.	.	A	.	G	.	.	.	A	.	.	.	.	T	.	.	.	.	1	0	3	2	0	0	0	6
Ecap5	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	C	.	.	.	.	.	.	1	1	3	0	1	1	3	10
Ecap6	.	.	G	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	1	0	0	0	0	0	0	1
Ecap7	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	C	1	0	0	0	0	1	0	2
Ecap8	.	A	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	0	1	0	0	0	0	0	1
Ecap9	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	0	1	0	0	0	1	0	2
Ecap10	.	.	.	.	.	.	A	.	G	.	.	.	A	.	.	.	.	.	.	.	.	.	0	1	0	0	2	1	0	4
Ecap11	.	.	.	.	.	.	A	.	G	.	.	G	A	.	.	.	.	.	.	.	.	.	0	0	1	4	0	0	0	5
Ecap12	T	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	0	0	0	0	1	0	0	1
Ecap13	.	.	.	.	.	.	.	G	.	.	G	A	.	.	.	C	.	.	.	.	.	.	0	0	0	0	0	2	0	2
Ecap14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	0	0	0	0	0	0	3	3
Ecap15	.	.	.	.	.	A	G	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	2	2
Ecap16	.	.	.	A	.	.	.	G	.	.	.	A	.	.	.	C	.	.	.	.	.	.	0	0	0	0	0	0	2	2
Ecap17	.	.	.	.	A	.	A	G	C	A	.	A	.	C	.	.	.	T	A	.	.	.	0	0	0	0	0	0	2	2
Ecap18	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	C	.	.	.	C	.	.	0	0	0	0	0	0	1	1
Totals																							30	30	30	30	30	30	13	193

Table 5. Haplotype and nucleotide differentiation (F_{ST} below diagonal) and uncorrected pairwise sequence divergence (Dxy, above diagonal) between populations based on observed mitochondrial DNA sequence variation at 888 base-pairs of the *ND1* gene for *Epioblasma brevidens* and *E. capsaeformis* at multiple sites located in the Big South Fork (BSF) Cumberland River, Scott County, TN, the Clinch River (CR) at three sites Wallen Bend (WB), Frost Ford (FF) and Swan Island (SI) and the Powell River (PR), where all of these sites are located in Hancock County, TN and the Nolichucky River (NR) located in Hamblen County, TN. "—" no comparison made. Pairwise in bold indicate a statistically significant comparison $p<0.05$. Pairwise comparisons shaded gray indicate negative F_{ST} values, which are interpreted as 0, i.e., no differentiation between populations.

Epioblasma brevidens	WB 2004	FF 2004	SI 2004	WB 2013	FF 2013	SI 2013	BSF 2004	PR 2013	CR 2004	CR 2013
WB 2004	—	0.00159	0.00126	0.00132	0.00152	0.00149	0.00292	0.00249	0.00139	0.00144
FF 2004	-0.00951	—	0.00158	0.00162	0.00176	0.00174	0.00269	0.00241	0.00167	0.0017
SI 2004	-0.02434	0.01051	—	0.00134	0.00147	0.00142	0.00261	0.00224	0.00136	0.00141
WB 2013	-0.03206	-0.00479	0.01589	—	0.00158	0.00157	0.00308	0.00263	0.00144	0.00149
FF 2013	-0.00935	-0.02631	-0.01492	0.01222	—	0.00164	0.00252	0.00224	0.0016	0.00163
SI 2013	-0.00389	-0.01743	-0.02315	0.02601	-0.03485	—	0.00243	0.00217	0.00157	0.0016
BSF 2004	**0.74819**	**0.5973**	**0.73732**	**0.72589**	**0.59203**	**0.6086**	—	0.00068	0.00274	0.00268
PR 2013	**0.46273**	**0.29463**	**0.43067**	**0.47597**	**0.28321**	**0.2808**	**0.27039**	—	0.00238	0.00235
CR 2004	-0.02342	-0.01115	-0.01474	-0.00575	-0.01506	-0.01234	**0.61283**	**0.38417**	—	0.00153
CR 2013	-0.01666	-0.01549	-0.01001	-0.0001	-0.01855	-0.01577	**0.57974**	**0.35185**	0.01195	—

Epioblasma capsaeformis	WB 2004	FF 2004	SI 2004	WB 2013	FF 2013	SI 2013	NR 2013	CR 2004	CR 2013
WB 2004	—	0.00175	0.00228	0.00213	0.00179	0.00197	0.01148	0.00194	0.00197
FF 2004	-0.02856	—	0.00224	0.00213	0.00173	0.00192	0.01149	0.00189	0.00193
SI 2004	0.0059	0.01369	—	0.00243	0.00225	0.00239	0.01131	0.00237	0.00235
WB 2013	**0.10666**	**0.13583**	0.04684	—	0.0021	0.00217	0.01093	0.00223	0.00205
FF 2013	-0.02872	-0.0285	-0.00389	**0.1**	—	0.00195	0.01138	0.00193	0.00194
SI 2013	-0.01867	-0.01021	-0.01226	**0.055**	-0.01989	—	0.0113	0.00209	0.00207
NR 2013	**0.37475**	**0.39595**	**0.23208**	**0.25881**	**0.36473**	**0.2994**	—	0.01143	0.01121
CR 2004	-0.01738	-0.0147	-0.00351	0.09247	-0.01952	-0.01308	**0.36908**	—	0.00208
CR 2013	0.00578	0.01863	-0.00013	0.02625	0.00086	-0.01396	**0.33439**	0.00786	—

Table 6. Summary of genetic variation among ten microsatellite DNA loci examined in this study in 2004 and 2013 for *Epioblasma brevidens* and *E. capsaeformis* in the Big South Fork (BSF) Cumberland River River at Parchcorn Creek (PC) shoal, Scott County, TN, the Clinch River (CR) at three sites Wallen Bend (WB), Frost Ford (FF) and Swan Island (SI) and the Powell River (PR) at the Parkey Property (PP) all located in Hancock County, Tennessee and in the Nolichucky River (NR), Hamblen County, TN, where N = number of individuals genotyped per locus, H_o = mean observed heterozygosity, H_e = mean expected heterozygosity, A = mean number of observed alleles per locus, Ap = total number of private alleles observed per locus, mean M-ratio = ratio of A and number of alleles possible within the range of nucleotide base-pair differences between the shortest and longest microsatellite alleles observed per locus, Bottleneck = probability value of a one-tailed Wilcoxon sign-rank test of heterozygosity excess (p-values <0.05 indicate excess heterozygosity and hence a possible population bottleneck) conducted in program BOTTLENECK, and mean F_{IS} = inbreeding coefficient. Total values were obtained by pooling and analyzing data together from all three sites.

| Species	Year	River	Site	N	H_o	H_e	A	Ap	M-ratio	Bottleneck	F_{IS}
E. brevidens	2004	BSF	PC	18	0.57	0.77	7.4	19	0.33	p=0.191	0.27
	2004	CR	WB	23	0.70	0.77	9.0	1	0.56	p=0.348	0.09
			FF	28	0.72	0.76	8.1	2	0.50	p=0.001	0.06
			SI	28	0.64	0.77	8.4	3	0.51	p=0.053	0.14
			Total	79	0.68	0.77	10.5	6	0.62	p=0.009	0.10
	2013	CR	WB	30	0.66	0.76	8.8	1	0.52	p=0.065	0.13
			FF	30	0.73	0.79	9.8	4	0.60	p=0.001	0.08
			SI	30	0.75	0.79	9.7	4	0.59	p=0.065	0.05
			Total	90	0.71	0.78	11.8	9	0.72	p=0.052	0.09
	2013	PR	PP	5	0.64	0.68	3.9	2	0.27	p=0.213	0.07
E. capsaeformis	2004	CR	WB	30	0.81	0.85	11.3	2	0.55	p=0.116	0.04
			FF	30	0.80	0.85	12.8	5	0.60	p=0.385	0.06
			SI	30	0.82	0.83	12.2	6	0.58	p=0.161	0.02
			Total	90	0.81	0.85	15.2	13	0.72	p=0.065	0.04
	2013	CR	WB	30	0.66	0.85	12.2	2	0.59	p=0.116	0.23
			FF	30	0.75	0.85	12.4	4	0.60	p=0.116	0.12
			SI	30	0.80	0.86	12.8	4	0.61	p=0.313	0.07
			Total	90	0.74	0.85	15.9	10	0.76	p=0.216	0.14
	2013	NR	Total	39	0.69	0.79	11.4	7	0.54	p=0.461	0.13

Figures
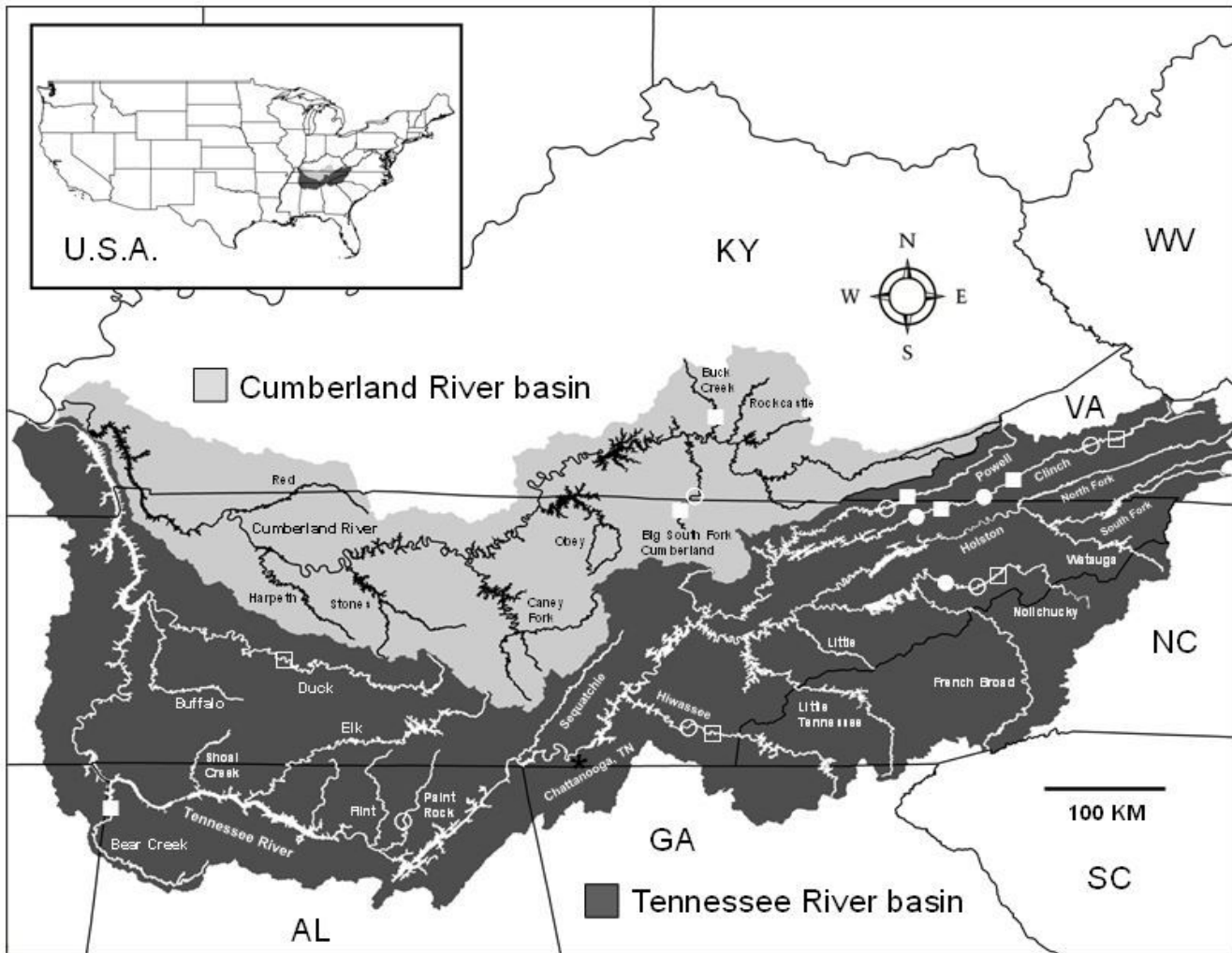
Figure 1

Sampling locations for extant populations of Epioblasma brevidens and E. capsaeformis in streams of the Cumberland and Tennessee river basins, where tissue was collected for DNA analysis from live individuals in 2004 and 2013. GPS coordinates of sampling locations are available in Table 1. Current distribution of endangered Epioblasma brevidens (squares) and E. capsaeformis (circles) in the Tennessee and Cumberland River systems, USA. Open squares and circles denote recently reintroduced populations within the last 10 years. Historically, both species occurred throughout each river system, including most of the larger tributaries shown.
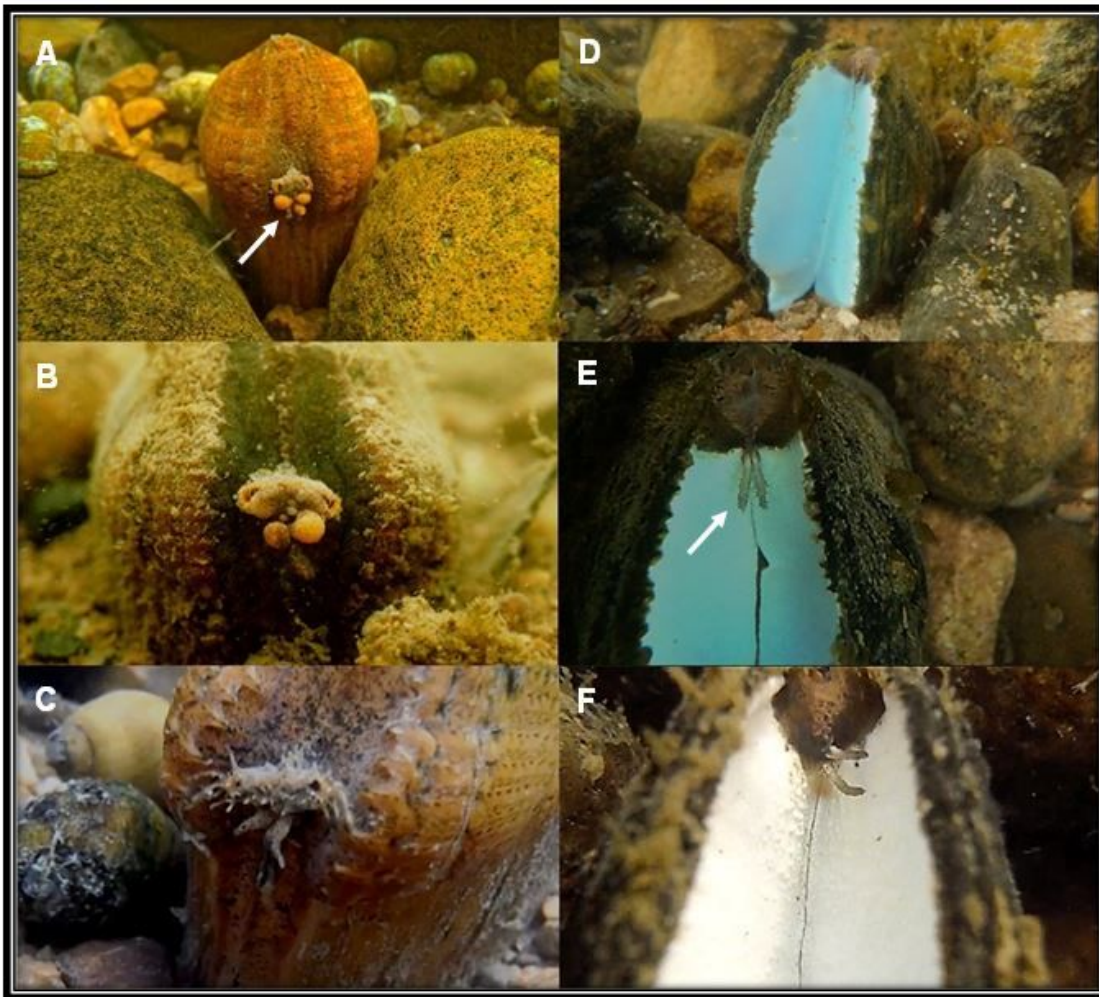
Figure 2

Mantle and micro-lure lure display of female Epioblasma brevidens: (A) Clinch River, Tennessee (TN) at Wallen Bend on September 24, 2012; white arrow is pointing to what appears to be several egg mimics, (B) Clinch River, TN at Sneedville on September 28, 2015 showing close up but of only two egg mimics, and (C) Powell River, TN at upper Brooks on October 1, 2013 showing micro-lure with numerous small papillae-like structures but without egg mimics. All three photographs highlight the morphological diversity observed in the micro-lure display of the species. Mantle and micro-lure lure display of female Epioblasma capsaeformis: (D) Clinch River, TN at Wallen Bend on May 16, 2013 showing normal blue colored mantle lure, (E) Clinch River, TN at Kyles Ford on May 30, 2013 with white arrow pointing to micro-lures mimicking cerci of an aquatic insect such as a mayfly, and (F) Nolichucky River, TN at Beech Bottoms Island on April 13, 2014 showing atypical white colored mantle lure. The blue-colored mantle lure of E. capsaeformis is common in the Clinch River population, whereas the white colored lure is more common in the Nolichucky River population. All photographs taken by Tim Lane.
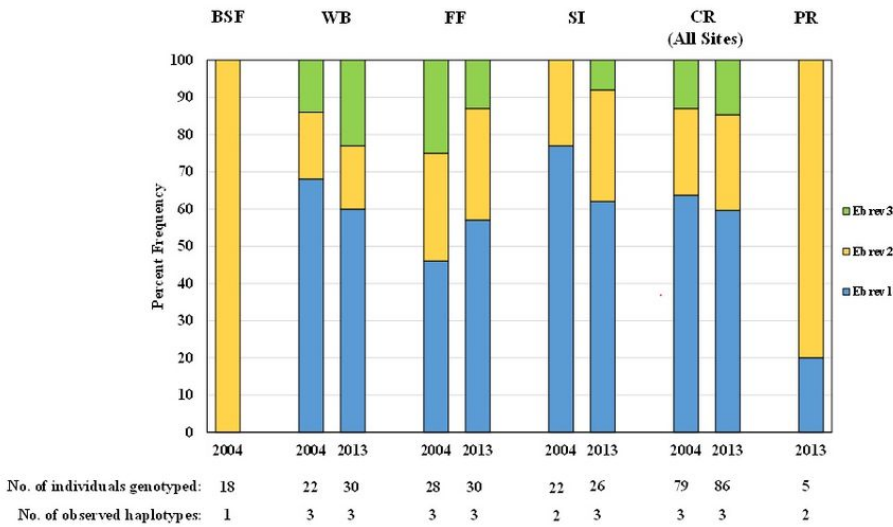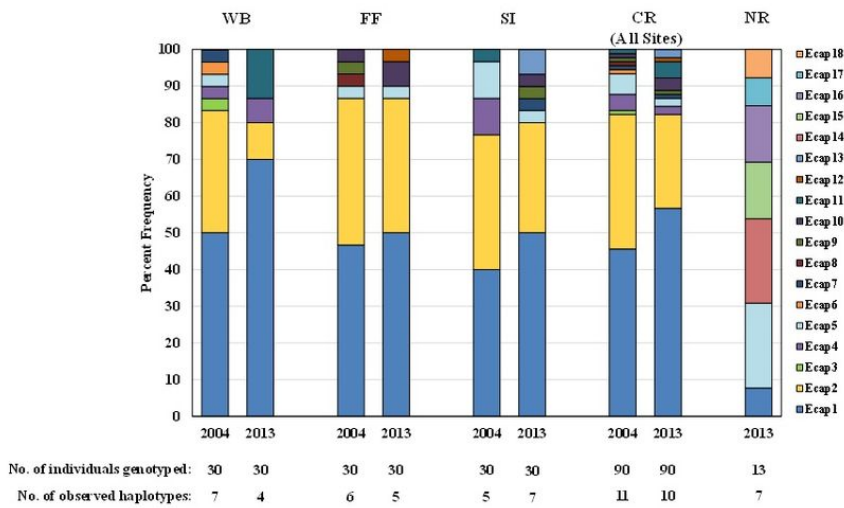
Figure 3

Frequencies of mitochondrial DNA haplotypes per population for Epioblasma brevidens (Panel A) and E. capsaeformis (Panel B) in the Clinch River, Hancock County, TN at Wallen Bend (WB), Frost Ford (FF) and Swan Island (SI), the Big South Fork (BSF) Cumberland River, Scott County, TN and the Nolichucky River (NR), Hamblen County, TN. Sampling was conducted in 2004 and 2013.
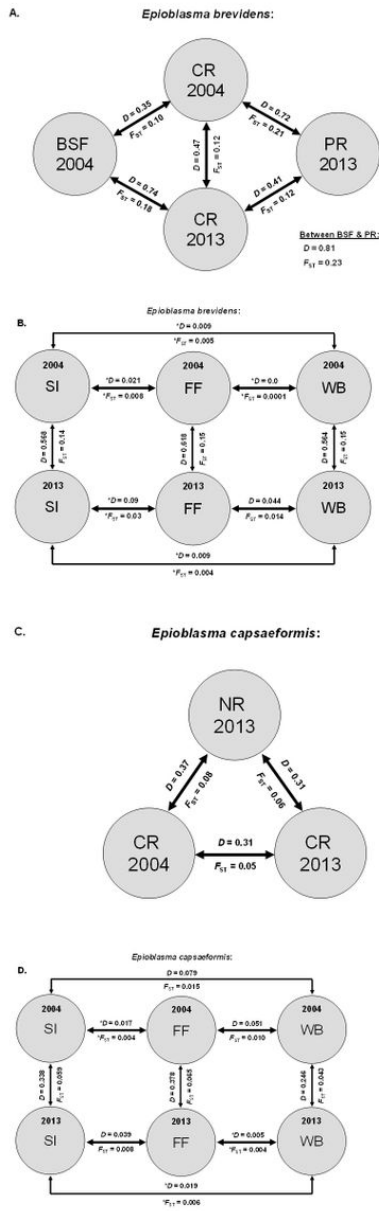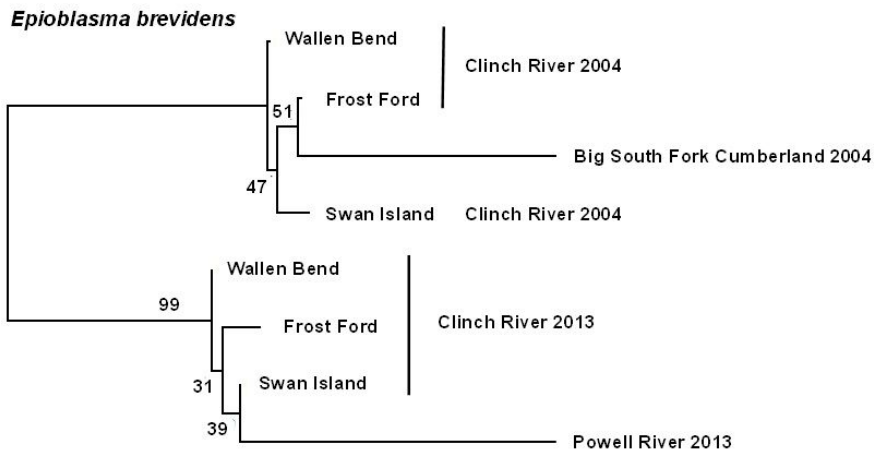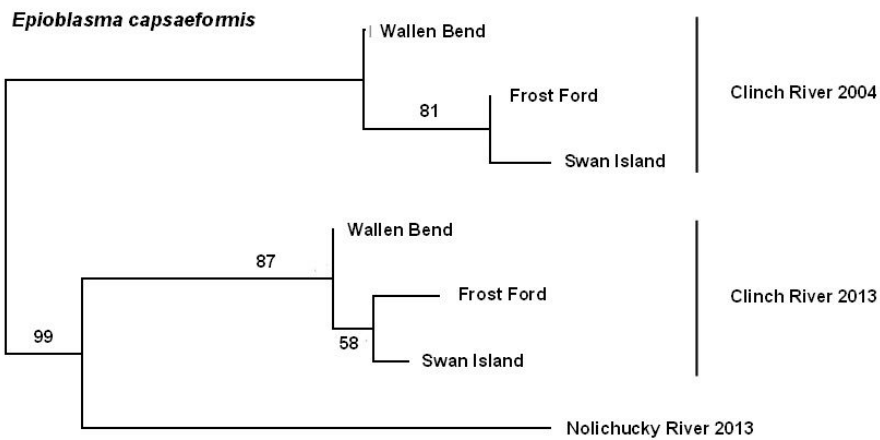
Figure 4

Estimates of genetic differentiation based on Jost's D and FST showing spatial and temporal variation among populations and years for Epioblasma brevidens (Panels A and B) and E. capsaeformis (Panels C and D). Abbreviations are Wallen Bend (WB), Frost Ford (FF), and Swan Island (SI), all sites located in the Clinch River (CR), Hancock County, TN, Powell River (PR), Claiborne County, TN, Big South Fork Cumberland River (BSF), Scott County, TN and Nolichucky River (NR), Hamblen County, TN. Differentiation values were estimated using 10 DNA microsatellite loci except for comparisons made with BSF, which were estimated using 8 loci (see Table 2). All values among populations and years are statistically significant (p < 0.05) unless marked with an asterisk*.

Figure 5

Mid-point rooted neighbor-joining (NJ) phylogenetic trees showing relationships among investigated populations of Epioblasma brevidens (Panel A) and E. capsaeformis (Panel B); trees were constructed from DNA microsatellite allele frequency data using Nei's standard genetic distance (DST).
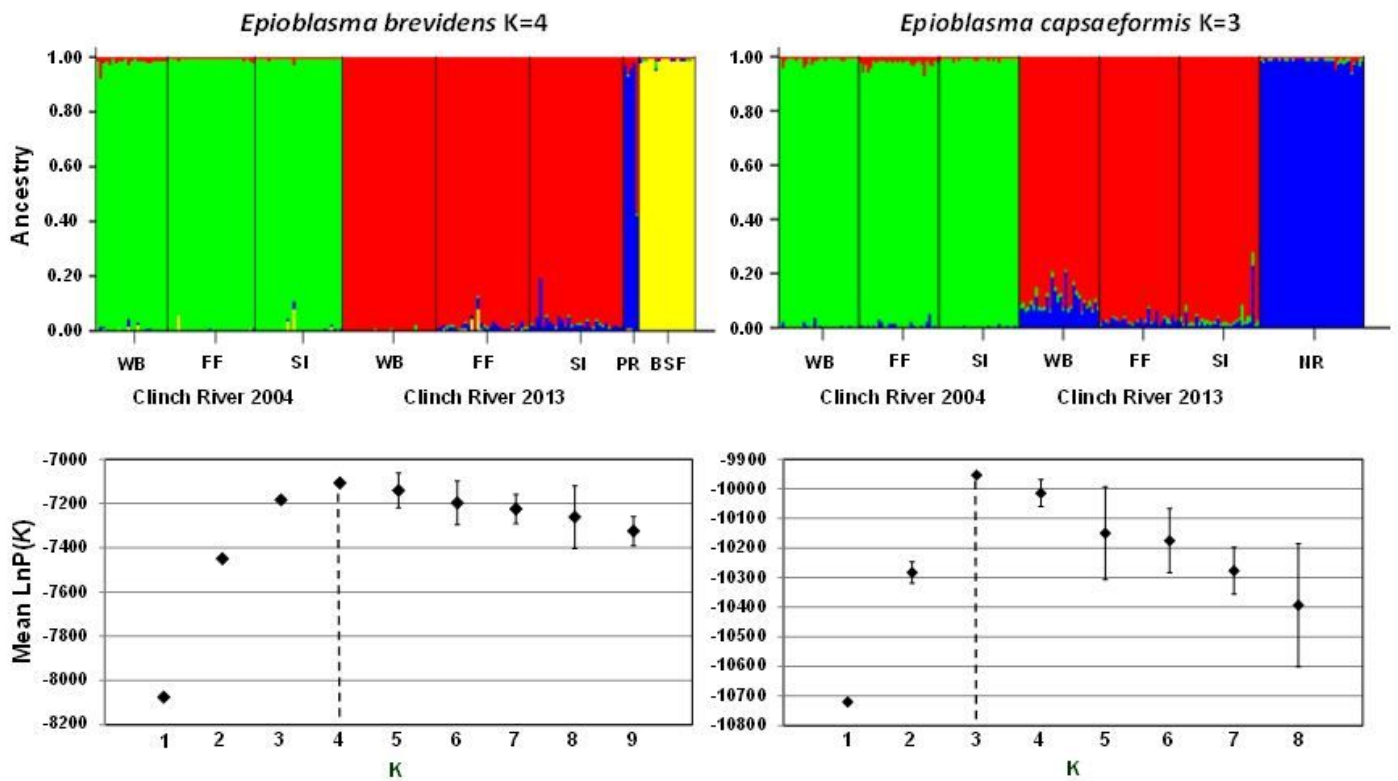
Figure 6

Top graph for each species shows individuals per population on the x-axis and population ancestry, i.e., assignment probability to populations on the y-axis. Assignment test posterior probability results were produced by program STRUCTURE version 2.3.4. Bottom graph for each species shows mean log probability estimates for Epioblasma brevidens of K = 1 through K = 8 clusters indicating strongest support for K = 4 biologically informative clusters, and for Epioblasma capsaeformis of K = 1 through K = 9 clusters indicating strongest support for K = 3 biologically informative clusters with error bars (SD = standard deviation) shown. Dashed lines in bottom graphs indicate the value of K with strongest support for each species.
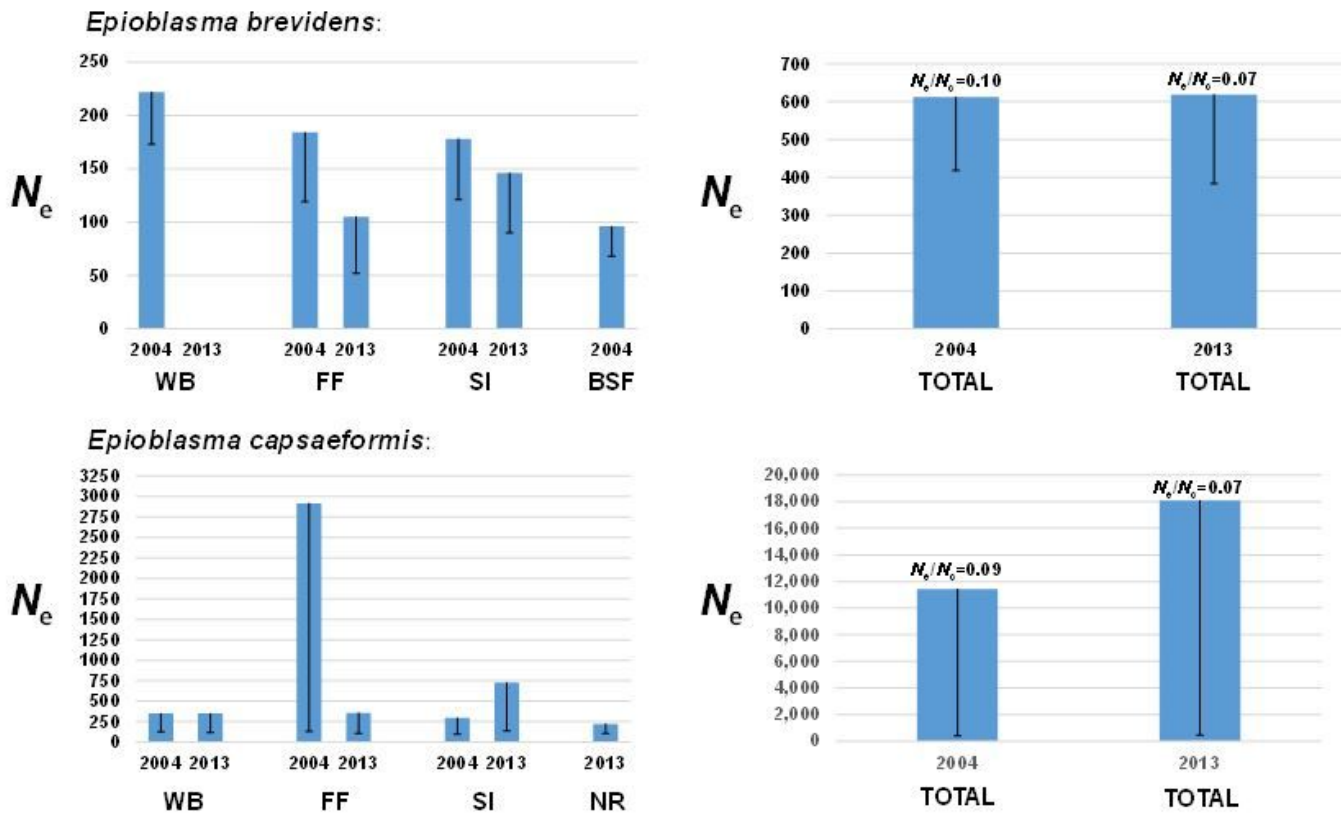
Figure 7

Effective population sizes (Ne) for Epioblasma brevidens and E. capsaeformis in the Clinch River, Hancock County, TN at Wallen Bend (WB), Frost Ford (FF) and Swan Island (SI), the Big South Fork (BSF) Cumberland River, Scott County, TN and the Nolichucky River (NR), Hamblen County, TN. The lower 95% confidence interval error bars are shown; however, the upper error bars are not shown because they are very large or "infinite" and could not be estimated. Total values were estimated by combining samples of DNA microsatellite genotypes from WB, FF, and SI. Sampling was conducted in 2004 and 2013.
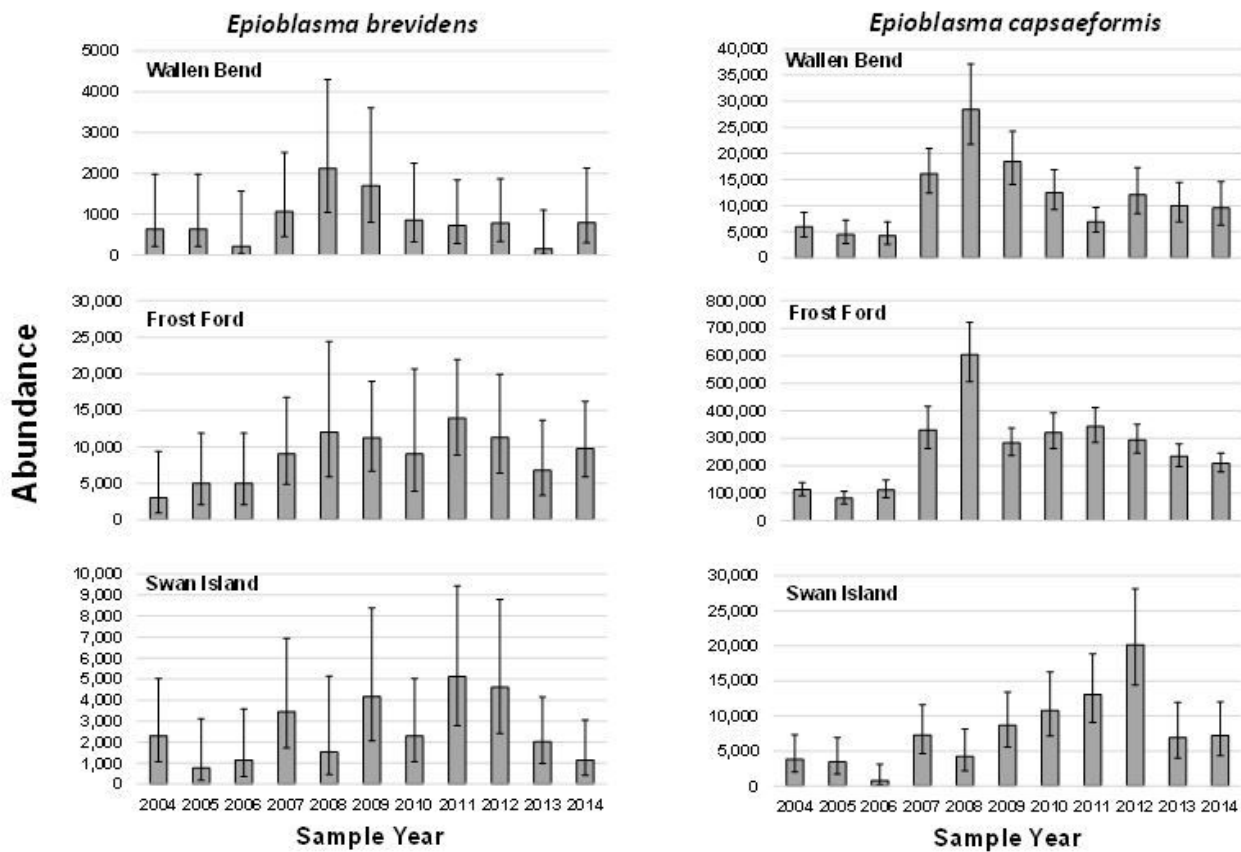
Figure 8

Time-series of mean mussel abundance of Epioblasma brevidens and Epioblasma capsaeformis estimated annually from 2004–2014 at Swan Island, Frost Ford, and Wallen Bend in the Clinch River, Hancock County, TN. None of the time series were significant over the study period indicating either an increasing or declining trend. However, error bars represent 95% confidence intervals, where non-overlapping intervals among sites or sample years indicate significant ($p < 0.05$) differences. Data from 2004–2008 for Epioblasma capsaeformis are from Jones and Neves (2011), data from 2004–2009 are from Jones et al. (2014), and data from 2010-