Genetic Diversity in the Threatened Freshwater Mussel Lampsilis powellii

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REGULAR ARTICLE

GENETIC DIVERSITY IN THE THREATENED FRESHWATER MUSSEL *LAMPSILIS POWELLII*

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ABSTRACT

North America is home to the greatest share of the world's freshwater mussel diversity; however, more than 70% of its ~300 species are endangered or threatened. *Lampsilis powellii*, the Arkansas Fatmucket, is endemic to Arkansas and is now restricted to upstream reaches of the Ouachita and Saline rivers, but the species is declining within this small range. Conservation actions such as augmenting or reintroducing populations may be necessary, but they require knowledge of the distribution of genetic variation within and among extant populations. We analyzed population structure between the South Fork Ouachita River and Saline River using a 607-base-pair region of the mitochondrial *COI* gene and 14 microsatellites designed for *Lampsilis abrupta*. *COI* sequences showed little variation, and the most common haplotype was present in both rivers. Our mtDNA sequences were indistinguishable from those of *L. siliquoidea* deposited on GenBank, but we were unable to make conclusions about the taxonomic distinctiveness of *L. powellii*. Microsatellites showed heterozygote deficiencies for most loci and revealed little evidence of population structure between the two rivers. Overall, our results show low genetic diversity in *L. powellii*, which may reflect its small population size due to its long history of geographic isolation compounded by anthropogenic habitat destruction and fragmentation. Further genetic analyses of lampsiline taxa are needed to establish species limits for *Lampsilis* in the Interior Highlands.

KEY WORDS: unionid, lampsiline, mtDNA, microsatellites, population genetics

INTRODUCTION

Small, isolated populations lose rare alleles through genetic drift, and such reductions in genetic diversity can make species more vulnerable to extinction because greater diversity increases adaptability and long-term population persistence (Reed and Frankham 2003; Hoffman et al. 2017). As a result, imperiled species face increased probabilities of extinction because small populations leave them vulnerable to the interacting effects of genetic drift, demographic change, and environmental stochasticity. Such species are at risk of entering a so-called extinction vortex (Gilpin and Soulé 1986). More than 70% of North American freshwater mussel species (families Unionidae and Margaritiferidae) are imper-

iled, and many survive only in small, isolated populations (Williams et al. 1993).

Lampsilis powellii (Lea, 1852), the Arkansas Fatmucket, is a federally threatened unionid mussel with a narrow historical distribution restricted to Interior Highlands portions of the Ouachita, Saline, and Caddo rivers in south-central Arkansas, USA (U.S. Fish and Wildlife Service 1990). Populations of upland fishes and mussels in many Interior Highlands streams are proposed to have been isolated from each other historically by long stretches of lowland habitat in the Mississippi Embayment, created when the lower reaches of these streams were buried under sediment deposited by high sea levels during interglacial periods (Mayden 1988; Haag 2010). The range of *L. powellii* has been fragmented further by dams, which destroyed suitable habitat for this species. Dams also restrict fish movement, which, in turn, restricts mussel dispersal and gene flow because mussel larvae are obligate parasites on fishes. *Lampsilis powellii* currently survives in

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only two isolated populations separated by impoundments, in the upper Ouachita and upper Saline river systems.

We examined population genetic structure and genetic variation of *L. powellii* from remaining populations using a 607-base-pair (bp) region of the mitochondrial *COI* gene and 14 nuclear microsatellite loci. Given historical and recent barriers to dispersal, we expected to observe reduced genetic diversity and limited connectivity among populations compared to more widespread mussel species. We identified patterns of genetic variation and population structure in *L. powellii*, which will be useful for assessing its conservation status and implementing population restoration efforts.

METHODS

We obtained swab samples from the mantle or foot of 42 individuals: 15 individuals from one site on the South Fork of the Ouachita River, and 27 individuals from four sites on the Saline River (Fig. 1). Genomic DNA was extracted using ArchivePure DNA Cell/Tissue kits (5 Prime, Gaithersburg, MD, USA). The mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene was amplified with primers LCO22me2 and HCO700dy2 using conditions developed by Walker et al. (2006) following the specifications included with GoTaq Master Mix (Promega Corporation, Madison, WI, USA) in 20 μL reactions. Clean-up of polymerase chain reactions (PCRs) was performed with a QIAquick gel extraction (Qiagen, Inc., Germantown, MD, USA) with 35 μL of the PCR product. We used the above primers for cycle sequencing and carried out reactions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Waltham, MA, USA) with the default protocol. Cycle sequencing products were purified with the EDTA/sodium-acetate/ethanol protocol included with the BigDye kit and analyzed on an ABI Genetic Analyzer (Applied Biosystems).

We assembled, edited, and aligned sequences with Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) and verified an open reading frame and absence of primer sequences. DNASP v5.10 (Librado and Rozas 2009) was used to estimate population genetic indices from mtDNA sequences, including number of haplotypes (H), mean number of bp differences (K), and mean nucleotide diversity (π) over the pooled data set and within each river. A 95% confidence parsimony network was created in PopArt v1.7 (Leigh and Bryant 2015), and multiple connections between haplotypes were simplified using methods described by Fetzner and Crandall (2003).

Fourteen of 15 microsatellite primers designed for *Lampsilis abrupta* (Eackles and King 2002) were successfully optimized in *L. powellii*. Forward primers for each PCR were labeled with a 5′ fluorescent tag (6-FAM, NED, PET, or VIC) for visualization. We amplified microsatellite loci in 10 μL reactions using GoTaq Master Mix (Promega), 0.5 μM of fluorescently labeled forward and reverse primer, and 10 ng of DNA template carried out under the following conditions: initial denaturing at 94°C for 2 min; 35 cycles of 94°C for 40 s, variable annealing temperature for 40 s (Appendix A1), 72°C for 1 min; and a final extension at 72°C for 5 min. We performed fragment analysis on an ABI 3730 Genetic Analyzer with LIZ600 size standard (Applied Biosystems). PEAKSCANNER v1.0 (Applied Biosystems) was used to score alleles, and TANDEM v1.07 (Matschiner and Salzburger 2009) was used to assign integer numbers to DNA fragment sizes.

We estimated null allele frequencies using the Brookfield I (1996) method as calculated in MICROCHECKER (van Oosterhout et al. 2004). We used GenAlEx v6.3 (Peakall and Smouse 2006) to estimate mean number of observed alleles per locus (N_A) and number of private alleles (N_P) and to calculate observed and expected heterozygosities (H_O and H_E) for each population. Allelic richness (A_R) was computed via rarefaction using FSTAT v2.9.3 (Goudet 1995) to estimate the total number of alleles in the population given the sample size. GENEPOP v4.0.10 (Rousset 2008) was used to conduct exact tests of pairwise linkage disequilibrium and to test whether genotype frequencies met Hardy–Weinberg expectation.

Population genetic structure was visualized by conducting a principal coordinate analysis (PCoA) in GenAlEx using a covariance matrix created from all polymorphic loci. A permutational multivariate analysis of variance (PERMANO-VA; $n = 9999$ permutations) was used to determine the significance of the PCoA clusters utilizing the adonis function within the vegan package (Oksanen et al. 2017) in R (R Core Team 2016). FreeNA was used to assess the degree of genetic differentiation among rivers by calculating pairwise F_{ST} values employing the ENA (excluding null alleles) correction, which has been shown to effectively correct for the positive bias of F_{ST} that may result from the presence of null alleles (Chapius and Estoup 2007).

RESULTS

We successfully sequenced mitochondrial DNA for 29 individuals and recovered nine *COI* haplotypes with a length of 607 bp. Sequences are available on GenBank (accession nos. MT762680–MT762708).

Three clusters of haplotypes were present, separated by 34–46 mutational steps (>5% of total sequence length; Fig. 2). The first cluster consisted of a common haplotype found in 13 individuals from the Saline River and seven individuals from the Ouachita River, two individuals of a second haplotype limited to the Saline River that differed from the common haplotype by two bp, and a singleton haplotype from the Ouachita River that differed from the common haplotype by four bp. Our most common haplotype was identical to a GenBank *L. siliquoidea* haplotype from the upper Mississippi River (accession no. MK672781.1; see Inoue et al. 2019).

A second cluster contained three singleton haplotypes from two sites in the Saline River that differed from each other by four or five bp. These haplotypes differed from those in the first cluster by 34–39 bp and corresponded to *Lampsilis hydiana* sequences on GenBank (accession no. MK672683.1).
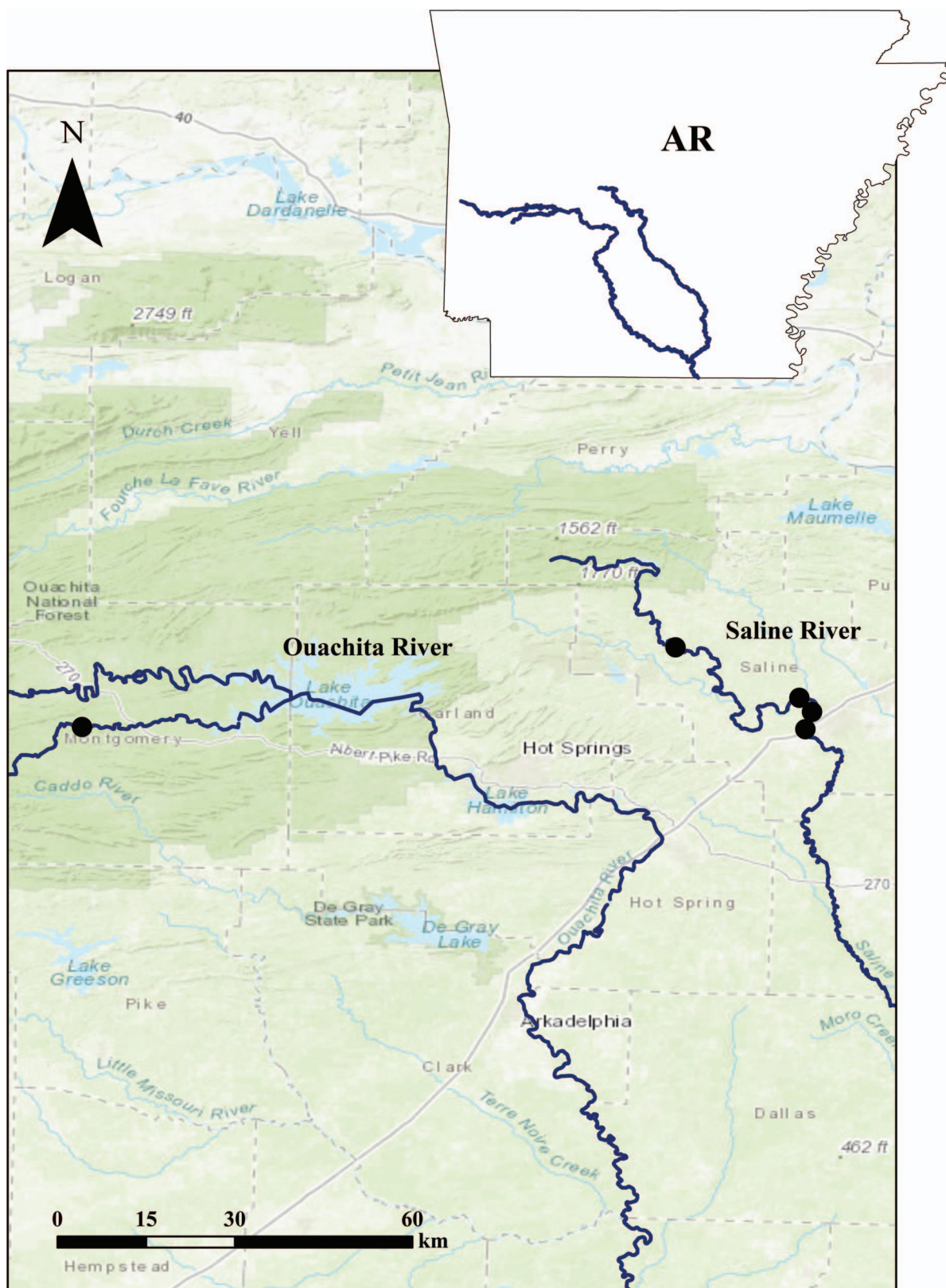
Figure 1. Sampling localities for *Lampsilis powellii* in Arkansas, USA.

Table 1. Summary statistics for *COI* sequences and 14 microsatellite loci for *Lampsilis powellii* in the Ouachita and Saline rivers, Arkansas, USA. Individuals identified as *Lampsilis hydiana* and *Actinonaias ligamentina* based on *COI* sequences are not included (see text). N = number of individuals sampled. Genetic diversity metrics are number of haplotypes (H), mean number of bp differences between all pairs of individuals (K), nucleotide diversity (π), mean number of observed alleles (N_A), rarefied allelic richness (A_R), mean number of private alleles (N_P), mean observed heterozygosity (H_O), and mean expected heterozygosity (H_E).

	COI				Microsatellites				
River	n	H	K	Π	N_A	A_R	N_P	H_O	H_E
Ouachita	8	2	0.75	0.001	5.77	3.82	3.21	0.42	0.68
Saline	15	2	0.25	0.000	9.07	4.97	6.50	0.44	0.75
Overall	23	3	3.00	0.002	7.43	4.30	NA	0.43	0.72

A third cluster contained three singleton haplotypes from the Saline River that differed from each other by six to nine bp. These haplotypes differed from those in the first cluster by 43–46 bp and were most similar to GenBank sequences for *Actinonaias ligamentina* (accession no. MK672757.1). We omitted from further analysis the six individuals in clusters two and three representing *L. hydiana* and *A. ligamentina*.

There was little genetic diversity among the 23 remaining *COI* sequences representing *L. powellii*, and patterns were similar for both rivers (Table 1). There were two haplotypes in each river, the mean number of base-pair differences between all individuals was three, and nucleotide diversity was low.

Microsatellite analyses also showed low genetic variation. We scored a total of 172 alleles over the 14 loci examined, ranging from four to 19 per locus (Tables 1 and 2). Microsatellite genotypes in GENEPOP format are deposited
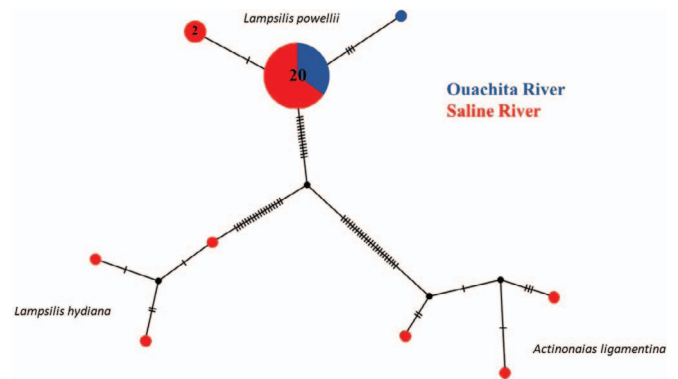

Figure 2. Haplotype network for samples identified as *Lampsilis powellii* derived from 607 bp region of the mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene. Each circle represents a unique haplotype. The number within each circle represents the number of individuals having that haplotype; circles without numbers represent a single individual. Black dots and tick marks represent missing haplotypes.

in Dryad (https://doi.org/10.5061/dryad.3r2280gd6). Mean rarefied allelic richness and heterozygosity across the 14 loci were similar between rivers (Table 1). The mean number of private alleles was twice as high in the Saline than in the Ouachita. We found considerable evidence of null alleles (present at 82.1% of all river-by-locus pairs) and no evidence of linkage disequilibrium. Eighteen of 28 locus-by-river combinations deviated from Hardy–Weinberg expectations after Bonferroni correction, with all showing a heterozygote deficiency consistent with the presence of null alleles.

Populations of *L. powellii* showed little evidence of significant divergence. The first PCoA axis separated the two rivers but explained only 11.6% of total microsatellite

Table 2. Genetic diversity at 14 microsatellite loci in *Lampsilis powellii* from the Ouachita and Saline rivers, Arkansas, USA. N = number of individuals genotyped. Genetic diversity metrics are allelic richness (N_A), observed and expected heterozygosity (H_O and H_E), and null allele frequency. * indicates deviation of heterozygosity from Hardy–Weinberg expectation after sequential Bonferroni correction. Bold values indicate null allele frequencies that are significantly different from zero at $P \geq 0.05$.

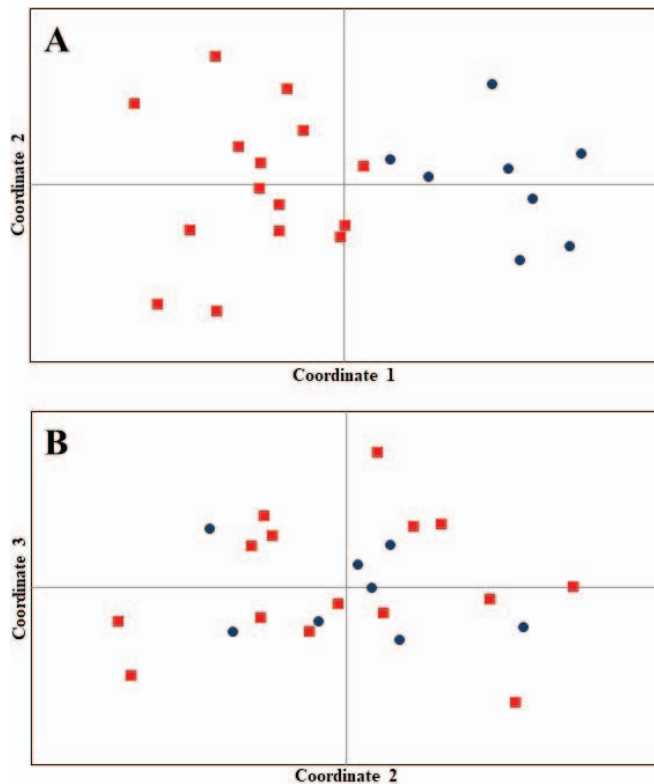|	Ouachita River					Saline River				
Locus	N	N_A	H_O	H_E	Null allele	N	N_A	H_O	H_E	Null allele
LabC02	8	5	0.63	0.72	**0.05**	15	6	0.20*	0.49	**0.19**
LabC23	8	4	0.38	0.60	**0.14**	15	2	0.20	0.49	**0.14**
LabC24	8	3	0.38	0.57	**0.12**	15	4	0.47*	0.61	**0.09**
LabC67	8	3	0.88	0.62	0.00	15	11	0.40*	0.86	**0.25**
LabD10	8	7	0.50*	0.77	**0.15**	14	9	0.50*	0.83	**0.18**
LabD29	8	7	0.38*	0.80	**0.23**	14	10	0.50*	0.81	**0.18**
LabD31	7	6	0.14*	0.81	**0.37**	15	15	0.40*	0.92	**0.27**
LabD70	8	1	0.00*	0.00	0.00	15	6	0.27*	0.51	**0.16**
LabD71	8	7	0.25*	0.81	**0.31**	15	12	0.40*	0.89	**0.26**
LabD99	8	5	0.00*	0.70	**0.40**	13	10	0.23*	0.85	**0.33**
LabD111	8	11	1.00	0.87	0.00	15	15	0.93	0.92	0.00
LabD187	8	5	0.50	0.63	**0.08**	10	10	0.80	0.88	0.04
LabD206	8	6	0.13*	0.76	**0.36**	14	6	0.80	0.60	**0.11**
LabD213	8	11	0.75*	0.89	**0.07**	14	11	0.43*	0.88	**0.24**

Figure 3. Results of principal coordinates analysis (PCoA) for 14 microsatellite loci of *Lampsilis powellii* from Arkansas, USA. Red squares represent individuals from the Saline River, and blue circles represent Ouachita River individuals.

variation (Fig. 3a). The second and third axes accounted for 20.5% and 27.3% of the variation, respectively; however, we could not infer any geographical patterns from these axes (Fig. 3b). The ENA-corrected estimate of pairwise F_{ST} among rivers was 0.076, while the PERMANOVA failed to reject the null hypothesis of no difference between rivers ($F = 1.72$, $P = 0.11$). The uncorrected estimate of F_{ST} (0.085) was similar to the ENA-corrected estimate; its slightly higher value was likely due to the presence of null alleles.

DISCUSSION

Lampsilis powellii seems to have maintained only a limited amount of intraspecific genetic diversity at both mtDNA and nDNA loci compared with other mussel taxa (Elderkin et al. 2007, 2008; Jones 2009). This limited amount of genetic diversity may be a result of the small sample sizes in our study, which is a common issue with rare species. However, the number of mtDNA haplotypes we found was lower than the number found in other endangered mussel species, even though samples sizes were comparable (Zanatta and Murphy 2008; Jones 2009; Menon et al. 2019). In the Ouachita River, *Cumberlandia monodonta* showed similarly low levels of mtDNA diversity with the presence of a single *COI* haplotype (Inoue et al. 2014b). Additionally, genetic diversity in *L.*

powellii was lower than in other unionid studies that employed the Eackles and King (2002) microsatellite primers (Kelly and Rhymer 2005; Menon et al. 2019). For example, genetic diversity was lower in *L. powellii* than in Great Lakes populations of *L. cardium* (N_A range: 3.3–13.7; H_O range: 0.42–0.85; Hewitt et al. 2019). Additionally, mean allelic richness was lower in *L. powellii* than for *L. siliquoidea* in the St. Clair River ($A_R = 8.7$–11.4; Rowe and Zanatta 2015). The latter two comparisons are noteworthy because those populations are geologically young and colonized the Great Lakes region only after Pleistocene glaciation. Mussel populations in the Interior Highlands are likely much older and would be expected to contain higher genetic diversity. The low genetic diversity of *L. powellii* may reflect its small population size due to its long history of isolation compounded by anthropogenic habitat destruction and fragmentation.

The Interior Highlands of North America is a biodiversity hotspot with high endemicity for a variety of aquatic organisms including mussels, fishes, crayfishes, and salamanders (Mayden 1985; Haag 2010). The region's distinctive aquatic fauna is believed to have been formed by vicariant pre-Pleistocene events, followed by repeated periods of isolation due to Pleistocene glaciations (Mayden 1988), resulting in genetically divergent populations. Not only do isolated populations with restricted gene flow lose genetic diversity, they also can exhibit an increase in population structuring (Paetkau et al. 1995). However, the limited genetic divergence between rivers that we found suggests a single panmictic population in relatively recent times. Despite the presence of the potential dispersal barrier of lowland habitats in the lower reaches of the Saline and Ouachita rivers, a mobile fish host may have allowed some dispersal across this barrier (e.g., Galbraith et al. 2015). Host fishes for *L. powellii* are currently unknown, but most *Lampsilis* are specialists on black basses (*Micropterus*), which can undertake substantial movements (Haag 2012; Schall et al. 2019). Identification of fish hosts of *L. powellii* is needed for inferring its potential dispersal ability and gene flow.

Interestingly, we were unable to differentiate *L. powellii* from *L. siliquoidea COI* sequences from the Mississippi River (Inoue et al. 2019). Whole mitochondrial genome sequencing identified a divergent protein-coding region, the *f-orf* gene, which distinguished between *L. siliquoidea* and *L. powellii* (Robicheau et al. 2018). However, when we examined the *COI* region of the whole mtDNA genome submitted to GenBank by Robicheau et al. (2018; accession no. NC_037720) and a partial *COI* gene sequence (Breton et al. 2011; accession no. HM849705) labeled *L. powellii*, they matched sequences identified as *L. hydiana* from another study (Inoue et al. 2019). At this time, we are unable to make conclusions about the taxonomic distinctiveness of *L. powellii*. Because the available genetic data are limited to mtDNA sequences and a few microsatellite loci, a high-resolution genomic approach may be necessary to elucidate the taxonomic position of *L. powellii* among other *Lampsilis* species from this region.

Lampsilis siliquoidea and *L. powellii* are difficult to

distinguish morphologically (Harris et al. 2004; Harris et al. 2010; Krebs et al. 2013). Geometric morphometric analyses can be used to differentiate morphologically similar species (Inoue et al. 2014a; Riccardi et al. 2019), and this technique also may be useful for differentiating *L. powellii* from other co-occurring lampsilines from the Interior Highlands. The presence in our dataset of samples referable to *A. ligamentina* and *L. hydiana* from individuals originally identified in the field as *L. powellii* underscores the difficulty of identifying lampsiline species in this region of high evolutionary diversification.

Our results have important conservation implications. The lack of a clear genetic signal for differentiating *L. powellii*, *L. siliquoidea*, and *L. hydiana* shows that further research is necessary to evaluate the phylogenetic status of these species. Our results suggest that between-river genetic divergence in *L. powellii* is very low, although both populations contain private microsatellite alleles. However, our small samples sizes make this conclusion preliminary; population genomic methods are likely necessary to determine whether significant between-river divergence exists for *L. powellii*. Resolution of these issues is critical for designing conservation strategies for the small populations of *L. powellii* that remain in the Saline and upper Ouachita basins.

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Appendix A1. Annealing temperature (T$_A$) for 14 microsatellite loci for *Lampsilis powellii*.

Locus	Primer Sequences	T$_A$ (℃)
LabC02	F: ATGGACACCAGAAAGAAAAGG	52.9
	R: GAAGTCACAAGGTCAGGATCTC	
LabC23	F: CAGTTGTTCCACTGTCGTAAAG	59.1
	R: TGGGACTAACATGGTGGTTAAG	
LabC24	F: TGGACCTATTCTTGCTTGTTG	59.1
	R: GTTCTTTCGCCTCCATGTATAG	
LabC67	F: AGTCTCTGGCTCAACCAACTC	55.7
	R: CAAATCAATTACTGCCTTTTC	
LabD10	F: TTGTATAAACGGTCATGGAAAAC	59.1
	R: CCGTGACCACTTCTTCTAAAAC	
LabD29	F: TGTTCTTAGTTTATATTTATGGTTTGC	55.7
	R: GCAGAAAATCTCCAGTTTATGG	
LabD31	F: CTGCAGAACATCGAATGC	59.1
	R: AAATGACAACAAAGTGAGGTTATATG	
LabD70	F: GACCGCCTCTTCTAAAACTCTC	59.2
	R: AACATCGCTTTCCATTAATCAC	
LabD71	F: GAAGGACATCAAGGTCAATCAG	59.1
	R: GGACACGGTCAAGTACAAAATAC	
LabD99	F: TTGAATGAGTCACGAGTATTTAATG	55.7
	R: TTAAGAATCGAAAATGCTCAATC	
LabD111	F: TGCATCAACTCTATTCACAACC	55.7
	R: CAATGATAATGTAAATGTAAGCCTATC	
LabD187	F: TCAAGTCTGCAATTTATTTTATGC	55.7
	R: TGATTCTTTCCCTAGAAAATACAAG	
LabD206	F: AAGTGTAGAGGCAGAGAACTGAC	55.7
	R: TCACTGATACAGCATACATAATATAC	
LabD213	F: ATACACAGGGTGCTCTAAATGC	59.1
	R: TTGCCAAAACAACATAGTTCC	