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Riverscape genetic variation, migration patterns, and morphological variation of the threatened Round Rocksnail, *Leptoxis ampla*

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Abstract

Within riverine systems, headwater populations are hypothesized to harbour higher amounts of genetic distinctiveness than populations in the main stem of a river and display increased genetic diversity in large, downstream habitats. However, these hypotheses were mostly developed with insects and fish, and they have not been tested on many invertebrate lineages. Pleuroceridae gastropods are of particular ecological importance to rivers of eastern North America, sometimes comprising over 90% of macroinvertebrate biomass. Yet, virtually nothing is known of pleurocerid landscape genetics, including whether genetic diversity follows predictions made by hypotheses developed on more mobile species. Moreover, the commonly repeated hypothesis that intraspecific morphological variation in gastropods results from ecophenotypic plasticity has not been well tested on pleurocerids. Using 2bRAD-seq to discover single nucleotide polymorphisms, we show that the threatened, Cahaba River endemic pleurocerid, *Leptoxis ampla*, has limited gene flow among populations and that migration is downstream-biased, conflicting with previous hypotheses. Both tributary and main stem populations harbour unique genomic profiles, and genetic diversity was highest in downstream populations. Furthermore, *L. ampla* shell morphology was more correlated with genetic differences among individuals and populations than habitat characteristics. We anticipate similar genetic and demographic patterns to be seen in other pleurocerids, and hypotheses about gene flow and population demographics that were based on more mobile taxa often, but not always, apply to freshwater gastropods. From a conservation standpoint, genetic structure of *L. ampla* populations suggests distinctive genetic diversity is lost with localized extirpation, a phenomenon common across the range of Pleuroceridae.

KEYWORDS
2bRAD-seq, Cahaba River, freshwater gastropods, pleuroceridae, population genomics, threatened and endangered species

1 | INTRODUCTION

Many factors influence genetic diversity across a landscape including life history, physical dispersal barriers and anthropogenic activity. Compared to terrestrial or marine organisms, animals occupying fluvial ecosystems have gene-flow constraints associated with inherent hierarchical properties of drainage networks, unidirectional water flow and relatively limited lateral connectivity (Davis, Epps, Flitcroft, & Banks, 2018; Hughes, Schmidt, & Finn, 2009; Malmqvist, 2002; Vannote, Minshall, Cummins, Sedell, & Cushing, 1980). However, these fluvial characteristics have unequal influence on different organismal groups. For example, overland dispersal facilitates population connectivity of many aquatic insect groups (Geismar, Haase, Nowak, Sauer, & Pauls, 2015; Razeng et al., 2017), whereas such connectivity is improbable for fish and other species permanently bound to flowing rivers. Indeed, much of our understanding of gene flow and population genetic patterns in fluvial systems comes from studies of fish and aquatic insects (e.g. Faulks, Gilligan, & Beheregaray, 2011; Finn & Adler, 2006; Finn, Blouin, & Lytle, 2007; Fluker, Kuhajda, & Harris, 2014a; Fluker, Kuhajda, Lang, & Harris, 2010; Kang, Ma, & He, 2017; Lecaudey, Schliewn, Osinov, Taylor, Bernatchez, Schliewn, & Osinov, 2018; Robinson, Simmons, Williams, & Moyer, 2013; Stobie, Oosthuizen, Cunningham, & Bloomer, 2018). Moreover, hypotheses that predict diversity patterns across riverine landscapes have been mostly developed with data on freshwater insects and fish (reviewed by Davis et al., 2018; Hughes et al., 2009). These hypotheses include the River Continuum Concept that predicts lower species diversity in headwaters compared to main stem populations (Vannote et al., 1980), the Mighty Headwaters Hypothesis that postulates headwater or tributary populations will harbour greater genetic uniqueness than main stem river segments (Finn, Bonada, Múrria, & Hughes, 2011), and the Stream Hierarchy Model that states populations will demonstrate a pattern of isolation by distance along a stream network path (Meffe & Vrijenhoek, 2011). Many riverine species also demonstrate a pattern of increased genetic diversity in downstream river reaches (i.e. downstream increase in intraspecific genetic diversity hypothesis; Paz-Vinas, Loot, Stevens, & Blanchet, 2015). Broad patterns seen in some aquatic groups, however, may not be applicable to those found in dispersal-limited fluvial taxa such as freshwater gastropods. For instance, limited gene flow between populations may result in genetically distinct main stem populations, a pattern that would conflict with the Mighty Headwaters Hypothesis. Population genomic studies are required to better understand gene flow and population dynamics of low-dispersing taxa, increase broad understanding of riverine ecology and enhance conservation efforts.

Freshwater gastropods generally exhibit a high degree of endemism with individual species often constrained to one or a few rivers, making them particularly susceptible to anthropogenic pressures such as pollution, hydrologic alteration and geomorphic degradation (Johnson et al., 2013; Neves, Bogan, Williams, Ahlstedt, & Harftfield, 1997). In particular, pleurocerid gastropods are one of the most imperilled groups of organisms in North America (Johnson et al., 2013) even though they are essential components of many freshwater ecosystems in eastern North America, making up greater than 90% of macroinvertebrate biomass in some streams (Newbold, Elwood, O'Neill, & Sheldon, 1983). As a result, they can significantly regulate biomass of primary producers (Rosemond, Mulholland, & Elwood, 1993) and influence food webs and ecosystem function (Morales & Ward, 2000; Mulholland, Steinman, Palumbo, Elwood, & Kirschtel, 1991). However, the current dearth of genome-scale molecular studies on freshwater snails in general, and pleurocerids in particular, limits our understanding of a critical component of North American riverine ecosystems. Lack of information also hinders management efforts. Prevailing conservation plans for pleurocerids include captive propagation for re-introductions (USFWS, 2005), but propagation and re-introduction efforts can cause more harm than good if genetic information is not used to inform management efforts (Edmands, 2007; George et al., 2009; Huff, Miller, Chizinski, & Vondracek, 2011; Jennings et al., 2010).

Analyses that explore historical demography, population connectivity and genetic diversity have been much less common on freshwater gastropods (but see Glow, Noble, Rollinson, Mimpfoundi, & Jones, 2004; Pfenninger, Salinger, Jaun, & Feldmeyer, 2011) than other freshwater groups such as mussels (e.g. Galbraith, Zanatta, & Wilson, 2015; Inoue, Lang, & Berg, 2015; Inoue, Monroe, Elderkin, & Berg, 2014; Stoeckle et al., 2017), fish (e.g. Fluker et al., 2014a; Fluker, Kuhajda, & Harris, 2014b; Fluker et al., 2010; Prunier, Dubut, Loot, Tudesque, & Blanchet, 2017; Thomaz, Malabarba, & Knowles, 2017; Van Leeuwen, Dalen, Museth, Junge, & Vøllestad, 2018) and insects (e.g. Finn & Adler, 2006; Finn et al., 2007; Geismar et al., 2015; Múrria et al., 2017; Razeng et al., 2017). Of the few molecular studies that have employed population-level sampling of pleurocerids, none have revealed fine-scale landscape genetic patterns. For instance, Whelan and Strong (2016) examined population-level mitochondrial heterogeneity, but they did not assess population connectivity or nuclear genome diversity among populations. Recently, Minton, McGregor, Hayes, Paight, and Inoue (2017) used inter-simple sequence repeats to examine population structure in *Elimia potosiensis*, but resolution across its range was limited. Research is needed to inform management decisions (Allendorf, 2017; McMahon, Teeling, & Höglund, 2014) and assess whether predictions of genetic diversity patterns across riverine landscapes (Finn et al., 2011; Meffe & Vrijenhoek, 2011; Paz-Vinas et al., 2015; Vannote et al., 1980) are applicable to low-dispersing, nonarthropod, invertebrates.

Fine-scale population genetics, coupled with morphological analyses, could also recover a better understanding of intraspecific shell shape variation displayed by many pleurocerid species. A common theme of many studies examining conchological variation within pleurocerid species is the hypothesis that shell shape variation is only, or primarily, the result of ecophenotypic plasticity (Dillon, 2011, 2014; Dillon, Jacquemin, & Pryon, 2013; Dunithan, Jacquemin, & Pyron, 2011; Minton, Lewis, Netherland, & Hayes, 2011; Minton, Norwood, & Hayes, 2008, but see Whelan, Johnson,

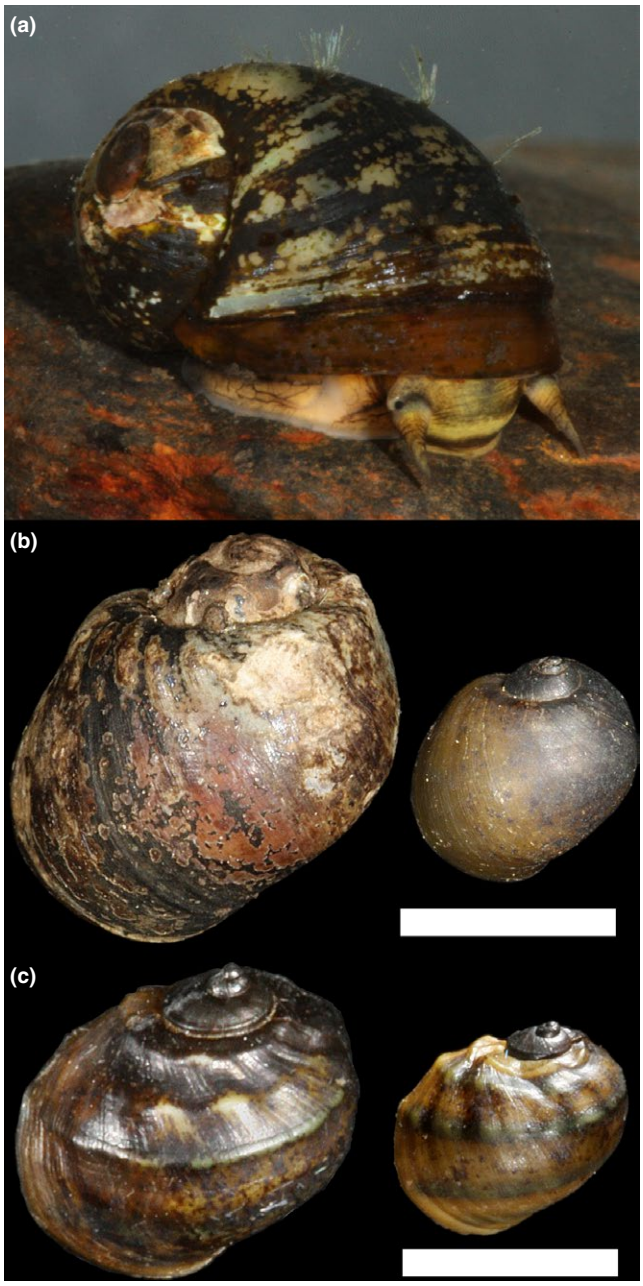

FIGURE 1 *Leptoxis ampla*. (a) Live individual, (b) common, smooth shell shape, (c) carinate shell shape found in the lower reaches of the Little Cahaba River. Scale bars = 5 mm. Photographs by Thomas Tarpley [Colour figure can be viewed at wileyonlinelibrary.com]

& Harris, 2012). This hypothesis states that intraspecific shell variation is not heritable and is instead the result of stream size (Dillon, 2011), or possibly other environmental factors. Consequently, shell shape differences among populations are not predicted to correlate with genetic patterns. Instead, individuals from the same population are predicted to have similar morphologies and individuals from different reaches with similar geomorphology (e.g. multiple headwater tributaries in a river network) should be more similarly shaped than individuals from reaches with disparate geomorphology (e.g. headwater tributaries vs. main stem). Despite the prevalence of studies claiming that shell shape differences among pleurocerid populations are the result of ecophenotypic plasticity (Dillon, 2011, 2014; Dillon et al., 2013; Dunithan et al., 2011; Minton et al., 2011, 2008), no study has examined shell shape in conjunction with high-resolution, population genomic data.

One pleurocerid of particular interest is *Leptoxis ampla*, or the Round Rocksnail (Figure 1), which is listed as threatened under the United States Endangered Species Act (Clark, 1998; Johnson et al., 2013). *Leptoxis ampla* is a small, riverine species that reaches sexual maturity within 1 year and has an iteroparous lifestyle (Whelan, Johnson, & Harris, 2015). Even though *L. ampla* was historically reported from the Coosa and Cahaba River basins in central Alabama, United States (Goodrich, 1922), recent work has suggested that records outside the Cahaba River were misidentifications and *L. ampla* is a Cahaba River endemic (Whelan, Johnson, Garner, & Strong, 2017; Whelan et al., 2012; Whelan & Strong, 2016). The headwaters of the Cahaba River begin in the Valley and Ridge Physiographic Province where the river is characterized by shallow, fast-flowing shoal habitat, interspersed with deeper, slower flowing pools (Supporting Information Figure S1). When the river crosses the fall line near Centreville, Alabama (Figure 2), the river gradient lowers and substrate is more characterized by fine sand and silt than the rocks and boulder substrates that are more common in the upper and middle reaches of the river. The Cahaba River is the only river in the Mobile River Basin, and one of few in the southeastern United States more broadly, to not be extensively modified for hydropower or navigation (Ward, Harris, & Ward, 2005). Furthermore, low-head dams in the main stem and most tributaries of the Cahaba River have been removed in the last 15 years (Bennett, Howell, Kuhajda, & Freeman, 2015; USFWS, 2016). Thus, the Cahaba River is an excellent system for gaining a better understanding of natural dispersal abilities of fluvial species. *Leptoxis ampla* is an ideal species for studying at the population level as its current range is relatively continuous in the headwaters and middle reaches of the Cahaba River drainage above the fall line, and there are no artificial impoundments within the current range of *L. ampla*. Furthermore, this species displays geographically associated morphological variation (Figure 1; Whelan et al., 2012), making it an appropriate species to examine whether genetic patterns are associated with morphological diversity. *Leptoxis ampla* is also found in multiple tributaries and main stem reaches of the upper and middle Cahaba River (Figure 2), allowing us to test whether populations lower in the river system have increased genetic diversity.

Given past work on broad patterns of riverscape genetic variation (e.g. Finn et al., 2011; Paz-Vinas et al., 2015), we hypothesized that genetic patterns of pleurocerids would generally follow the hierarchical nature of their drainage network with low genetic diversity, but high genetic uniqueness, in headwater reaches and a concomitant increase in diversity downstream. Support for these hypotheses would indicate genetic patterns across a riverine landscape can be predicted for even understudied groups like freshwater gastropods as these are general predictions based on the Mighty Headwaters Hypothesis, Stream Hierarchy Model and extensions of the River Continuum Concept. On the other hand, if these hypotheses are rejected then the field may

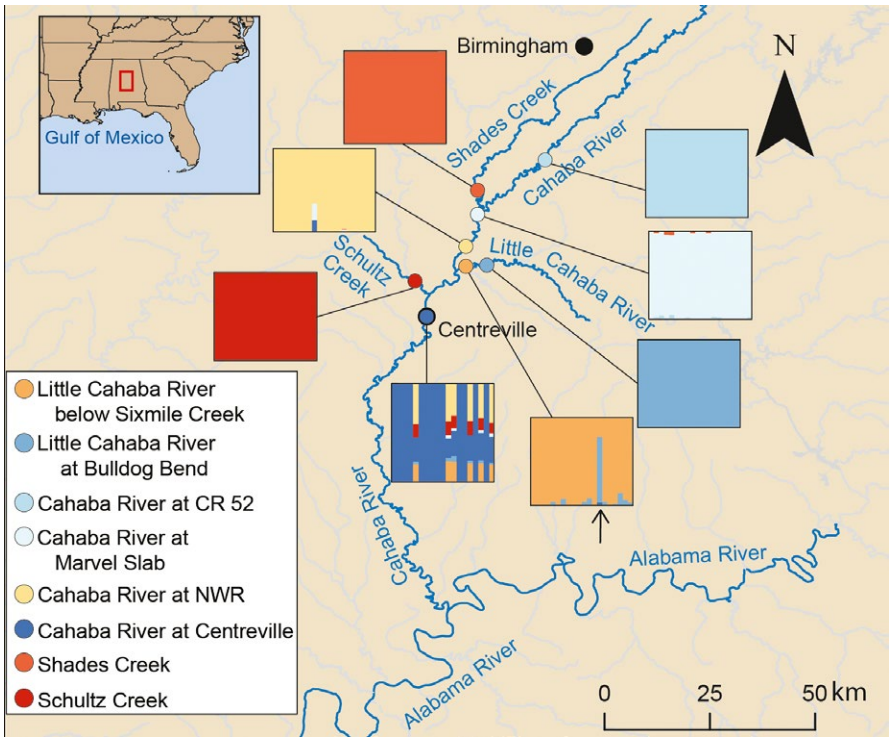

FIGURE 2 Map of *Leptoxis ampla* collection localities. Locations are labelled with ADMIXTURE plots ($K = 8$) of individuals from each site, inferred with data set MMAF 0.05. Each column indicates percentage of inferred shared ancestry with any given genetic cluster. Since the most likely K was the number of populations sampled, colours approximately correspond to the most prominent cluster at any given collection site. Arrow points to the one individual from Little Cahaba River below Sixmile Creek with a smooth shell; all others at this site had a carinate shell (Figure 1) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Characteristics of collection localities and catalog numbers for shell vouchers

Collection site	Number of individuals genotyped	Latitude	Longitude	Distance from centreville (km)[a]	AUMNH catalog numbers
Little Cahaba River below Sixmile Creek	20	33.0541	−87.0598	19.66	44090–44105, 44107–44109, 44254
Little Cahaba River at Bulldog Bend	20	33.0576	−87.024	27.51	44171–44189
Cahaba River at County Road 52	20	33.2845	−86.8826	76.01	44150–44169
Cahaba River at Marvel Slab	20	33.1653	−87.0296	36.21	44130, 44131, 44133–44149, 44255
Cahaba River at National Wildlife Refuge	21	33.0839	−87.0643	22.29	44110–44129, 44251, 44252
Cahaba River at Centreville	19	32.9462	−87.1402	0.00	44189–44204, 44208, 44209, 44257, 44258
Shades Creek	20	33.2202	−87.0334	47.84	44230–44249
Schultz Creek	20	33.0022	−87.1484	8.41	44208–44210, 44212–44229, 44259

[a]Distance measured by river path.

need to reevaluate whether broad predictions that apply to many insect and fish groups are applicable for low-dispersing invertebrates. Using *L. ampla* as a model, we test these hypotheses with a genomic data set generated with 2bRAD sequencing (Wang, Meyer, McKay, & Matz, 2012) and a morphological data set to assess whether intraspecific shell shape variation follows genetic patterns.

2 | MATERIALS AND METHODS

2.1 | Sample collection

An initial survey to identify suitable *L. ampla* collection sites was conducted throughout the Cahaba River system in August 2016 (Figure 2; Supporting Information Figure S1). We identified eight

localities spanning the historical range of *L. ampla*, ranging from the Cahaba River south of Birmingham, Alabama, to the fall line near Centreville, Alabama. These sites were selected because of network position (four tributary and four main stem localities spanning the species range), high snail abundance, and accessibility. Sampled sites also encompass the upstream and downstream extents of the species range and all tributaries known to harbour *L. ampla*. Initial survey work indicated that *L. ampla* was present at places in the Cahaba River system between chosen collection sites, but sampling was limited to eight sites because continuous sampling across the species range was not feasible, and we were interested in broad patterns across the range of *L. ampla*. In October 2016, 20-22 *Leptoxis ampla* individuals were collected by hand at each of the eight sites (Figure 2, Table 1). All *L. ampla* individuals were collected under federal permit TE130300 and a United States Fish and Wildlife Service Section 6 agreement with the Alabama Department of Conservation and Natural Resources.

At each site, snails were collected haphazardly, and we ensured sampling was not isolated to a single spot (e.g. one boulder). All samples were preserved following Fukuda, Haga, and Tatara (2008) and placed in 96%–100% ethanol. A 3 mm^3 of foot tissue was removed for DNA extractions, and all shells were deposited in the Auburn University Museum of Natural History (AUMNH). Shells were cataloged in such a fashion that all DNA extractions can be matched to individual shells.

2.2 | Genomic data set generation

DNA was extracted from foot tissue using the Qiagen DNeasy Plant Kit with slight modifications to accommodate a proteinase K tissue digestion. A plant kit was used to remove mucus polysaccharides and other PCR inhibitors that can be common in pleurocerid DNA extractions. To recover a reduced representation genome, we utilized the 2bRAD protocol of Wang et al. (2012; protocol available http://people.oregonstate.edu/~meyere/docs/2bRAD_25Aug2016.pdf) with the *Alf*I restriction enzyme. Pleurocerids have a genome size of approximately 2.1 Gigabases (Dillon, 1989) so a 1/16 reduction scheme was used during sample preparation to target ~4,800 RAD loci with expected coverage of ~20×. All samples were dual-barcoded to facilitate multiplexing on two Illumina flow cell lanes. Sequencing was done at Hudson Alpha Institute for Biotechnology (Huntsville, Alabama) on an Illumina HiSeq 2000 using 50 bp single-end chemistry.

Raw Illumina reads were demultiplexed by sample, trimmed to remove the first and last base pairs, and quality-filtered to remove reads where <90% of nucleotides had Phred scores >20 and those that had any Ns, homopolymer tracks of 10 or more nucleotides, and/or sequences of <34 bp; sequences were further filtered for the presence of an *Alf*I recognition site (scripts from https://github.com/Eli-Meyer/2brad_utilities). Quality-filtered raw reads were processed with *Stacks* 1.37 (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011; Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). The *denovo_map.pl* pipeline was selected because a reference genome was not available for any pleurocerid. Default parameters were used, except distances allowed between stacks (M = 3) and distance allowed between catalog loci (n = 3). These values and the use of the default value for minimum stack depth (m = 3) were chosen following recommendations of Paris, Stevens, and Catchen (2017). Assembled loci were processed with the *Stacks populations* program. We generated two data sets, one with a minimum minor allele frequency (MMAF) of 0.05 and one with a MMAF of 0.01. We consider the data set with MMAF of 0.05 to be our primary data set as false alleles are more likely to be present in the MMAF of 0.01 data set. Nevertheless, both data sets were used in most analyses so we could determine whether the presence of rare alleles affected results. Maximum observed heterozygosity of any given locus was set to 0.5 for both data sets to minimize the chance of retaining paralogous loci. To minimize missing data, only loci that were present in seven out of eight collection sites and 75% of individuals per collection site were retained. Loci with <20X coverage were discarded. Only one SNP per RAD locus was used in downstream analyses as most analyses assumed no linkage disequilibrium among SNPs. File formats for downstream analyses were either output by *Stacks* or converted from *Stacks* output to necessary file formats using *PGDSpider* (Lischer & Excoffier, 2012).

2.3 | Tests for loci under selection

We used *BayeScan* 2.1 (Foll & Gaggiotti, 2008) to identify loci that may be under selection. Given that we expected a large amount of genetic structure among populations, we ran analyses with prior odds of neutrality set to 1,000 and 10,000; we used a false discovery rate of 0.05. We ran a *blastn* analysis of the two loci that were putatively under selection (see results) against the NCBI nonredundant nucleotide database to see whether the function of the two loci possibly under selection could be inferred. *Blastn* analyses were done using default parameters, except for an e-value cut-off of 1.0.

2.4 | Genetic diversity, population structure, and gene flow

Standard population genetic parameters such as number of private alleles, nucleotide diversity and heterozygosity were calculated for each collection site with *Stacks*. Allelic richness was calculated with the R (R Core Development Team, 2017) package *diveRsity* (Keenan, McGinnity, Cross, Crozier, & Prodöhl, 2013). An analysis of molecular variance (AMOVA; Excoffier, Smouse, & Quattro, 1992) was calculated with the R package *Poppr* (Kamvar, Tabima, & Grünwald, 2014); individuals were classified by which site they were sampled from, a missing data cut-off of 0.3 was applied, and significance was tested with a 500 permutation randomization test.

Discriminant analysis of principal components (DAPC) was done with the R package *adegenet* (Jombart & Ahmed, 2011) to examine broad patterns of genetic clustering in our data. For DAPC, the number of clusters was chosen based on Bayesian information criteria. Finer-scale patterns of genetic admixture were assessed with the

TABLE 2 Pairwise geographical distance (km) and F_{ST} values for data set MMAF 0.05. Geographical distances between sampling sites, measured by stream network path, below diagonal. Pairwise F_{ST} values above diagonal

	Little Cahaba River below Sixmile Creek	Little Cahaba River at Bulldog Bend	Shades Creek	Schultz Creek	Cahaba River at County Road 52	Cahaba River at Old Marvel Slab	Cahaba River at National Wildlife Refuge	Cahaba River at Centreville
Little Cahaba River below Sixmile Creek	–	0.3774	0.7218	0.4973	0.7213	0.5790	0.5307	0.4628
Little Cahaba River at Bulldog Bend	7.85	–	0.7733	0.6051	0.7690	0.6162	0.5639	0.5130
Shades Creek	34.02	41.89	–	0.7417	0.7494	0.4458	0.5609	0.5869
Schultz Creek	17.32	25.17	45.59	–	0.7366	0.5923	0.5408	0.4760
Cahaba River at County Road 52	62.19	70.06	49.65	73.67	–	0.4115	0.5709	0.5919
Cahaba River at Old Marvel Slab	22.39	30.26	11.63	33.87	39.80	–	0.2730	0.3583
Cahaba River at National Wildlife Refuge	8.47	16.34	25.55	19.95	53.72	13.92	–	0.2119
Cahaba River at Centreville	19.66	27.51	47.84	8.41	76.01	36.21	22.29	–

model-based program *ADMIXTURE* 1.3 (Alexander, Novembre, & Lange, 2009; Shringarpure, Bustamante, Lange, & Alexander, 2016). The best-fitting number of genetic clusters for our data was identified with 10-fold cross-validation calculated in *ADMIXTURE*, and we performed ten independent *ADMIXTURE* replicates using the best-fit cluster number. *ADMIXTURE* results were visualized with *CLUMPAK* (Kopelman, Mayzel, Jakobsson, Rosebnberg, & Mayrose, 2015).

We also examined how geography influences patterns of gene flow. First, distances between collection sites were measured by tracing river path between sites in Google Earth (Table 2). River path was used because the most likely dispersal route of *L. ampla* is via water and traversing straight line distances over land is virtually impossible for a gilled pleurocerid. We calculated pairwise F_{ST} (Weir & Cockerham, 1984) as a measure of genetic differentiation between sites, using the *R* package *Hierfstat* (Goudet, 2005). A Mantel test of correlation between geographical distance and F_{ST} values was calculated with 999 random permutations in the R package ade4 (Dray & Dufour, 2007). F_{ST} values were also plotted against geographical distance, and we calculated a linear regression to further measure the extent to which geography could explain genetic differentiation. Given the significant signature of isolation by distance in both analyses on both data sets ($p < 0.010$; see results), we also used a recently developed method, *conStruct* (Bradburd, Coop, & Ralph, 2018), to examine fine-scale population structure in a spatially aware context. *conStruct* is conceptually similar to *ADMIXTURE*, except geographical distances between sampling localities are included in the model used for inferring population structure. We used 10-fold cross-validation implemented in *conStruct* to test the best-fit number of genetic clusters when geography was modelled with genetic data. Using the best-fit number of clusters, three independent *conStruct* analyses were ran for 50,000 Markov chain Monte Carlo (MCMC) generations. Convergence of independent analyses was assessed using trace plots generated by *conStruct*, and results were visualized using plots generated by *conStruct*. Computational demands of conStruct prevented us from analysing the larger MMAF 0.01 data set with this method.

Past studies have suggested that freshwater snails, including pleurocerids, have a net upstream movement (Houp, 1970; Huryn & Denny, 1997; Krieger & Burbank, 1976; Stewart, 2007). To test this hypothesis with *L. ampla*, we used *Migrate-n* 3.6.11 (Beerli, 2006; Beerli & Palczewski, 2010) to calculate the likelihood of our data under three demographic models: unrestricted movement between sites, only downstream movement, and only upstream movement. As a result of computational demand of *Migrate-n*, we randomly chose 100 variable loci for analyses from both MMAF 0.05 and MMAF 0.01 data sets. DNA sequences, rather than only SNPs, were used for migrate-n analyses as the SNP model in Migrate-n is not well tested (see Migrate-n manual). Distances between sites were measured as above (Table 2). *Migrate-n* was run under Bayesian inference using the DNA sequence model, a uniform prior distribution for mutation-scaled effective population size (θ) with a minimum value of 0.0 and a maximum value of 2.0, and a uniform prior distribution for migration rate with a minimum value of 0.0 and maximum value

of 20,000; prior distributions were chosen based on preliminary analyses. Mutation rates were allowed to vary. Analyses with each of the three demographic models were done with four independent replicates and four heated chains per replicate. Each replicate consisted of 140,000,000 MCMC steps, sampling every 100 steps, and the first 100,000,000 replicates were discarded as burn-in. Default values were used for all other parameters. To compare each model, log marginal likelihood values of each analysis were calculated by Bezier approximation with *Migrate-n*, and log Bayes factors (BF) were calculated by taking the difference between the log marginal likelihood of each model (Beerli & Palczewski, 2010).

2.5 | Geographical effects on genetic diversity

We assessed whether *L. ampla* displays a downstream increase in genetic diversity with linear regressions of network position against allelic richness, observed heterozygosity, expected heterozygosity and nucleotide diversity. Following Paz-Vinas et al. (2015), network position was measured as distance from Centreville, which is the most downstream point in the Cahaba River that we sampled and all sites pass through Centreville by river network path (Figure 2). All response variables of both data sets met assumptions of normality except observed heterozygosity. We used the R package bestNormalize (https://github.com/petersonR/bestNormalize/) to determine the best transformation technique for observed heterozygosity and subsequently transformed the variable with arcsinh transformation. Linear regressions were done in R.

2.6 | Geometric morphometrics

Using individuals sampled for genetic analyses, nine homologous landmarks (Figure 3) were assessed from all specimens via Procrustes analysis to measure gross shell shape. Morphometric landmarks (LM) followed methods of Cazenave and Zanatta (2016) and included the columella (LM 1), maximum width of the shell aperture (LM 2, 3), suture of the outer aperture lip and body whorl (LM 4), median of first suture (LM 5), width of second suture spire (LM 6,7), apex (LM 8) and the widest margin of the body whorl (LM 9) (Figures 3 and 4). Specimens were photographed using a copy stand and a Canon EOS Rebel digital camera with an 8-48 mm lens; the standard resolution of all images was 3,800 x 2,600 pixels at 30 cm distance and included a scale bar. All specimens were positioned with the aperture facing up as in Figures 3 and 4. Images were processed in *tpsDig* (Rohlf 2017) and employed the Procrustes superimposition method to account for the influences of size and rotation on the digitized landmarks.

To examine shape differences across the range of *L. ampla*, we analysed relative-warp scores obtained on the symmetric component from Procrustes superimposition procedures with MANOVA using collection site and whether the site was in the main stem Cahaba

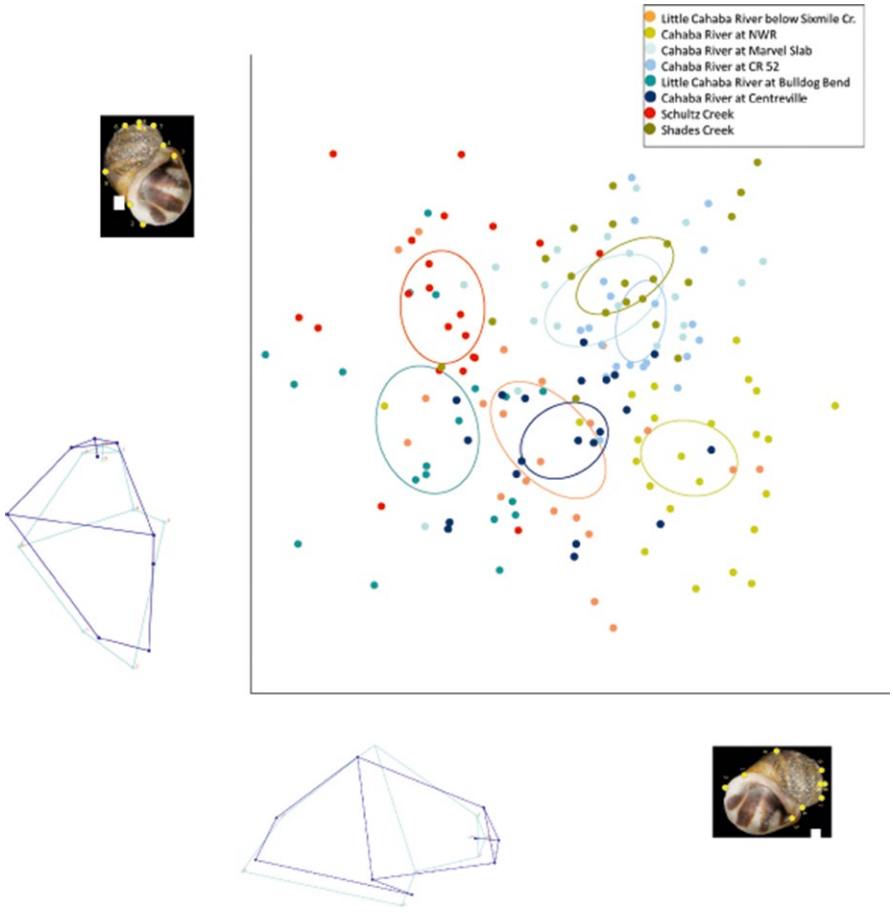

FIGURE 3 Canonical variance analysis ordination of shell shape for *Leptoxis ampla* from eight collection sites across the species range in the Cahaba River with 95% confidence ellipses around respective site centroids. CV 1 accounts for 40.8% of the total variation, and CV 2 accounts for 27.8% of the total variation. Wiregraphs are coloured with light blue for starting shape (near axis origin) and dark blue for target shape (near axis extremity). Shell photographs are apertural view of *L. ampla* (Lectotype, MCZ 161,806) and landmarks used for geometric morphometric analyses [Colour figure can be viewed at wileyonlinelibrary.com]

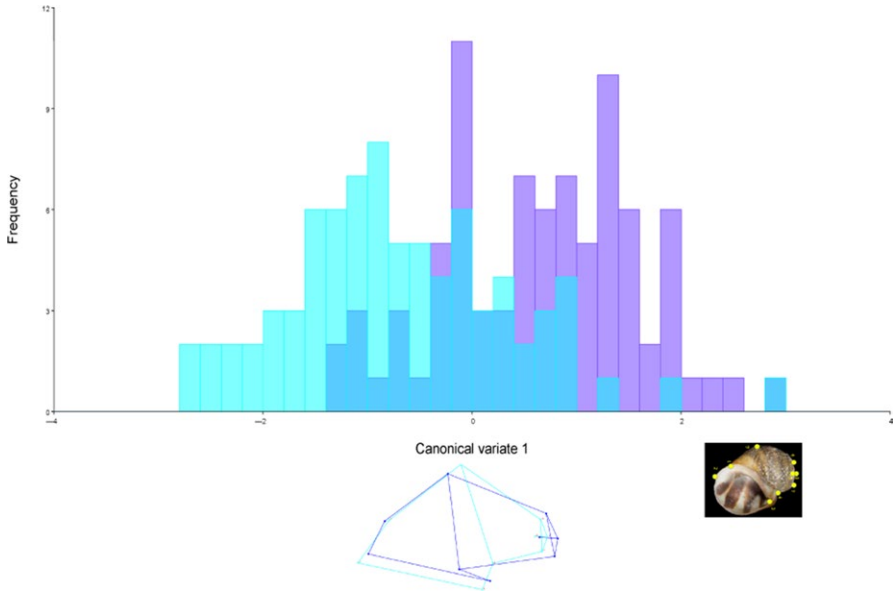

FIGURE 4 Canonical variance analysis frequency distribution for shell shape of *Leptoxis ampla* from pooled samples in tributary and main stem populations. Axis 1 accounts for 100% of the total variation. Wiregraph is coloured with light blue for starting shape (near axis origin) and dark blue for target shape (near axis extremity). Shell photograph is apertural view of *L. ampla* (Lectotype, MCZ 161,806) and landmarks used for geometric morphometric analyses [Colour figure can be viewed at wileyonlinelibrary.com]

River or a tributary as independent factors; these groupings were different from those used for AMOVAs (see above) because past work has suggested network position influences pleurocerid shell shape (Minton et al., 2011, 2008; Minton, Reese, Swanger, Perez, & Hayes, 2007), but we would not expect it to have much bearing on genetic similarity (i.e. populations in different tributaries are not predicted to be genetically more similar to each other than with a main stem population). Procrustes superimposition procedures account for differences in individual size, position and variations in image rotation on landmark coordinates (Klingenberg, 2011). A canonical variance analysis (CVA) was then conducted on the covariance matrix of the symmetric component of each site to determine whether there were any shape differences among specimens from different sites. The same procedure was repeated for network location. A permutation test of pairwise Procrustes distances between groups was computed using 1,000 iterations per comparison to test for significant differences between groups associated with each CVA. Wireframe graphs were utilized for visualization of morphological variation along each axis. All geometric morphometric analyses were conducted in *MorphoJ* software (Klingenberg, 2011).

3 | RESULTS

3.1 | Sample collection

We collected *L. ampla* from across its historical range (Figure 2), but we are not aware of a previous record of *L. ampla* from Schultz Creek. All but one individual from Little Cahaba River below Sixmile Creek had carinate shells, whereas all other sampled individuals had smooth shells (Figure 1). DNA was successfully extracted with enough yield for sequencing from 19 to 21 individuals from each site. All shells have been deposited at the Auburn University Natural History Museum under catalog numbers AUM44090-AUM44260 (Table 1).

3.2 | Molecular analyses

Sequencing failed in one individual from Shades Creek, so 159 individuals were included in the final molecular data set with individuals averaging 3,342,850 reads (range 1,302,566-6,500,245). The number of assembled RAD loci ranged from 20,796 to 89,593, with an average of 64,296, after filtering loci for assembly errors, possible paralogs, sequencing coverage, and missing data. A total of 7,607 loci were retained in data set MMAF 0.05, and 9,098 loci were retained in data set MMAF 0.01. A total of 4,999 loci in data set MMAF 0.05 and 5,020 in data set MMAF 0.01 were invariant across all individuals, and only one SNP was retained for each variable locus. This resulted in data set MMAF 0.05 having 2,608 SNP loci and data set MMAF 0.01 having 4,078 loci.

BayeScan analyses on both data sets indicated that two loci were under balancing or purifying selection when ran with prior odds of neutrality equalling 1,000. No loci were identified as under selection when prior odds of neutrality were equal to 10,000. *Blastn* analyses of the two loci possibly under selection against the NCBI nonredundant nucleotide database resulted in no hits with an e-value <1.0, but this result may be due to loci being only 34 bp in length. Given that outlier methods like *BayeScan* are known to be susceptible to high type I error rates for loci under balancing selection (Beaumont & Balding, 2004; Narum & Hess, 2011) and that no loci were considered under selection with prior odds of 10,000, we did not remove any loci from subsequent analyses.

TABLE 3 Population genetic diversity estimates from data set MMAF 0.05. Allelic richness, observed heterozygosity, expected heterozygosity and nucleotide diversity are population averages

Population	Private alleles	Allelic richness (*SD*)	Observed heterozygosity (*SD*)	Expected heterozygosity (*SD*)	∏ (*SD*)	Average θ (95% CI)
Little Cahaba River below Sixmile Creek	41	1.4970 (0.5274)	0.1647 (0.2032)	0.1683 (0.1860)	0.1728 (0.1908)	0.00126 (0.00000–0.00440)
Little Cahaba River at Bulldog Bend	11	1.3166 (0.4913)	0.1227 (0.2071)	0.1163 (0.1805)	0.1194 (0.1852)	0.00043 (0.00000–0.00347)
Shades Creek	17	1.1130 (0.3532)	0.0508 (0.1587)	0.043 (0.1233)	0.0441 (0.1269)	0.00037 (0.00000–0.00320)
Schultz Creek	120	1.3685 (0.5161)	0.1501 (0.2175)	0.1468 (0.1980)	0.1507 (0.2032)	0.00130 (0.00000–0.00440)
Cahaba River at county road 52	10	1.1311 (0.3556)	0.0524 (0.2289)	0.0464 (0.2154)	0.0477 (0.1304)	0.00273 (0.00000–0.00573)
Cahaba River at old Marvel Slab	4	1.4172 (0.4926)	0.1423 (0.2025)	0.1394 (0.1841)	0.1431 (0.1889)	0.00505 (0.00147–0.00840)
Cahaba River at National Wildlife Refuge	24	1.4637 (0.5238)	0.1566 (0.2005)	0.1552 (0.1841)	0.1591 (0.1887)	0.00603 (0.00240–0.00947)
Cahaba River at Centreville	23	1.5862 (0.4801)	0.1506 (0.1741)	0.1828 (0.1847)	0.1878 (0.1897)	0.00291 (0.00000–0.00600)

Note. ∏ = nucleotide diversity. θ = mutation-scaled effective population size.

The number of private alleles present at each sampling locality with the MMAF 0.05 data set ranged from 4 at Cahaba River at Marvel Slab to 120 at Schultz Creek (Table 3). Allelic richness ranged from 1.113 in Shades Creek to 1.5856 at Cahaba River at Centreville (Table 3). Average observed heterozygosity and expected heterozygosity ranged from 0.0508 and 0.043 to 0.1647 and 0.1683, respectively, with the lowest amount of heterozygosity present in Shades Creek and the highest amount in Little Cahaba River below Sixmile Creek (Table 3). Average nucleotide diversity ranged from 0.0441 at Shades Creek to 0.1878 at the Cahaba River at Centreville (Table 3). Similar patterns were seen with the MMAF 0.01 data set (Supporting Information Table S1), except overall number of private alleles was much higher and some main stem populations had more private alleles than tributary populations. Linear regressions of network positions (as measured by distance from Cahaba River at Centreville) against all four genetic diversity estimates of both data sets were significant (p = 0.004–0.014; Table 4 and Supporting Information Table S2; Supporting Information Figures S2 and S3), and at least 65% of the variation of each metric was explained by river position (Table 4 and Supporting Information Table S2).

TABLE 4 Results of linear regressions for the effects of network position on metrics of genetic diversity from the MMAF 0.05 data set

Genetic metric	Distance from centreville	R^2	p
Allelic richness	−0.0059	0.70	**0.010**
Observed heterozygosity	−0.0352	0.71	**0.009**
Expected heterozygosity	−0.0020	0.78	**0.004**
Nucleotide diversity	−0.0020	0.78	**0.004**

Note. Values for distance to Centreville are regression coefficients; each coefficient was significant in every model. R^2 and p-values are for the full model. Bold values represent significant p-values.

Considerable genetic structure was recovered among populations, and results were very similar between the two data sets with different MMAF. AMOVAs on both data sets were significant. For the MMAF 0.05 data set, 58.0% of genetic variation was explained by collection site (p = 0.002), and for the MMAF 0.01 data set, 53.7% of genetic variation was explained by collection site (p = 0.002). DAPC analyses on both data sets indicated eight distinct clusters

TABLE 5 MANOVA results of partial warp scores from the Procrustes procedure for *Leptoxis ampla* to determine general shape differences between sites and network position

Source	Wilks' Λ	*F*	*df*	*p*
Site	0.102	4.07	98, 919	**<0.001**
Position	0.687	4.862	14, 150	**<0.001**

Bold values represent significant p-values.

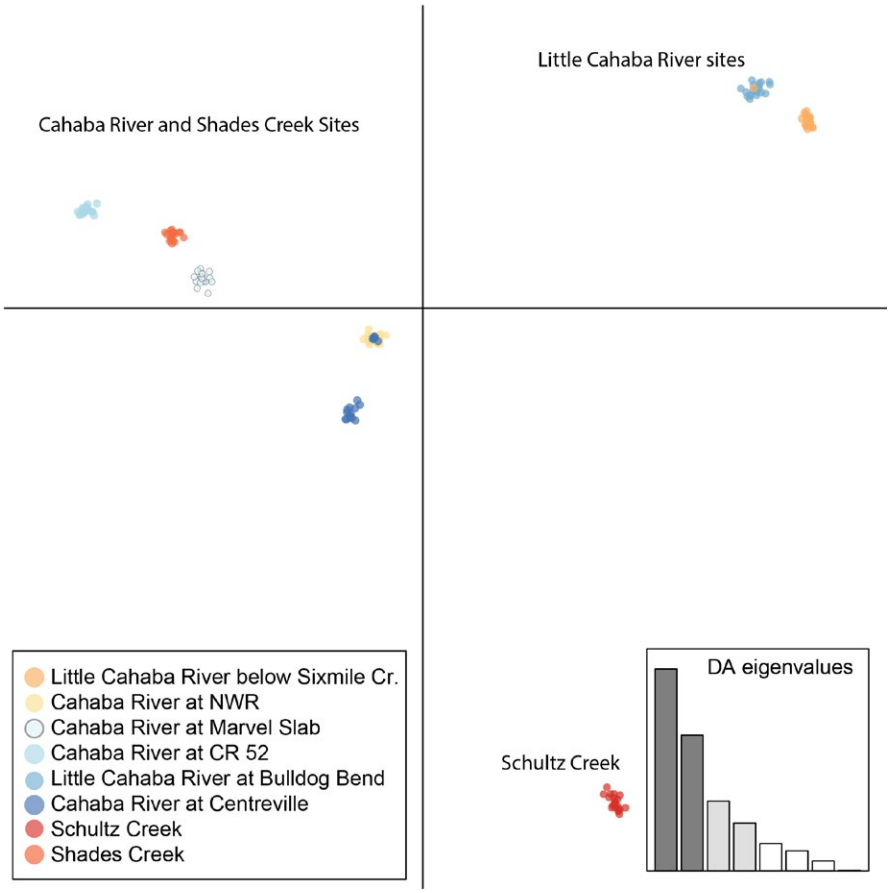

FIGURE 5 DAPC plot of individuals with data set MMAF 0.05, coloured by sampling locality [Colour figure can be viewed at wileyonlinelibrary.com]

of individuals, generally associated with population (Figure 5 and Supporting Information Figure S4). However, one individual from the Little Cahaba River below Sixmile Creek clustered with individuals from Little Cahaba River at Bulldog Bend, and six individuals from Cahaba River at Centreville clustered with Cahaba River at National Wildlife Refuge (Figure 5 and Supporting Information Figure S4). Pairwise F_{ST} values ranged from 0.2119 between the Cahaba River at National Wildlife Refuge and Cahaba River at Centreville to 0.7733 between Shades Creek and Cahaba River at Bulldog Bend (Table 2); similar, but lower overall, pairwise F_{ST} values were seen for data set MMAF 0.01 (Supporting Information Table S3). A significant relationship was seen between pairwise F_{ST} and geographical distance between populations (MMAF 0.05: $p = 0.0006$, $R^2 = 0.347$; MMAF 0.01: $p = 0.0003$, $R^2 = 0.394$; Figure 6 and Supporting Information Figure S5), and Mantel tests were also significant ($p < 0.008$), indicating a pattern of isolation by distance and corroborating the Stream Hierarchy Model.

ADMIXTURE analyses on both data sets also showed clear structure among populations. Eight was the most likely number of clusters (K) based on cross-validation error, equalling the number of sampled populations. Most individuals showed little evidence of genetic admixture with other clusters, and instances for which an individual did have mixed ancestry, the vast majority of the mixed ancestry was shared with an upstream population (Figure 2 and Supporting Information Figure S6). Notably, the one smooth-shelled individual from Little Cahaba River below Sixmile Creek had over 50% genetic

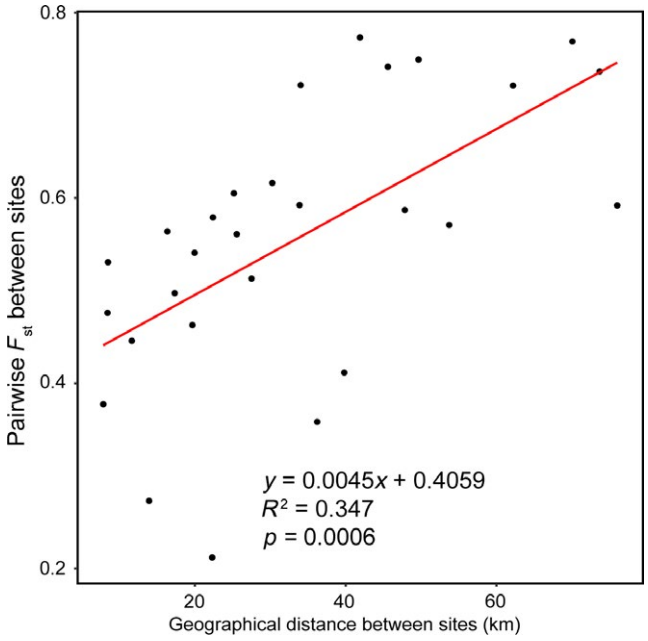

FIGURE 6 Linear regression of pairwise Fst of data set MMAF 0.05 and geographical distance between collection sites [Colour figure can be viewed at wileyonlinelibrary.com]

admixture with the upstream population. Conversely, all other individuals from Little Cahaba River below Sixmile Creek had a carinate shell form and small amounts of inferred admixture. *conStruct* analyses that incorporated a geographical layer into analyses indicated a

most likely *K* of 5. This lower *K* value than that inferred with ADMIXTURE further indicates a pattern of isolation by distance (Bradburd et al., 2018). Greater genetic admixture among clusters was inferred by *conStruct* (Figure 7), which we expected given the significant pattern of isolation by distance seen among populations. In both ADMIXTURE and conStruct analyses, each population was inferred to harbour individuals with genomic profiles not seen in other populations.

Demographic modelling with *Migrate-n* revealed that migration patterns among populations of *L. ampla* were significantly downstream-biased. For data set MMAF 0.05, a model that only allowed downstream migration was greatly favoured over both a model that allowed for unrestricted movement among populations and a model that allowed only upstream migration with BF of 2,742 and 6,385, respectively. The unrestricted movement model was also strongly favoured over the upstream model with a BF of 3,642. Results were similar to the MMAF 0.01 data set with the downstream model being favoured over other models with BF greater than 2,500. According to the best-fit migration model, θ was inferred to be much lower in tributaries (0.00037–0.00130) than in the main stem Cahaba River (0.00273–0.00603; Table 3).

3.3 | Geometric morphometrics

We digitized and analysed 166 individuals across the eight sites; a slightly higher number of individuals were used in morphometric analyses than genetic analyses as we included individuals where DNA extractions failed to produce enough yield. MANOVA indicated a significant difference in shape across sites ($p < 0.001$; Table 5). Despite significant differences indicated by MANOVA, CVA indicated considerable overlap in shell shape associated with each site (Figure 3), but axes 1 and 2 of the CVA explained only 68.6% of the total variation seen among collection sites (40.8% and 27.8%, respectively, Figure 3). Generally, axis 1 reflected a negative gradient with first whorl width and axis 2 reflected a positive gradient with first whorl width (Figure 3). MANOVA also indicated significant shell shape differences between main stem and tributary populations ($p < 0.001$; Table 6). Axis 1 of the CVA explained 100% of the variation between tributary and main stem sites; thus, results are displayed as a frequency histogram (Figure 4). There was overlap in shape between main stem and tributary individuals, but Procrustes distances among groups were significantly different ($p = 0.0163$). In general, aperture height varied slightly and body shape was slightly more fusiform in main stem as opposed to tributary individuals (Figure 4).

Permutation tests for differences in Procrustes distances among each site revealed that specimens from many sites were morphologically unique relative to each other (Table 6). Notable exceptions include those from Little Cahaba below Sixmile Creek and Cahaba River at Marvel Slab, which were generally not unique in overall shape (Table 6). Even though both of these sites showed broad overlap and no significant shape difference with most other sites, GMM with this landmark set would not have discriminated morphological differences associated with carinate and smooth shell forms. Schultz Creek snails were morphologically different in shape from every other site, and Shades Creek snails were morphologically different in shape from every other site except Cahaba River at Marvel Slab (Table 6). Notably, shell shape was significantly different among most tributary sites.

4 | DISCUSSION

Genetic patterns seen in *L. ampla* conform to broad predictions made by the downstream increase in intraspecific genetic diversity hypothesis (Paz-Vinas et al., 2015) and the Stream Hierarchy Model (Meffe & Virjenhoek, 1988). Neutral genetic diversity estimates such as allelic richness and nucleotide diversity were higher the lower a site was in the Cahaba River system, and a significant pattern of isolation by distance was inferred with multiple analyses. Even though headwater populations are relatively distinct, our results suggest that main stem populations also harbour individuals with unique genomic profiles, conflicting in part with the Mighty Headwaters Hypothesis. Our findings are also not fully predicted by the River Continuum Concept as some tributary populations had higher genetic diversity than main stem populations, indicating that genetic diversity patterns across a riverscape cannot be predicted based solely on stream order. However, network position does appear to predict genetic diversity as more downstream populations generally had greater genetic diversity (Supporting Information Figure S1). Contrary to suggestions of

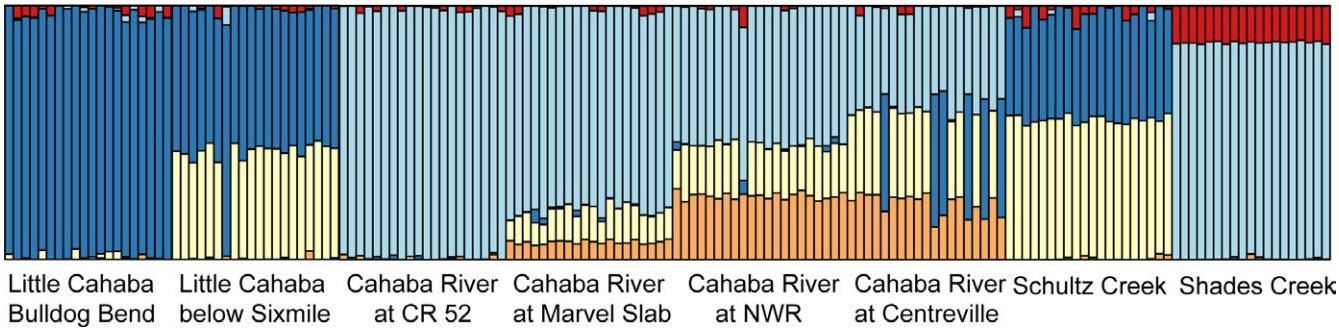

FIGURE 7 Genetic admixture inferred with conStruct (*K* = 5) with data set MMAF 0.05. Each column indicates percentage of inferred shared ancestry with any given genetic cluster and is colour-coded by genetic cluster [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 6 *p*-values from permutation tests (10,000 permutation rounds) for Procrustes distances among sites for *Leptoxis ampla*

	Little Cahaba River at Bulldog Bend	Cahaba River at CR 52	Cahaba River at Centreville	Cahaba River at Marvel Slab	Cahaba River at NWR	Shultz Creek	Shades Creek
Cahaba River at CR 52	**0.0046**						
Cahaba River at Centreville	0.0513	**<0.0001**					
Cahaba River at Marvel Slab	0.0615	0.3035	0.0685				
Cahaba River at NWR	**0.0317**	**0.0099**	**0.0008**	**0.0375**			
Shultz Creek	**0.0035**	**<0.0001**	**0.0098**	**0.0367**	**<0.0001**		
Shades Creek	**<0.0001**	**0.0001**	**<0.0001**	0.117	**<0.0001**	**<0.0001**	
Little Cahaba River below Sixmile Creek	0.576	0.0722	0.0632	0.1297	0.0778	**0.0168**	**0.0006**

Note. Bold values denote significantly different ($p < 0.05$) shapes between sites.

past studies on pleurocerids (Houp, 1970; Huryn & Denny, 1997; Krieger & Burbank, 1976; Stewart, 2007), *L. ampla* movement between populations was found to be significantly downstream-biased. We also found that even without artificial impoundments, populations in tributaries are rather isolated, and mutation-scaled effective population size was also consistently lower in tributaries than main stem populations regardless of tributary position in the overall system. We expect findings to be applicable to other pleurocerids given general similarities in life history among pleurocerids (Dazo, 1965; Whelan et al., 2015).

Generally, population isolation appears to be a natural part of pleurocerid biology. Given this, populations of pleurocerid species separated by artificial impoundments (e.g. *Leptoxis coosaensis*; Whelan et al., 2017) are probably not as affected as organisms with greater dispersal abilities such as fishes (Fluker et al., 2014a). Impoundments are often considered a primary cause of species decline in the Mobile River Basin (Johnson et al., 2013; Neves et al., 1997; Williams, Bogan, & Garner, 2008), but they may not be the biggest risk to remaining species that have survived initial impoundment. That is, no major impoundments will probably be constructed in the Mobile River Basin over the next 50 years, but human population growth is predicted to be higher in the southeastern United States than most other regions (Pendall, Martin, & Astone, 2015). Thus, we should expect increased pressures on freshwater organisms in the southeastern United States, and tributary populations may be particularly susceptible to population decline. Without conservation efforts and/or sustainable development, continued urban sprawl could result in decreased genetic diversity and higher extinction risk of slow-dispersing freshwater invertebrates.

4.1 | Downstream-biased movement patterns

Our findings broadly conflict with past studies on pleurocerids (Houp, 1970; Huryn & Denny, 1997; Krieger & Burbank, 1976; Stewart, 2007) and other freshwater gastropod lineages (reviewed by Kappes & Hasse, 2012) that suggested freshwater snails have movement patterns that strongly favour upstream migration over downstream movement (but see Yeung & Dudgeon, 2015). *Migrate-n* analyses strongly indicated that migration patterns in *L. ampla* are downstream-biased. ADMIXTURE and conStruct analyses also indicated that migration and population admixture are largely unidirectional as in the few instances that an individual had a large proportion of shared ancestry, it was always seen in an individual that had genetic admixture with an upstream population (Figures 2 and 7, Supporting Information Figure S6). A similar pattern was seen with the DAPC analysis where if an individual clustered with a different population from where it was collected, that individual was always clustered with an upstream population (Figure 5 and Supporting Information Figure S4).

Upstream migration has been argued to be necessity for freshwater snails to maintain population sizes in the most upstream reaches of a species range. This upstream hypothesis has been most strikingly supported by the behaviour of neritid snails where coordinated, mass upstream movement has been documented (Pyron & Covich, 2003; Schneider & Frost, 1986; Schneider & Lyons, 1993). However, nerite snails have a planktonic veliger larval stage that is carried downstream by natural flows, almost guaranteeing the inability of population persistence without considerable upstream movement by adults (Schneider & Frost, 1986). In contrast, pleurocerids lay eggs on rocks and do not have a pelagic larval stage, making them less susceptible to being carried downstream by natural water flows (Whelan et al., 2015).

Previous mark–recapture studies (Houp, 1970; Huryn & Denny, 1997; Krieger & Burbank, 1976; Stewart, 2007) that documented upstream movement in pleurocerids did not include *L. ampla*, but we still predict our findings to be broadly applicable to other pleurocerids because of similar life histories and dispersal abilities (Huryn, Benke, & Ward, 1995; Huryn, Koebel, & Benke, 1994; Whelan et al., 2015; Whelan & Strong, 2014). Moreover, past studies focused on river reaches spanning hundreds of metres, whereas the closest two populations we examined were separated by 7.85 kilometres (Table 2). The greatest movement distance observed by Huryn and Denny (1997) for an individual snail was less than 300 metres. They observed virtually no downstream movement, but we cannot rule

out that snails were washed downstream and not recaptured, as over 80% of tagged snails were not recaptured. Huryn and Denny (1997) provided evidence that snails face upstream to reduce drag from water moving over a snail's shell, resulting in upstream movement of individual snails. Such movement is likely necessary for habitat colonization given an appropriately long timescale. Limited upstream movement, especially at small spatial scales, surely occurs in pleurocerids. However, predominately downstream movement demonstrated here likely results in higher genetic admixture seen in downstream populations (as shown in Figures 2 and 7, Supporting Information Figure S6) by facilitating gene flow across larger distances than any one snail probably ever moves upstream.

Broadly, our findings emphasize the need to consider local and range-wide processes when assessing landscape ecology of low-dispersing invertebrates, and we view movement patterns of riverine organisms to be an area of research need. There are some species with well-documented migration patterns (e.g. anadromous fish), but many groups, particularly molluscs and crustaceans, have not been thoroughly studied. Information on movement patterns is essential for better understanding how species respond to natural and anthropogenic disturbances, and to understand historical range expansion or contraction following geological events like glaciation. Future research will also be important for determining whether broad predictions can be made about how different animal groups disperse across a riverine landscape.

4.2 | Gene flow and genetic distinctiveness between populations

When considered in full, our results indicate that populations in the main stem Cahaba River are more genetically diverse and have greater population connectivity than populations found in tributaries (Table 3 and Supporting Information Table S1; Figures 2 and 7, Supporting Information Figure S6). *ADMIXTURE* and conStruct analyses indicated that populations in the Cahaba River main stem have more shared ancestry with other main stem populations than with tributary populations (Figures 2 and 7, Supporting Information Figure S6). This pattern is more prominent the further downstream a population was in the Cahaba River, further indicating that gene flow among *L. ampla* populations is considerably influenced by the geographical landscape. Genetic diversity estimates such as observed heterozygosity and nucleotide diversity were also generally higher in main stem populations, but exceptions exist including in the most upstream population, Cahaba River at County Road 52 near Birmingham, Alabama, which had relatively low genetic diversity estimates and the Little Cahaba River below Sixmile Creek, which has the highest observed heterozygosity levels of any sampled site. These exceptions suggest that genetic diversity patterns across the riverine landscape are shaped by factors in addition to stream size.

Patterns of genetic diversity seen in *L. ampla* conform to only some aspects of the Mighty Headwater Hypothesis of diversity in stream networks (Finn et al., 2011). In agreement with the Mighty Headwaters Hypothesis, headwater populations are rather distinct from other populations, which is exemplified by relatively high numbers of private alleles and limited genetic admixture seen in Schultz Creek and Little Cahaba River below Sixmile Creek populations. However, unique shared ancestry profiles of individuals from higher order reaches of the Cahaba River drainage, compared to headwater populations, are not predicted by the Mighty Headwater Hypothesis. That is, by consisting of individuals with shared ancestry profiles unique to any given population (Figures 2 and 7, Supporting Information Figure S6), the main stem populations are more genetically unique than predicted. One likely explanation for why our observations fall partly outside the Mighty Headwaters Hypothesis predictions is that only mitochondrial genetic data were used in the original study that developed the hypothesis (Finn et al., 2011). Analyses with such data would have failed to reveal unique nuclear genetic admixture profiles required to fully understand the genetic uniqueness of populations and individuals.

Studies on freshwater fish have shown that genetic distinctness of tributary populations and gene flow among tributaries are highly dependent on life history, dispersal ability, and the presence of artificial dispersal barriers like reservoirs and dams (Bessert & Ortí, 2008; Fluker et al., 2014a, 2014b). Research focusing on low-vagility aquatic invertebrates found in the Mobile River Basin (e.g. endemic crayfishes) have demonstrated high levels of population structure and different levels of genetic diversity within the same drainage, although not explicitly in a hierarchical network arrangement (Helms, Vaught, Suciu, & Santos, 2015). Whether the fine-scale genetic patterns we observed are unique to freshwater snails or are extendable to other low-vagility aquatic taxa is unknown. Nevertheless, our data reject the idea that headwater, or tributary, populations are more important than main stem populations for maintaining genetic distinctness across pleurocerid species.

4.3 | Genetic diversity and population size vary with riverine landscape

Our analyses revealed a pattern of increased intraspecific genetic diversity in *L. ampla* the lower a site was in the Cahaba River system. To our knowledge, such a genetic pattern has not been previously found in freshwater snails, but is common in other freshwater groups (Paz-Vinas et al., 2015). The primary driver of this pattern is likely downstream-biased migration by facilitating greater genetic diversity through gene flow to downstream populations. However, upstream populations (e.g. Shades Creek and Cahaba River at County River 52) are also the populations closest to the Birmingham, Alabama, metropolitan area and may be negatively influenced by anthropogenic activities like pollution. Most likely, a complex interplay of factors influences the downstream increase in genetic diversity seen here. Future studies on pleurocerid species where upstream populations are not near metropolitan areas may be necessary to fully understand the causes of the pattern seen in *L. ampla*.

Mutation-scaled effective population size (θ) was lower and similar across all tributary populations, even despite high levels of overall genetic diversity in some populations (e.g. Little Cahaba River below Sixmile Creek; Table 3 and Supporting Information Table S1).

Notably, the populations with the highest θ, Cahaba River at Marvel Slab and Cahaba River at National Wildlife Refuge, were near the centre of *L. ampla*'s range, possibly supporting the abundant-centre hypothesis (reviewed by Sagarin, Gaines, & Garylord, 2006). We are not aware of other studies examining this hypothesis on freshwater snails, and our data suggest that a abundant-centre pattern applies to *L. ampla* and may apply to other pleurocerids given similar life histories among species.

Unlike *L. ampla*, many pleurocerid species have been affected by river channel modification for navigation and hydropower, often resulting in extinctions or fragmented populations that remain only in tributaries (Johnson et al., 2013). Given patterns observed here, we expect populations of these fragmented species to have relatively low effective population sizes and possibly low genetic diversity if remaining populations are in upstream tributaries. Populations with lower effective population sizes are more likely to experience drift that causes loss of adaptive alleles (Wright, 1931). Thus, pleurocerid populations that once existed in both tributaries and main stem reaches, but now only persist in tributaries, may be at high extinction risk. On the other hand, gene flow between populations of *L. ampla*, particularly among tributaries, is low. This pattern was surprising given a lack of artificial dispersal barriers (e.g. reservoirs) across the range of *L. ampla*. Pleurocerid species affected by impoundment probably had similarly low population connectivity prior to anthropogenic habitat modification. Thus, artificial dispersal barriers, particularly on the timescale they have existed, may not affect unnaturally disjunct pleurocerid populations as much as they have been shown to affect animals with greater dispersal abilities like fish (Bessert & Ortí, 2008; Fluker et al., 2014a).

4.4 | Ecophenotypic plasticity alone does not explain variation in shell morphology

The role of genetic variation in influencing shell morphology is exemplified by the two populations from the Little Cahaba River. Unlike other populations sampled, the population at Little Cahaba River below Sixmile Creek is dominated by individuals with a carinate shell shape (Figure 1). However, the one individual from Little Cahaba River below Sixmile Creek with a smooth shell form had a relatively large amount of shared ancestry with the upstream population, Little Cahaba River at Bulldog Bend, which is dominated by individuals with smooth shells (Figures 1, 2 and 7, Supporting Information Figure S6). This individual also clustered with individuals from the Little Cahaba River at Bulldog Bend in DAPC analysis (Figure 5 and Supporting Information Figure S4). This suggests a strong genetic component to shell shape. Although such a distinct association with genetic profile and a qualitative shell shape character was observed in only one individual, it corroborates the findings of Whelan et al. (2012), Whelan et al. (2015) that ecophenotypic plasticity is not the primary cause of intraspecific shell shape variation in pleurocerids.

Although sampling above and below the Little Cahaba below Sixmile Creek allowed us to address differences in two distinct shell forms, geometric morphometric analyses allowed us to examine shape change across the range of *L. ampla*. Geometric morphometric analysis revealed that there were significant shape differences across the Cahaba River drainage. Shape differences were subtle (if at all detectable) to the naked eye, but were manifest as slight gradients in whorl width and shell height across the sites. At a broad scale, tributary populations were more similar to each other than main stem populations (Figure 4), but most tributary populations were significantly different from other tributary populations (Figure 3; Table 6). In contrast to patterns seen among tributaries, main stem populations (aside from at CR 52) were generally similar in shell shape (Table 6). This similarity could be a result of adaptation to similar environmental conditions (i.e. those of the main stem Cahaba River), a result of higher gene flow between main stem sites compared to other populations, or both. Notably, shell morphology of individuals from Shades Creek was not significantly different from individuals from Cahaba River at Marvel Slab. Shades Creek is a smaller river than Cahaba River at Marvel Slab, but Shades Creek is genetically more similar to Cahaba River at Marvel Slab than any other site based on pairwise F_{st} values (Table 2 and Supporting Information Table S3) and *conStruct* results (Figure 7). This directly rejects the hypothesis that genetics plays no role in intraspecific shell shape and stream size alone causes intraspecific shell shape variation (Dillon, 2011).

Limited gene flow among sites likely facilitates shell morphology associated with sampling locality and genetic patterns. That is, evolutionary theory suggests that even low selective pressures on shell shape would be expected to facilitate location-specific shell morphology in the light of low migration between populations (Haldane, 1930; Hämälä, Mattila, & Savolainen, 2018; Lenormand, 2002; Yeaman & Otto, 2011; Yeaman & Whitlock, 2011). Such selection on shell morphology was recently demonstrated in another Cerithioidean, *Melanoides tuberculata* (Abdelhady et al., 2018), and we predict it may be more common than currently appreciated. Future studies that examine genes involved in pleurocerid shell morphology, rather than anonymous 2bRAD loci, will be needed to better assess the influence of selection vs. drift on shell shape differences among populations. Nevertheless, ecophenotypic plasticity should no longer be the null hypothesis for explaining intraspecific shell shape differences in pleurocerids, especially in regard to discrete characteristics like presence and absence of shell sculpture.

4.5 | Conservation implications

The genetic distinctness of sampled populations suggests that all populations are important for conserving genetic diversity of *L. ampla*, rather than emphasizing either tributary or main stem populations. That is, if any one population is extirpated, unique genomic profiles would be lost. Given that sampled populations of *L. ampla* were rather distinct, we caution against population augmentation, either through translocation or captive propagation efforts, so outbreeding depression can be avoided. We expect similar genetic patterns to be seen among other pleurocerids, and without data suggesting otherwise, we suggest that managers also avoid

augmentation for recovery of other pleurocerid species. However, re-introductions to sites from which a species has been completely extirpated are likely a viable management strategy. The observed pattern of greater genetic diversity in downstream habitats for *L. ampla* indicates that downstream populations, particularly main stem populations, are likely to be ideal broodstock populations from a genetic diversity standpoint. We expect similar patterns to exist for other imperilled pleurocerids.

Our results suggest that *L. ampla* is present across its historical range (see Goodrich, 1922; Whelan et al., 2017 for more details on the historical range of *L. ampla*). However, we are not aware of any previous reports of the species being found in Schultz Creek, indicating that past survey work overlooked at least one population. The Cahaba River is still threatened by increasing urbanization in the Birmingham, Alabama, metropolitan area and other anthropogenic stressors, but *L. ampla* appears to have either never suffered a range contraction or naturally expanded its range in recent years. Given limited amounts of gene flow and inferred migration, we think the more likely explanation is that previous surveys for *L. ampla* did not adequately sample Cahaba River tributaries. Together, these suggest that the threatened status of *L. ampla* needs reevaluation.

5 | CONCLUSIONS

Despite their ecological importance to freshwater ecosystems in eastern North America, little is known about pleurocerid genetics and migration. Our findings demonstrate that widely held hypotheses such as net upstream movement of riverine gastropods and ecophenotypic influences on shell shape fail to fully describe pleurocerid biology. As an understudied group, it should not be surprising that detailed study of *L. ampla* conflicted with past hypotheses. However, some broader hypotheses did predict patterns seen in *L. ampla*, including a downstream increase in genetic diversity and the Stream Hierarchy Model. Nevertheless, patterns did not fully conform to established stream diversity hypotheses like the Mighty Headwaters Hypothesis that were derived from relatively vagile organisms like insects. Even though our study focused on *L. ampla*, we expect our findings to be broadly applicable to other pleurocerid species and possibly other freshwater groups with similarly low dispersal abilities.

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AUTHOR CONTRIBUTIONS

N.V.W., B.S.H., P.D.J. and K.M.H. designed the study. N.V.W., B.S.H., B.N.S., and P.D.J. collected samples. N.V.W., M.P.G and B.N.S. performed molecular laboratory work and analyses. B.S.H and J.M.W. carried out geometric morphometrics. N.V.W. and B.S.H. wrote the manuscript. All authors edited the manuscript.

DATA ACCESSIBILITY

Shell vouchers have been deposited to the Auburn University Museum of Natural History (Table 1). Raw sequence data have been submitted to NCBI SRA database under BioProject PRJNA517492. The final SNP data set in various file formats and shell photographs have been deposited to FigShare doi: http://doi.org/10.6084/m9.figshare.7054523.v1. Scripts written for this study are available at http://github.com/nathanwhelan/population_genomics_scripts

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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