Gene family amplification facilitates adaptation in freshwater unionid bivalve *Megalonaias nervosa*

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Abstract
Freshwater unionid bivalves currently face severe anthropogenic challenges. Over 70% of species in the United States are threatened, endangered or extinct due to pollution, damming of waterways and overfishing. These species are notable for their unusual life history strategy, parasite–host co-evolution and biparental mitochondrial inheritance. Among this clade, the washboard mussel *Megalonaias nervosa* is one species that remains prevalent across the Southeastern United States, with robust population sizes. We have created a reference genome for *M. nervosa* to determine how genome content has evolved in the face of these widespread environmental challenges. We observe dynamic changes in genome content, with a burst of recent transposable element proliferation causing a 382 Mb expansion in genome content. Birth–death models suggest rapid expansions among gene families, with a mutation rate of $1.16 \times 10^{-8}$ duplications per gene per generation. *Cytochrome P450* gene families have experienced exceptional recent amplification beyond expectations based on genome-wide birth–death processes. These genes are associated with increased rates of amino acid changes, a signature of selection driving evolution of detox genes. Fitting evolutionary models of adaptation from standing genetic variation, we can compare adaptive potential across species and mutation types. The large population size in *M. nervosa* suggests a 4.7-fold advantage in the ability to adapt from standing genetic variation compared with a low diversity endemic *E. hopetonensis*. Estimates suggest that gene family evolution may offer an exceptional substrate of genetic variation in *M. nervosa*, with $P_{sgv} = 0.185$ compared with $P_{sgv} = 0.067$ for single nucleotide changes. Hence, we suggest that gene family evolution is a source of ‘hopeful monsters’ within the genome that may facilitate adaptation when selective pressures shift. These results suggest that gene family expansion is a key driver of adaptive evolution in this key species of freshwater Unionidae that is currently facing widespread environmental challenges. This work has clear implications for conservation genomics on freshwater bivalves as well as evolutionary theory. This genome represents a first step to facilitate reverse ecological genomics in Unionidae and identify the genetic underpinnings of phenotypic diversity.
1 | INTRODUCTION

As organisms are faced with intense rapidly changing selective pressures, new genetic material is required to facilitate adaptation. Among sources of genetic novelty, gene duplications and transposable elements (TEs) offer new genes or new regulatory patterns that can facilitate evolutionary change (Conant & Wolfe, 2008; Feschotte, 2008; Ohno, 1970; Oliver & Greene, 2009). These mutations can offer a substrate of novel genetic variation that may produce new functions to help organisms adapt when innovative changes are needed for survival. In evolutionary systems that are faced with sudden shifts in selective pressures, these sources of new genes are expected to be all the more essential as large-effect mutations facilitate moves to novel fitness peaks (Orr, 2005).

Historically, duplicate genes and TEs have been well studied in model organisms where it has been possible to estimate prevalence (Adams et al., 2000; Drosophila 12 Genomes Consortium et al., 2007; Goffeau et al., 1996; International Human Genome Sequencing Consortium et al., 2001), mutation rates (Adrion et al., 2017; Bennett et al., 2004; Demuth et al., 2006; Hahn et al., 2007; Rogers et al., 2009), and identify adaptive changes (Aminetzach et al., 2005; Han et al., 2009; Schmidt et al., 2010). With advances in genome sequencing, it is possible to gain a broader view of how gene family proliferation and TE content evolve across the tree of life. Most model organisms are either short lived with large, persistent populations (Drosophila, yeast) or long lived with modest population sizes and lower genetic diversity (Humans, mammals) (Lynch, 2007). It has only recently become tractable to generate reference genomes and evaluate the role of genetic novelty in alternative evolutionary systems. An understanding of how genomes evolve in populations that have recently faced strong shifts in selective pressures and sharp population declines that amplify genetic drift will clarify whether these mutations might play an outsized role in specific evolutionary scenarios.

The tempo of evolution in response to selection depends directly on population sizes, mutation rates and generation time (Gillespie, 1991; Hermisson & Pennings, 2005; Maynard Smith, 1971). It is therefore important to understand how genetic novelty responds in systems with alternative demography and life history in order to discern how these loci contribute to rapid evolutionary change. Moreover, the potential for species to respond to selective challenges may depend on life history, as maturation time, growth and reproductive strategies may contribute to risk of extinction (Haag, 2012; Haag & Rypel, 2011; Johnson, 2002; Stearns, 2000). Freshwater unionid bivalves (Unionidae) offer a long-lived evolutionary model (Haag, 2012; Haag & Rypel, 2011), with intense changes in population sizes and strong selective pressures due to environmental change (Haag & Williams, 2014; Strayer et al., 2004). These bivalves have experienced systemic decline due to human-mediated habitat alteration to freshwater ecosystems, with approximately 70% of North American species considered threatened, endangered or extinct (Haag & Williams, 2014; Strayer et al., 2004; Williams et al., 1993). Understanding of how duplications and TEs have evolved as these taxa face modern anthropogenic challenges may explain how they might contribute to species survival during sudden evolutionary changes.

Unionid bivalves are benthic filter feeders that form the backbone of freshwater ecosystems (Lopez et al., 2020; Vaughn, 2018; Vaughn et al., 2008; Williams et al., 2008). They are notable for their unusual life history and mitochondrial biology. Unionidae are known for biparental mitochondrial inheritance, where females receive mitochondria solely from the maternal lineage but males also receive germline mitochondria from paternal lineages, a potential genetic factor in sex determination (Breton et al., 2007, 2011; Liu et al., 1996; Wen et al., 2017). While adults are sessile filter feeders, all species have a larval stage that parasitizes fish gills, facilitating dispersal (Barnhart et al., 2008; Williams et al., 2008). Because of this unusual life history, Unionidae serve as an interesting evolutionary model system for parental care, parasite–host co-evolution and evolution of life history strategies. It may also offer a system to study the genetic response to strong and suddenly shifting selective pressures (Lopez et al., 2020; Williams et al., 2008) in a long-lived species (Haag, 2012; Haag & Rypel, 2011) that departs from the standard population genetic models.

Given the magnitude of shift in freshwater ecosystems, Unionidae are an ideal model to study how new gene formation creates ‘hopeful monsters’ within the genome and facilitates rapid adaptive change. Genome sequencing offers a means to bring these fascinating organisms into the modern genetic era, where reverse ecological genomics may reveal the genetic responses to these changing environmental and co-evolutionary pressures (Marmeisse et al., 2013). Many studies have surveyed genetic diversity using a small number of loci, microsatellites, mtDNA or more recently transcriptomes or SNP capture arrays (Campbell et al., 2005; Corman et al., 2014; González et al., 2015; Pfeiffer et al., 2019; Wang et al., 2012). However, full genome sequencing is not yet widespread, limiting genetic analysis. As a first step to facilitate evolutionary genetics with sufficient power to resolve genetic patterns of adaptation in Unionidae, sequencing and assembly of reference genomes can help fill this gap. Advances in genome annotation and assembly will have broad impacts on conservation and management for imperilled Unionidae.

Among Unionidae, Megalonaias nervosa is one widespread, prevalent species whose populations have remained robust, so far, in the face of environmental challenges. Megalonaias nervosa is thick-shelled, spawns in the fall and produces large brood sizes of up to 1...
million glochidia per individual (Haggerty et al., 2005; Williams et al., 2008). Earliest maturation is between 5 and 8 years of age (Woody & Holland-Bartels, 1993), and the species is long lived with individuals identified up to 43 years of age (Haag & Rypel, 2011). M. nervosa larvae are generalist parasites that use multiple prevalent fish species as hosts (Williams et al., 2008; Woody & Holland-Bartels, 1993). These mussels are present throughout the Southeastern United States, in the Mississippi, Ohio and Tennessee River waterways (Williams et al., 2008). The Tennessee River offers a focal location where 79 species of Unionidae have been reported, though as many as 32 of those are extirpated or extinct (Garner & McGregor, 2001). In Northern Alabama, The Tennessee Valley Authority (TVA) has documented as much as 6700 tons of shells harvested in a single year at peak demand (Williams et al., 2008) and up to 45% of these were M. nervosa (Ahlstedt & McDonough, 1992).

To determine how the genome of M. nervosa has responded to recent selective pressures, we have generated a reference genome for a single individual collected from Pickwick Lake, Muscle Shoals, Alabama. We explore the relative contribution of gene family expansion, TE proliferation and single nucleotide mutations to genetic variation and its influence on evolutionary potential. This reference genome shows strong signatures of TE proliferation and adaptation through gene family expansion in M. nervosa. We further analyse genetic diversity in comparison with recently sequenced bivalves, and how it is expected to influence subsequent evolutionary processes in this species.

2 METHODS

2.1 Assembly and annotation

A single gravid female approximately 11 years of age based on ring counts was collected from Pickwick Lake in Muscle Shoals, Alabama, in 2017 (JGPickwickLake2017 Specimen #3). The individual was 15.8 cm long (anterior to posterior), 11 cm high (dorsal to ventral), and 5.7 cm wide (between outer surfaces of the two shell valves), with large growth rings identified in the earliest years. The individual was shipped to Charlotte, North Carolina, where it was dissected and tissues were flash-frozen in liquid nitrogen immediately on collection. DNA from compact adductor tissue using the Qiagen DNeasy kit. Illumina TruSeq DNA PCR-free libraries were prepared manually following the manufacture's protocol targeting 350 bp inserts. Size selection was optimized using Ampure XP beads. The libraries were sequenced (2 lanes) on the Illumina HiSeq 4000 platform using the 150 bp paired-end Cluster kit (DUGSIM).

We assembled transcriptomes using the Oyster River Protocol version 2.3.1 (ORP) (MacManes, 2018), then aligned to the reference genome using blat version 36 (Kent, 2002). Alignments were converted to hints using blat2hints and filtered for unique matches using filterPSL from Augustus. ORP is able to assemble transcripts with TPM <1.0, better performance than previous de novo assemblers, including Trinity (MacManes, 2018). Their OrthoFuser program combines transcripts that are homologous, again with better performance than any single assembler alone (MacManes, 2018).

These alignment hints were used as input for de novo gene prediction in Augustus version 3.3 (Stanke & Waack, 2003), allowing isoform prediction with evidence (Stanke et al., 2008). Out of 709,458 putative transcripts identified across six tissues, 641,750 were aligned to a single contig. Species-specific gene prediction models for M. nervosa produced during BUSCO training were used for Augustus gene prediction. We used Augustus gene models to create CDS and protein fasta files using gffread (Pertea & Pertea, 2020). We produced functional annotation for gene models using Interproscan version 5.34-73.0 (Quevillon et al., 2005) based on identified Pfam (El-Gebali et al., 2019) conserved domains.

Annotated sequences were compared to the RepBase database (Jurka et al., 2005) of TEs in a BLASTN search at an E-value of 10^{-10}. Annotations with BLAST hits in RepBase or with Interproscan results suggesting TE or viral activities were removed from the annotations. We note that these criteria could allow for some novel selfish genetic elements if sufficiently distant from previously annotated TEs in other species. Sequences were compared against gene models from Crassostrea gigas (the Pacific Oyster; Zhang et al., 2012) using a
BLASTP at an E-value of $10^{-20}$. One-to-one orthologs were defined as reciprocal best hits. To establish a final high quality annotation data set, we required that de novo predictions be supported by at least one line of evidence: results from Interproscan, BLAST comparison against C. gigas or support in the ORP transcriptome assembly.

Differences in gene annotations could potentially be caused by methodological differences in studies on various species. To generate putative annotations that are directly comparable, we generate transcriptome-based annotations for six species of Unionidae: M. nervosa, U. tetratalamus, E. complanata, E. crassidens, L. lienosus and V. ellipsiformis (see Supporting Information).

### 2.2 Gene families

Gene family expansion is an important mode of new gene formation that can lead to evolutionary innovation (Conant & Wolfe, 2008; Hahn, 2009; Hahn et al., 2007; Ohno, 1970). The rates at which new genes are formed and lost dictate evolution of gene content and the substrate of genetic novelty (Hahn, 2009; Lynch & Conery, 2000; Rogers et al., 2009). To determine the genomic responses of new gene formation in M. nervosa, we identified gene families and used birth–death models to identify rates of evolution through new gene formation (Lynch & Conery, 2000; Rogers et al., 2009). These models assume new genes form in clock-like fashion according to Poisson processes. The history of gene birth and death is recorded in the demographic history of gene family members, where gene ages are recorded in genetic sequence data. These methods offer a more accurate estimate of new gene formation than simple accounting where the number of new genes observed is divided by branch lengths (Lynch & Conery, 2000). They are also tractable to study birth–death processes for duplicate genes in a single genome.

We used a Fuzzy Reciprocal Best Hit algorithm (Hahn et al., 2007) on an all-by-all BLASTN comparison at an E-value of $10^{-20}$ of gene annotations within M. nervosa to identify gene families. We allowed for a difference in E-values within two orders of magnitude. If orthogroup order was ambiguous, the maximum rank was chosen. The distribution of duplicate gene ages is recorded in the divergence among copies at synonymous sites (dS), and the relative rate of amino acid changing mutations is recorded in divergence at non-synonymous sites (dN).

We collected sequences for each orthogroup, translated them, then aligned amino acid sequences in clustalw2 (Larkin et al., 2007; Thompson et al., 1994). Alignments were back-translated to the original nucleotide sequence to produce in-frame alignments. We used PAML’s codeml (Yang, 1997) to estimate dN and dS, estimating codon frequencies from nucleotide frequencies (F1x4) under the guide tree from clustalw2. We excluded all genes past the mutation saturation point with dS > 1.0. We then fit a birth–death model using maximum likelihood to the distribution of dS for gene family members according to $\lambda e^{-\lambda s}$ similar to previous work on duplicate genes (Lynch & Conery, 2000; Rogers et al., 2009). We established the 95% CI using a jackknife of 75% resampling with 10,000 replicates. Mutation rates for duplicate gene formation and the half-life of mutations were inferred based on birth–death parameters. These can be rescaled to years assuming copies diverge by twice the mutation rate of $5 \times 10^{-9}$ (Pogson & Zouros, 1994). Interproscan conserved domain annotations identified orthogroups belonging to large gene families. We fitted separate birth–death models for the most common gene families in the M. nervosa genome: Cytochrome P450 genes, Hsp70s, Mitochondria-eating genes, Chitin metabolism genes, von Willebrand proteins, ABC transporters and opsin genes.

### 2.3 TE content

Transposable elements are known as sources of innovation that can remodel genomes (Feschotte, 2008; Oliver & Greene, 2009). They are often associated with genome remodeling not solely because of new insertions but also due to their ability to facilitate rearrangements and copy gene sequences. To determine how TEs might have reshaped genome content in M. nervosa, we identified putative transposable elements in M. nervosa using a three pronged approach. RepeatScout 1.0.5 (Price et al., 2005), RepDeNovo (Chu et al., 2016) and homology-based detection using either Interproscan 5.34-73.0 (Quevillon et al., 2005) or BLAST comparisons to RepBase (Bao et al., 2015). RepeatScout identifies repetitive sequences in genomes where TE copies have sufficient divergence from one another to successfully assemble into independent contigs. We then mapped these repeats back to the M. nervosa reference assembly using a blastN at an E-value of $10^{-20}$ with dust filters turned off. These methods effectively identify repeats with >2% divergence. They do depend on genome assembly quality and may not be appropriate for cross-species comparisons when assembly quality is variable.

RepDeNovo does not rely on pre-existing genome assemblies but rather assembles contigs from high copy number reads in raw fastq files. This program identifies recently proliferated transposons that have <2% divergence from one another. We mapped Illumina sequencing reads onto these repetitive contigs identified with RepDeNovo and estimated copy number using sequence depth. TE types were identified using a tblASTx against the RepBase Database. Finally, we identified transposon proteins among gene annotations using homology-based searches in Interproscan or using blastN comparisons with the RepBase database. Together, these methods generate a comprehensive portrait of TE content in the genome of M. nervosa.

For comparison, Illumina sequences are incidentally available for Venustaconcha ellipsiformis (42X coverage, SRR6689532; Renaut et al., 2018) and Elliptio hopetonensis (14X coverage), both originally collected for other purposes. Venustaconcha ellipsiformis is present throughout the northern Mississippi River, while E. hopetonensis is an endemic species with limited numbers located in the Altamaha River. These distantly related taxa offer the nearest comparisons among unionid mussels where genetic data are available. We used the assembly free methods of RepDeNovo (Chu et al., 2016) to assay TE content in these two species. These data offer directly comparable
estimates of TE content, independently from quality of genome assembly. We used downsampled data for *M. nervosa* at 14X coverage to ascertain that differences in TE content were not driven by sequence coverage.

2.4 Low coverage assembly for *E. hopetonensis*

Illumina sequencing data were incidentally available for *Elliptio hopetonensis* taken from a single individual collected from the Ocmulgee River in 2012 (Field ID JMW120329-2, Ocmulgee R, site 2 of day, JMWW, MUH, DAW). Mantle tissues was dissected and prepared for DNA sequencing on a HiSeq2000 at the University of Colorado sequencing core. Using this 14X coverage Illumina sequencing data for *E. hopetonensis*, we have generated a cursory reference assembly in Abyss using default parameters. The assembly N50 is only 1 kb, and the maximum contig size is 22 kb. Genome size estimates are 1 Gb, with 500 Mb in scaffolds 1 kb or larger. *busco* (Simão et al., 2015; Waterhouse et al., 2017) analysis suggests the assembly is largely incomplete with only 15% of conserved metazoan genes identified. However, in a tBLASTn, we can find partial matches for 15,010 *C. gigas* genes. The number of *C. gigas* orthologs identified in this cursory reference is similar to the number identified in *M. nervosa*. While this cursory reference genome is not of sufficient quality to anchor genes to regions or create syntenic maps, it can offer a comparison against *M. nervosa* for genome size, and effective population size, as well as surveys of TE content described above.

2.5 Effective population size

The tempo of evolution depends directly on the effective population size, mutation rate and generation times. To evaluate the timescale of adaptation, we used heterozygosity in the reference specimen to estimate population genetic parameters such as \( \theta = 4N_e \mu \), the expected coalescent time for neutral alleles of 2N generations, and \( t_{\text{MRCA}} \) of 4N generations (Wakeley, 2009). To further identify the genetic potential available through standing genetic variation, we estimated the time to establishment of selective sweeps on new mutations \( T_s \); and the probability of adaptation from standing variation \( P_{\text{nev}} \). These parameters offer a description of theoretical expectations for how evolution should proceed, given current population sizes and estimates of diversity. They offer a means to determine the limits of what might be possible given strong selection acting on these species.

We mapped Illumina reads from the genome assembly to the *M. nervosa* reference genome using bwa aln with default parameters (Li & Durbin, 2009). We identified SNPs using GATK (McKenna et al., 2010) haplotype caller version 3.8-0-ge9d806836 assuming diploid genomes. We identified heterozygous sites to estimate genome-wide heterozygosity for *M. nervosa*. We performed identical analysis for the cursory assembly for *E. hopetonensis*. These data provide estimates of \( \theta = 4N_e \mu \) for these freshwater bivalves. Using these data, we calculate \( N_e \) based on a mutation rate of \( \mu = 5 \times 10^{-9} \) in bivalves (Pogson & Zouros, 1994). We are able to use this parameter to estimate coalescent timescale of 2N generations and the \( t_{\text{MRCA}} = 4N \) generations. Timescales are offered in generations and converted to years assuming an average 20-year generation time based on the long lifespan and late maturation of *M. nervosa* at 8 years of age (Haag & Rypel, 2011; Woody & Holland-Bartels, 1993). Alternative scaling from generations to years would scale linearly with generation time.

We estimate the time of establishment to a sweep on a new mutation \( T_s = 1/\theta \) (Gillespie, 1991; Maynard Smith, 1971) for each species. These models describe time for new mutations to arise de novo and escape stochastic forces of drift to establish selective sweeps. The alternative to adaptation from new mutation is adaptation from standing genetic variation. Population diversity depends directly on the effective population size and mutation rate (Watterson, 1975). Yet, many mutations present in populations will be at such low frequency that they are unable to establish deterministic selective sweeps in the face of genetic drift (Hermisson & Pennings, 2005). We estimated the probability of adaptive pressures is able to establish deterministic sweeps from standing variation \( P_{\text{nev}} = 1 - e^{-s \ln(1 + 2N_e \mu)} \) (Hermisson & Pennings, 2005). It depends on the population size, selective coefficient and mutation rate. These parameters are estimated assuming moderate selective coefficients of \( s = 0.01 \) and for very strong selection of \( s = 0.1 \).

2.6 Gene trees and evolutionary relationships

We generated transcriptome-based annotations for 6 species of Unionidae: *M. nervosa*, *U. tetralasmus*, *E. complanata*, *E. crassidens*, *L. lienosus* and *V. ellipsiformis*. For each of six assembled transcriptomes, we identified 5285 sets of reciprocal best hit orthologs using *busco* for *M. nervosa* across all transcriptomes. Sequences with a direct match across all six species were used to generate gene tree phylogenies and address Incomplete Lineage Sorting (ILS). We aligned sequences using *muscle* (Edgar, 2004) under default settings. Site trimming was conducted using trimAl 1.2 (Capella-Gutiérrez et al., 2009) with a no gap penalty among alignments of the annotated *M. nervosa* gene. We chose to use a no gap penalty criterion to mitigate potential bioinformatics artefacts and exon boundary uncertainties among the available transcriptomes. A total of 4475 gene sets met these criteria. We used these alignments to generate gene tree phylogenies using RAxML 8.2.12 (Stamatakis, 2006, 2014). The rapid bootstrapping function was selected using a GTR-Γ model across 100 randomly sampled tree replicates. Astral III (Zhang et al., 2018) estimated Normalized Quartet Scores (Sayyari & Mirarab, 2016) and constructed a consensus tree among all generated gene trees from RAxML. Tree topology was plotted in R package Phangorn (Schliep, 2011) using the DensiTree function (Bouckaert, 2010), using unscaled branch lengths for the purposes of visualization. Full Astral III consensus tree use coalescent scaled branch lengths \((t/2N)\) units and gene tree branch lengths in substitutions are reported in Supporting
Information. Gene trees from RAxML were used to identify discordance with the consensus phylogeny.

3 | RESULTS

3.1 | Genome assembly

We generated a reference genome sequence for *M. nervosa* to facilitate evolutionary and ecological genomics in Unionidae. We collected 40X Illumina short-read sequencing and 5X Oxford Nanopore long molecule sequences for a single gravid female *M. nervosa* from Pickwick Lake, Muscle Shoals, Alabama. Genome statistics prior to filtering were 2.7 Gb, with a scaffold N50 of 52.7 kb and a contig N50 of 51.5 kb (Table S1). These show higher contiguity than the previously assembled genome of *V. ellipsiformis* (Renaut et al., 2018).

We compared these results with one high-quality marine bivalve assembly for Pacific oyster, *Crassostrea gigas*. Divergence between *C. gigas* and *M. nervosa* is difficult to estimate with certainty, but ranges from 495 million (Kumar et al., 2017) to 200 Myr (Bolotov et al., 2017). While these marine and freshwater bivalves that diverged hundreds of millions of years ago are phenotypically and ecologically distinct, *C. gigas* is the nearest non-unionid bivalve with a high-quality assembly and annotation. The contig N50 is superior to the recent *C. gigas* genome of 19.4 kb, but poorer than the scaffold N50 of 401 kb (Zhang et al., 2012). The distribution of kmers is continuous with no ‘shoulders’ of high or low kmer frequency, indicating no sign of polyploidy or genomes misassembled in the face of heterozygosity (Figure S1).

Some 92% of the genome is assembled into contigs over 10 kb, and 50% is assembled into contigs 50 kb or larger. Contaminating sequences were removed from the bivalve reference assembly using a BLASTN, producing a total assembly of 2.36 Gb, 500 Mb (28%) larger than estimates for one other unionid mussel sequenced to date, *V. ellipsiformis*. *Venustaconcha ellipsiformis* has a genome size of 1.80 Gb (Renaut et al., 2018). BUSCO analysis (Simão et al., 2015) suggests 83% complete orthologs and 9% fragmented orthologs, consistent with previous genome assemblies for bivalves. Only 2.1% of BUSCOs are duplicated, again confirming the absence of polyploidy or high heterozygosity that would lead to separation of homologous chromosomes in the assembly. Such sequence quality captures the majority of gene content either wholly or partially, and is sufficient for many applications in evolutionary and population genomics.

3.2 | Gene annotations

To identify genes and gene families that have emerged in freshwater Unionidae, we generated gene annotations for *M. nervosa*. We produced gene annotations using RNA sequencing data for six tissues from the same individual: Gills, mantle, muscular foot, mucosal palps, adductor muscle and glochidia (offspring) (Figure 1). The Oyster River Protocol identifies 66.7%–87.1% of BUSCOs across each of the six transcriptomes. We identify 49,149 genes: 17,982 singleton genes with 21,596 transcripts and 31,167 members of gene families with 32,967 transcripts. A total of 801 transcripts belonging to 733 genes contain premature stop codons suggesting pseudogenes. Genes encompass approximately 477 kb of sequence. Average intron length is 3714 bp (min 7, max 145,991). In a reciprocal best hit BLAST comparison, we identify 8811 one-to-one orthologs between *M. nervosa* and *C. gigas*.

We placed no a priori criteria for what functional categories should be represented in the *M. nervosa* genome. However, among Interproscan annotations (Quevillon et al., 2005), we note large gene families related to biological processes expected to be important for Unionidae survival and reproduction. We identify 78 glutathione transferase genes, 44 thioredoxins, 143 cytochrome *P450* genes and 106 ABC transporters expected to interact with cytochromes (Figure S2). In BLAST searches, we observe over 500 fragmented copies each of cytochrome *P450* genes and ABC transporter interacting regions that are not of sufficient length for identification in Interpro scans of functional domains. Only one cytochrome *P450* gene overlaps with another annotation, a gene of unknown function. Thus, it is not likely that isoform splitting could be artificially inflating gene family sizes in the annotations. We find multiple copies of genes involved in anticoagulation processes in *M. nervosa*, consistent with blood-feeding during the parasitic larval stage. We find 138 von Willebrand factor proteins (99 type A and 39 type D), 15 Xa-binding genes and 33 fibrinogen binding proteins.

We further observe 96 *Hsp70s* and 6 *Hsp90s*, consistent with previously published results from *C. gigas* showing expansion of heat shock proteins (Zhang et al., 2012), but substantially increased over only 52 observed in *V. ellipsiformis* (Renaut et al., 2018). These genetic differences are consistent with thermal tolerance through *Hsp* activity in other organisms (Serensen et al., 2003), though pleiotropic functions of *Hsp70* genes may also influence gene family expansion. *V. ellipsiformis* has a range in the northern United States, cooler climates compared with *M. nervosa*. *Megalonaias nervosa* is found throughout the Southeastern United States, and the present
sample’s home location lies in Muscle Shoals, Alabama. We also note 238 rhodopsin genes and 117 Mitochondria-eating protein genes. These are in addition to large numbers of conserved domains with less clear functional implications such as zinc finger domains, WD-40 domains, short-chain dehydrogenase domains and Ankyrin repeats. In a targeted identification, we find 11 chitin synthase genes and 55 Chitin binding Peritrophin-A genes, putatively important for shell formation. We identify seven mucins, proteins important for defence and release of glochidia offspring. These numbers are many times higher than listed in official annotations in *V. ellipsiformis* (Table S2), though we note that annotation methods differ, especially with respect to repeat masking before annotation rather than after annotation.

Previously published annotations for *V. ellipsiformis* show fewer members of these large gene families (Table S2; Renaut et al., 2018). However, these annotations through different methods and with different assembly qualities and different tissues could lead to a false portrait of species difference. To generate annotations for direct comparisons independently from genome assembly, we annotated *de novo* assembled transcriptomes for *Megalonaia nervosa*, *Elliptio crassidens*, *Elliptio complanata*, *Uniomerus tetralasmus*, *Amblema plicata*, *Leaunio lienosus* (*Vilosa lienosa*) and *Venustaconcha ellipsiformis* (Supporting Information). Results of transcriptome only analysis point to large detox gene content across Unionidae, but with variation in the extent of gene family expansion (Figure S3, Table S3). *Megalonaia nervosa* does not appear to be an outlier with respect to any functional category (Table S3, Figure S3). These genome free transcriptome annotations validate results of large gene families from genome based annotations. They also suggest that copy number expansion is common across Unionidae, a prospect that deserves greater attention with whole-genome annotation in the future.

### 3.3 Gene family evolution

Gene family expansion through duplication can offer a substrate of genetic novelty that may facilitate adaptation to new environments. To identify signatures of adaptive changes and rapid evolution of copy number, we estimated dN/dS (Goldman & Yang, 1994; Nei & Gojobori, 1986) and fit birth–death models genome-wide and for large gene families in *M. nervosa*. We also evaluated putative tandem duplications that may be heterozygous in the reference genome alone by mapping Illumina reads to the reference and finding divergently oriented read pairs (Rogers et al., 2014). We identify 8507 cases where abnormally mapping read pairs would suggest tandem duplications in one of the two haplotypes, in addition to those gene duplications identified in the reference assembly here, though not all of these are expected to capture gene sequences.

*Cytochrome P450* family orthogroups have 26 branches suggesting no divergence at synonymous sites and 36 branches in the tree indicating dN/dS >> 1.0, a strong signature of natural selection driving amino acid substitutions (Goldman & Yang, 1994; Nei & Gojobori, 1986). These results suggest strong adaptation at detox gene families in *M. nervosa*. We also identify 11 incidences of Hsp70 formation, 21 branches for Opsin genes, 11 mitochondria-eating protein duplications, 15 branches for von Willebrand proteins and five chitin duplications associated with dN/dS > 1. These signatures of rapid expansion are fit with a birth-death model. The distribution of duplicate ages, given by dS, shows larger numbers of younger gene family members, tapering off exponentially as expected for a Poisson process. Model fit suggests a birth rate $\lambda = 40,859$ (95% CI 40,262–41,455) and death rate $\mu = 6.8$ (95% CI 6.7–6.9) per 1.0 dS. Model fit is independent of histogram binning or visualization.
amino acid substitution in excess of rates expected under neutrality indicate that new gene formation through gene family expansion makes a significant contribution to adaptation in *M. nervosa*. It is difficult to resolve evolutionary events in near time using a single genome. However, we can infer that gene copies where $dS < 10^{-6}$ occurred within the last 100 generations ($10^{-6}/(2 \times 5 \times 10^{-9})$) or 2000 years.

We fit a birth–death model to 6002 first-order paralogs for all gene families using $dS$ as a proxy for age according to $\lambda e^{-\mu dS}$ (Table S4, Figure 2). The distribution of $dS$ values follows a pattern of exponential decay as expected under birth–death processes (Figure 2). Using maximum likelihood, we fit a death rate, $\mu = 6.8 \pm (95\% \text{ CI } 6.7–6.9)$, and a birth rate, $\lambda = 40,859$ genes per 1.0 $dS$ (95\% CI 40,262–41,455). Assuming gene copies appear in clock-like fashion and each accumulate point mutations at a rate of $5 \times 10^{-9}$ (Pogson & Zouros, 1994), this birth rate scales to a mutation rate of $1.16 \times 10^{-8}$ duplications per gene per generation. Such mutation rates are equivalent to copying each gene in the genome once on average every 11 million generations. The half-life of a newly formed gene family member is 0.1 $dS$, corresponding to 1 million generations or approximately 200 million years. We note that this is far longer than we found for duplicate gene pairs in our previous work in deletion-biased *Drosophila* (Rogers et al., 2009). The distribution of $dS$ also does not show evidence of long-term preservation, suggesting an escape from birth–death processes (Figure 2).

We identified 88 cytochrome P450 genes that appear to be members of large gene families in a first-order Fuzzy Reciprocal Best Hit BLAST (Hahn et al., 2007). We estimated $dN$ and $dS$ in PAML and fit birth–death models to these large gene families (Figure 3). For cytochrome P450 genes, $\lambda = 898$, $\mu = 10.2$ (Table S4). Scaling birth rates to be proportional to the number of genes examined (143 cytochrome annotations vs. 49,149 total genes), this is a 7.6-fold higher rate of new gene formation and a 1.5-fold higher rate of gene family contraction in cytochromes than we would expect based on the genome-wide background (Table S4, Figure 3), well beyond the 95\% CI ($p < .0001$). Among other large gene families, von Willebrand factors, chitin metabolism genes, Hsp70 genes, mitochondria-eating proteins and opsins show birth rates 3.5–4.0 times higher than background levels (Table S4, Figure 3). ABC transporters and Hsp70 genes show reduced death rates, with a half-life up to 50\% longer than background (Table S4, Figure 3).

3.4 | Burst of recent transposable element activity

TEs are selfish genetic elements that copy themselves often at the expense of their host organisms. Transposable elements are key drivers of gene family expansion as they can facilitate ectopic recombination, create new retrogenes and duplicate adjacent gene sequences (Feschotte, 2008; Oliver & Greene, 2009). The way that these modes of mutation interact to reshape genomes during
adaptation to environmental challenges is key to understanding how organisms evolve in nature.

These high copy number repeats can be identified in next-generation sequence data independently from genome assemblies or in fully assembled reference genomes. We used RepDeNovo (Chu et al., 2016) to identify young, newly propagated transposable elements in three species where genomic sequence data are currently available: *M. nervosa*, *V. ellipsiformis*, and *E. hopetonensis*. We observe evidence of a burst of TE activity in *M. nervosa*, resulting in an expansion of genome content. We observe approximately twofold greater TE content in *M. nervosa* compared with the other species (Figure 4). Because these methods do not rely on genome assembly quality, results from RepDeNovo should be directly comparable across species. These TEs account for a 21% (382 Mb) increase in genome size compared with previously sequenced bivalve *V. ellipsiformis*.

A total of 21% of the increase in TE content in *M. nervosa* compared with *E. hopetonensis* is explained by expansion of *Polinton*-like DNA transposons, and 21% is explained by expansion of *Gypsy* LTR retroelements (Figure 4). Remaining classes each explain 0–5% of TE expansion. Repeat content in *V. ellipsiformis* approximately resembles that of *E. hopetonensis* with the exception of the 4-fold increase in *Polinton* DNA transposons and a reduced number of *hAT* repeats (Figure 4). Notably, the Neptune family of Penelope-like elements is identified in *M. nervosa* but absent in *E. hopetonensis* and *V. ellipsiformis*, explaining 5% of the expansion in TE content (Figure 4). Approximately 45% of the repetitive content in each genome could not be matched in a tBLASTx, suggesting some repeat families are highly diverged from annotated repeats in RepBase (Bao et al., 2015). The difference in genome size for *V. ellipsiformis* and *M. nervosa* is primarily explained by the proliferation of 382 Mb of repeats. *M. nervosa* and *E. hopetonensis* are from 42X and 14X coverage data, respectively. Downsampling genomic data to 14X, total TE content increases marginally to 465 Mb. Hence, we do not believe the observed differences in TEs between species can be explained by sequence depth but rather reflect biological differences between *M. nervosa* and the two other bivalve species.

RepeatScout identifies older repetitive elements with sufficient divergence to be assembled correctly into genome scaffolds (Price et al., 2005). We identify 3686 repeats in *M. nervosa*, 1827 of which are greater than 200 bp long. We are able to classify 573 of these into families using a tBLASTx comparison against RepBase at an E-value of $10^{-10}$. We find 98 *Polinton* elements, 75 *Gypsy* elements,

**FIGURE 4** TE content for recently proliferating repeat families found in three species of Unionidae using RepDeNovo, an assembly free repeat identifier. *Megaloniias nervosa* has the highest aggregate TE content across all families with 382 Mb of new genome content. *Gypsy* elements and *Polinton* elements each explain 21% of the expansion in genome content. A burst of *Polinton* amplification is observed in *V. ellipsiformis* [Colour figure can be viewed at wileyonlinelibrary.com]
73 hATs, 66 RTEs, 59 DIRS and 33 Mariner elements (Figure S4). Other families represented by multiple elements include Crack, Kolobok, Nimb, Chapaev, piggyBac and Penelope. These elements encompass 275 Mb of sequence. Diversity for these older groups of TEs compared with results of RepDeNovo would suggest that hAT, Mariner and RTE elements may have been more active in the ancient past but silent in more recent generations. Meanwhile, Gypsy and Polinton elements have maintained steady rates of activity. RepeatScout depends directly on the quality of genome assembly and may not be appropriate for cross-species comparisons unless assembly quality is equivalent.

We identify only seven LINE elements, with limited diversity to L1 and L2 retroelements, raising questions about when these selfish TEs may have appeared in the phylogeny of Unionidae. Interproscan analysis reveals hundreds of open reading frames for TE-associated protein domains, many with support in the transcriptome (Table S5). These ORFs have been removed from gene annotations described above. In total, the results of RepDeNovo and RepeatScout suggest that M. nervosa harbours a total of 575 Mb of transposons, approximately 25% of genome content.

3.5 | Effective population size

The tempo and outcomes of evolution via selection and drift depend directly on the effective population size. Coalescent theory suggests that heterozygosity, the number of differences between chromosomes in diploid individuals per site, will be distributed according to $H = \theta = 4N_e\mu$. Here, $N_e$ is the effective population size necessary to produce genetic diversity in an idealized model and $\mu$ is the mutation rate. Modern population genetic theory is based on these parameters and the ways that they shape the response to forces of drift and selection in natural populations. We can use theoretical models to explore the limits of current genetic diversity and the likelihood of adaptation from SNPs and gene duplications.

We have used heterozygosity in reference specimens for three species of bivalves to estimate $\theta$. We observe high heterozygosity suggesting $\theta_{M.nervosa} = 0.00777$, $\theta_{V.ellipsiformis} = 0.0060$ and $\theta_{E.hopetonensis} = 0.00576$ (Figure 5). Results from M. nervosa match well compared to within-population estimates for a small number of loci (Pfeiffer et al., 2018). Our results suggest that genetic diversity in these three species of bivalves is still high, even in a species that has a limited geographic range and more specialized fish host influencing dispersal (Johnson et al., 2012). E. hopetonensis is an endemic to Georgia and South Carolina with a limited range but with census sizes estimated to be over $10^6$ individuals. It is not surprising that it would have such lower $N_e$ than M. nervosa and V. ellipsiformis, which are widespread throughout the Eastern United States, if migration offers connectivity across drainages. These observed levels of heterozygosity lead to estimates for $N_e$ of 388,500 individuals in M. nervosa. In V. ellipsiformis, $N_e$ is estimated at 300,000 individuals and in E. hopetonensis 288,000 individuals. These estimates are substantially higher than estimates that have been observed in humans of approximately 15,000 individuals (Li & Durbin, 2011) but slightly lower than $10^5$–$10^6$ is seen in Drosophila (Karasov et al., 2010) or $10^7$ or greater in unicellular eukaryotes (Lynch, 2007).

We estimate coalescent times based on $2N_e$ generations of 777,000 generations for M. nervosa, 600,000 generations for V.
ellipsiformis, and 576,000 generations for *E*. hopetonensis (Table 1) with \( t_{\text{MRCA}} \) scaling by two times higher for 4\( N_e \) generations (Wakeley, 2009). This evolutionary system is unusual in comparison with life history of organisms commonly used for population genomics like yeast and *Drosophila* (large \( N_e \), short generation time) or humans (small \( N_e \), long generation time) (Lynch, 2007). It is rarer to find systems where populations have housed millions of individuals with long generation times. The observed high \( N_e \) in a long lived, late maturing species is expected to influence the tempo of evolution. Given a mutation rate of \( 5 \times 10^{-9} \) (Pogson & Zouros, 1994) and a modest estimate of 20 years per generation, the coalescent times under neutrality for such diversity would span up to 15 million years and total population diversity by 30 million years based on \( t_{\text{MRCA}} \).

### 3.6 Gene tree phylogenies for unionidae

The observed value of \( \theta = 0.0077 \) in *M*. nervosa raises the prospect of long coalescent timescales that may in fact be expected to transcend events of species separation. As a first step to examine relationships among Unionidae and capture information about potential incomplete lineage sorting, we produced gene-tree phylogenies for 6 species where transcriptome sequences were available and of sufficient quality to facilitate phylogenetics: *M*. nervosa, *U*. tetralasmus, *E*. crassidens, *E*. hopetonensis, *L*. lienosus and *V*. ellipsiformis. Using de novo assembled transcriptomes, we identified 4475 genes where 1:1 orthologs were present across all six species with high-quality sequence alignments. We placed the root of the tree at the divergence of Quadrulini, according to prior mtDNA-based phylogenies from Campbell et al. (2005). The consensus across all gene trees is consistent with the phylogeny represented in this previous work on mtDNA, indicating that the maternal haplotype tree reflects the species tree for these six species. A total of 2131 out of 4475 gene trees or 47.62% are fully consistent with the consensus species tree (Table S6, Figure 6). For any bifurcation at the terminal branches, 66.2%–73.3% of gene trees support the three terminal species splits (Table S6, Figure 6). Final normalized quartet score is 0.7605 from Astral III, where 76.05% of all gene quartets match species tree. Unscaled branch lengths are plotted for purposes of visualization, but scaled branch lengths are available in Supporting Information. A new species tree is generated in Supporting Information.

### 3.7 Adaptive potential in *M*. nervosa

We can estimate the time necessary for a new allele to appear and establish a selective sweep in a population, \( T_s \). We observe waiting times for strong selective sweeps (\( s = 0.01 \)) on new mutations ranging from 12,870 generations in *M*. nervosa and 56,180 generation in *E*. hopetonensis. Using an estimated generation time of 20 years, these numbers correspond to 257,000 years in *M*. nervosa and 347,000 years in *E*. hopetonensis (Table S7), exceptionally long wait times to respond to fluctuating environments. Under stronger selection coefficients of \( s = 0.10 \), we would expect wait times of 25,700 years in *M*. nervosa and 34,700 years in *E*. hopetonensis (Table S7). Hence, we would suggest that any response to modern anthropogenic selective pressures in Unionidae will necessarily proceed from standing variation.

| Species          | \( \theta = 4N_e \mu \) | \( N_e \)   | \( p_{\text{REV}} \) | \( T_s \) (gens) |
|------------------|-------------------------|-------------|---------------------|-----------------|
| *M*. nervosa     | 0.00770                 | 388,500    | .0672               | 12,870          |
| *V*. ellipsiformis| 0.00600                 | 300,000    | .0509               | 16,667          |
| *E*. hopetonensis| 0.00576                 | 288,000    | .0486               | 56,180          |

Assumes \( s = 0.01 \).
formation estimated from birth–death models (1.16 × 10⁻⁸ duplications per gene per generation) and identical parameters of \( N_s \) and \( s \), we would estimate \( P_{sgv} \) for gene family expansion to be 18.5%. Hence, gene family expansion is expected to be an important substrate of novelty in \( M. \) nervosa.

4 | DISCUSSION

4.1 | Gene family expansion and natural selection

Gene family evolution is an important source of novel genetic material that produces innovation in the face of shifting selective pressures (Conant & Wolfe, 2008; Hahn, 2009; Ohno, 1970). New genes arise through duplication, then accumulate mutations that modify original functions (Conant & Wolfe, 2008). They may also modify expression patterns, especially when regulatory elements are shuffled in the face of partial duplication (Rogers et al., 2017). In the face of rapidly changing selective pressures, these substrates of genetic novelty are expected to be all the more essential as organisms require adaptive innovation. In \( M. \) nervosa, we estimate rates of gene duplication equivalent to 1.16 × 10⁻⁸ per gene per generation. Such a high mutation rate combined with the large population sizes estimated for \( M. \) nervosa suggests a high probability of adaptation from standing genetic variation of \( P_{sgv} = 0.185 \). This estimate is 2.76-fold greater than \( P_{sgv} \) for single nucleotide changes (Table 1). Hence, the data suggest a greater likelihood of adaptation from standing genetic variation for any given gene duplication compared with any given SNP. This theoretical rate suggests that adaptation through gene family amplification may offer a rich substrate to respond to environmental changes, especially when new functions are needed.

The \( M. \) nervosa genome houses an unusually high rate of copy number expansion in detox genes compared with genome-wide background rates of duplication, with a birth rate 7.6 times higher. Hence, we conclude that cytochromes are operating under enhanced birth-death processes with rapid expansion (and possibly contraction) in \( M. \) nervosa compared with genome-wide expectations. This enhanced birth rate for new gene formation is likely to be responsible for the observed excess of cytochrome P450 genes compared with many other annotated animals. Humans have only 27 cytochrome P450 genes while mice have 72 (Nelson et al., 2004), half the number observed in \( M. \) nervosa. Moreover, 26 cytochrome P450 genes appear with no divergence at synonymous sites, suggesting recent amplification within the past 100 generations (approximately 2000 years) though timescales are difficult to resolve in the near term. Determining more precisely whether these changes preceded the modern industrial era and damming of the river in 1918 would require data from more individuals, potentially from historical specimens. Regardless it is clear that detox gene amplification in \( M. \) nervosa has expanded gene content and aided adaptation in recent evolutionary time, whether they appeared among standing variation before or after the anthropogenic era.

Alongside these newly formed copies, many of the remaining copies appear to be older, suggesting pre-adaptation or genetic priming for highly toxic environments. The cytochrome gene family houses 36 duplication events with high dN/dS > 1.0. Such signatures
indicate rapid amino acid substitutions in excess of expectations under neutrality, an indication of strong selective pressures and adaptive processes driving amino acid substitutions (Goldman & Yang, 1994; Nei & Gojobori, 1986). The Tennessee River has been historically subject to high pesticide loads from TVAs mosquito control programmes and from cotton farming (Woodside, 2004). Previous annotation of E. complanata has found amplification of 12 cytochrome P450 genes by annotation via homology with C. gigas, whose long divergence time is likely to limit the number of copies that can be identified (Cornman et al., 2014).

Such expansion occurs in addition to observed signatures of amino acid substitutions driven by selection. In aggregate, these results of gene family evolution point to widespread adaptation through gene family expansion, especially in detox genes. Toxicity assays in Unionidae, including M. nervosa, have identified high tolerance for multiple pesticides and herbicides (Loganathan et al., 1998; Milam et al., 2005), consistent with high rates of exposure expected in filter feeders that process large water volumes (Bril et al., 2017). Toxicity levels in bivalve tissues have been noted in excess of 0.2 ppm (Bedford et al., 1968), similar to concentrations that can be used as bait to kill insects (Schmidt et al., 2017).

Large gene families with new copies are also identified among anticoagulation genes consistent with blood-feeding in the parasitic larval stage, though these may be easier to identify in M. nervosa with transcriptomes for glochidia. We note expanded families of chitin metabolism genes, mitochondria-eating proteins, opsins and heat shock proteins. Unionidae are known for dual uniparental inheritance of mitochondria, where males inherit germ-line mitochondria from the father and females inherit them from the mother. The excess of mitochondria-eating genes and cytochromes that are part of electron transport chain seems intriguing in light of this unique biology. It is possible that these genes may play a role in regulating mitochondria, especially given the known heteroplasmacy in male Unionidae and processes of doubly uniparental inheritance (DUI) (Breton et al., 2007, 2011; Liu et al., 1996; Wen et al., 2017). Whether these genes are directly involved in DUI or rapidly evolving in response to DUI may offer an area of new exploration in Unionidae genetics. These large gene families have 3.5- to 4.0-fold higher birth rates than genome background rates suggesting rapid gene gains.

By identifying new genes and searching for genetic signatures of rapid evolutionary events, we can facilitate reverse ecological genomics and discover evolutionary processes important to the organism without pre-existing human biases. While we placed no prior constraints on functional categories, we note many large gene families associated with Unionidae biology. Globally, these results suggest that new gene formation has had a substantial impact on the genome of M. nervosa. It has experienced widespread creation of new genes with strong signatures of adaptive evolution and recent evolutionary changes. The ways that these mutations contribute to adaptive changes in the near term are well worth further study to determine how they contribute to Unionidae evolution as well as their importance in evolutionary theory.

### 4.2 Transposable elements and genome remodelling

TE mobilization can lead to rapid and dynamic changes in genomes (Feschotte, 2008; Oliver & Greene, 2009). Selfish elements can copy, modify or break gene sequences as well as induce regulatory changes in neighbouring genes (Feschotte, 2008; Oliver & Greene, 2009). These mutations are most often detrimental (Lynch, 2007). They can, however, incidentally create new genetic variation within genomes that shapes evolutionary outcomes. TEs are known to activate in times of organism stress. It has been proposed that TE activation can be a source of gene expression remodelling to modify suites of genes in few mutational steps (Laudencia-Chingcuanco & Fowler, 2012; Lynch et al., 2011; Oliver & Greene, 2009). These TEs are related to the high rates of gene family expansion observed in M. nervosa, as mobile elements are expected to facilitate new gene copies through gene capture, retrogene formation and ectopic recombination. The fact that both TEs and gene families exhibit dynamic evolution suggests that these mutations have an exceptional impact on genome remodelling and evolutionary change.

We observe a recent burst of TE mobilization for Polinton and Gypsy elements in M. nervosa that resulted in a 382 Mb expansion in genome size. Comparisons with older transposons in the genome assembly suggest that these families have had ongoing proliferation throughout the history of M. nervosa. In contrast, hAT, Mariner and RTE elements appear to have been active in the more ancient past but largely quiescent in the recent past. The causes and impacts of these changing TE dynamics deserve further exploration in future cross-species comparisons as well as searches for variation within populations.

It remains unclear to what extent the recent TE mobilization in M. nervosa may have produced beneficial changes, neutral variation or widespread damage to genomes, a prospect that might be addressed in future population genetic work. With modern population size reduction (Ahlistedt & McDonough, 1992), the nearly neutral coefficient will have changed by more than one order of magnitude. It is possible that this shift in nearly neutral impacts (Ohta, 1973) has allowed some TEs to proliferate unchecked (Lynch, 2007), even while absolute population sizes have remained very large. It is equally possible that strong selection has favoured some insertions or TE-mediated genome remodelling as a means to respond to environmental challenges. The observed adaptive signatures and rapid evolution of gene families may point to an interaction between TEs and adaptation through duplication, a prospect that deserves future exploration.

Regardless of whether this TE expansion has produced beneficial or detrimental changes, it is clear that recent proliferation of TEs has altered genome structure and expanded genome content in M. nervosa compared with its relatives. It is possible that TEs have proliferated in the face of environmental stressors, declining populations or even due to invasion via horizontal transfer from other organisms (Schaack et al., 2010). However, it is also possible that some of these new TE insertions reflect adaptive changes as selfish genetic
elements remodel genomes and incidentally create new genes or new expression profiles across the M. nervosa genome. Sorting out the adaptive TE insertions from detrimental TE insertions will require full population genomic data sets, a prospect that will become tractable with this new reference genome.

4.3 | Evolutionary relationships in unionidae

Phenotypic studies in freshwater bivalves have noted rampant convergence of forms across lineages (Inoue et al., 2013; Johnson et al., 2018; Pfeiffer et al., 2016; Smith et al., 2020; Williams et al., 2008), and phylogenetic analysis has been challenging in the face of variation rather than adherence to strict phenotypes (Campbell et al., 2005). Our preliminary analysis of gene trees based on transcriptome assemblies for six species is consistent with these previous challenges of phylogenetic placement based on both phenotypes and genetic analysis of few loci. The population genetic parameters for three species of Unionidae where whole genome sequences are available also imply that the coalescent time scale may be greater than many species splits. Under these circumstances, we may expect rampant trans-species polymorphism and widespread convergence from adaptation via shared ancestral variation.

In transcriptome data, we observe a clear consensus tree among all gene trees. However, over 50% of gene trees differ from the consensus in some way. Many discordant gene trees are symmetric with respect to species placement, a feature of ILS. However, we observe asymmetric relationships between gene trees and species trees at some nodes, raising the question of how much interbreeding may have occurred across species recently or in the past. Curiously, a 7th species, A. plicata, does not have a clear placement in the phylogeny. Phylogenetic discordance has been noted in the face of short branch lengths in Unionidae in broad phylogenetic surveys using bait pull-down sequencing (Pfeiffer et al., 2019).

Focusing on gene trees for six focal species, we are able to offer finer-scale resolution and a more focused account of incomplete lineage sorting among closely related species. The observed discordance even among these few species is consistent with predictions based on population genetic diversity measures and long coalescent times. Full resolution of how complex histories of ILS, introgression and convergence influence Unionidae remains an open question that will require the field to move beyond transcriptome level analysis. With future work developing high-quality genome assembly and annotation for more species across the diversity of Unionidae, it may be possible to place quantitative bounds on how these evolutionary forces have shaped the genetic underpinnings of phenotypic diversity in Unionidae.

4.4 | Tempo of evolution

The tempo of evolution depends directly on effective population sizes and generation times. We have observed high heterozygosity in Unionidae, with $\theta = 0.0077$ per site in M. nervosa. Under these parameters, we expect neutrally evolving variation will experience long coalescent times of 77,700 generations, up to 15 Myr assuming constant generation times of 20 years. Population genetic data can be used to extract information concerning population history up until the $t_{MRCA}$ of all individuals in the population, which is expected to be 4N generations (Wakeley, 2009), or 30 Myr. This timescale for neutral variation spans ice ages and mass extinctions, encompassing historical environmental changes that have reshaped the history of the earth. Previous work on a small number of loci has noted high levels of sequence variation within many populations of M. nervosa and limited differentiation across geographic areas (Pfeiffer et al., 2018).

It is suggested that the larval life history as generalist fish parasites may allow migration across multiple habitats, increasing diversity within populations to overcome geographic separation across its range (Pfeiffer et al., 2018), a prospect that is likely to be important for genetic diversity and conservation in largely sessile species (Archambault et al., 2018). We observe the highest genetic diversity in M. nervosa, and the least in the endemic species E. hopotenesis. M. nervosa is known to use lots of common fish as hosts (Williams et al., 2008; Woody & Holland-Bartels, 1993). However, not all Unionidae are broadcast spawners, such as some Elliptios, which cast mucus nets and use fewer fish species for dispersal (Johnson et al., 2012). This difference in host strategy within broadcast spawning mussels probably impacts dispersal. These life history factors likely play an additional role in shaping genetic diversity, especially differentiation across drainages within the species range.

Populations of bivalves from Northern and Southern Alabama are separated by the Fall Line, where river systems flow from rocky upland provinces to the less consolidated Coastal Plain (Williams et al., 2008). Periodic connections when rivers varied courses across waterways as glaciers worldwide formed and receded allowed species migration far in the past, potentially influencing genetic diversity. In light of these numbers, it appears likely that the historical and geological forces that have shaped these species may indeed still be written in their DNA. This long-standing genetic diversity may offer the chance to use population genetics to explore evolutionary theory in deeper timescales than most standard population genetic models.

The genetic diversity of M. nervosa and other Unionidae is shaped by ancestral variation and is expected to decay gradually over time as populations diminish or become fragmented (Wright, 1931, 1951). Still, in the face of modern anthropogenic selective pressures in Unionidae, this genetic variation will be essential for adaptation. Population genetic theory predicts that selective sweeps on new mutations will be slow to appear, requiring tens of thousands of generations or hundreds of millions of years for desired genetic variation to appear (Gillespie, 1991; Maynard Smith, 1971). For adaptation to occur on nearer timescales, standing variation is expected to offer the substrate for initial adaptive responses. The probability that a genetic variant exists and is able to establish a deterministic selective sweep in a population $P_{agg}$
depends directly on $\theta$ (Hermisson & Pennings, 2005). In these bivalves, *M. nervosa* has the highest level of genetic variation, 35% higher than endemic *E. hopelonensis*. This will result in a 33% higher chance of adaptation from standing variation, a key advantage in the response to ongoing selective events. With modern population reductions (Ahlstedt & McDonough, 1992), this ancestral standing variation will be even more essential than these estimates from historical population sizes might reflect.

Census of bivalves noted smaller classes of *M. nervosa* below the 4-inch commercial harvest limit were noted to be scarce in Wheeler Lake (Ahlstedt & McDonough, 1992), though the authors have noted healthy numbers of juveniles near Pickwick Lake. It is unlikely that $N_e$ fully reflects the ongoing mussel declines, nor do we expect it reflects equilibrium levels for reduced populations. It does, however, indicate that the species still house substantial genetic diversity, if populations can be preserved. We would expect a decay in this diversity over time as population sizes drop according to $H_t = H_0 \left(1 - \frac{1}{4t} \right)$ (Gillespie, 2004). Even assuming the minimum age of reproduction at 5 years of age, it has been fewer than 20 generations since the damming of the Tennessee River began in 1918 and even fewer since Pickwick Dam was established in 1938. Hence, we expect that genome-wide $H_0$ will reflect historical rather than modern diversity.

The extent to which ongoing trace migration across the geographic range, especially incidental or intentional transport with humans, might reseed population diversity remains largely understudied. Previous work has suggested that diversity in *M. nervosa* is well connected across most reservoirs, potentially benefiting from diversity of fish hosts that can transport offspring (Pfeiffer et al., 2018). Future work with whole-genome sequencing multiple individuals across the geographic range might clarify whether populations remain panmictic or if distinct genetic features may be emerging in specific geographic locations via drift or local adaptation.

Here, Unionidae offer the prospect to study how adaptation occurs in long-lived but numerous species that is notably different from many population genetic models. Yet, the implications of these differences go far beyond abstract concepts in population genetic theory. Thoseorganisms that house the greatest diversity may indeed have the best chances for adaptive evolution that could help them survive when new genetic variation is needed. If adaptation from new mutations requires long timescales, standing genetic variation is the major source of adaptive changes in the near term. If standing variation is sufficient to offer genetic solutions to selective pressures, organisms will be able to readily adapt. If genetic diversity is unavailable, animals faced with strong selection will be unable to realize adaptive change and may be at greater risk of extinction. Indeed, standing genetic variation has been shown to be essential as other marine bivalve species adapt to anthropogenic changes (Bitter et al., 2019). Evaluating the substrate of genetic variation is essential to understand the limits that these species face, especially given widespread extinction of freshwater Unionidae.

### 4.5 Genetic resources for freshwater unionidae

Unionidae currently face a modern day mass extinction (Haag & Williams, 2014; Lopes-Lima et al., 2018; Williams et al., 1993). They have faced strong recent selective pressures of pollution, barriers on waterways, invasive species and loss of fish hosts. These organisms badly need genetic resources to assess population health, monitor diversity and identify unique groups across geographic locations. Moreover, evolutionary analysis is currently limited by a lack of genetic resources. At present, a single fragmented whole-genome sequence exists across all Unionidae for comparison (Renault et al., 2018), and the nearest outgroup, *C. gigas* is 495 million (Kumar et al., 2017) to 200 million years divergent (Bolotov et al., 2017).

Previous work has used limited mtDNA sequencing, targeted single genes, transcriptomes or few microsatellite markers (Campbell et al., 2005; Cormon et al., 2014; González et al., 2015; Pfeiffer et al., 2019; Wang et al., 2012). In the absence of whole-genome sequencing, bait-based pulldowns of SNPs combined with sequencing have been useful in reconstructing phylogenies (Pfeiffer et al., 2019) (though these may be subject to some measure of ascertainment bias). Each of these approaches offer useful genetic data in cost-effective schemes. Future whole-genome sequencing will offer a more complete description of genomic changes, especially with respect to duplicate gene formation and TE proliferation across Unionidae. The reference genome for *M. nervosa* represents a step towards evolutionary genomics of gene families and TEs in a high-risk clade, offering a portrait of dynamically changing genomes that have responded to strong selection in the past. This work represents a first step to understanding of genetic responses in a robust species within a larger clade of vulnerable species.

In this work, we have identified high copy number for key gene families in Unionidae responsible for detox, mitochondria maintenance, heat shock and blood-feeding. Transcriptome-based analysis suggests that comparable gene family expansion is likely to be common among Unionidae. As these functions are directly related to the known life history and environment of the organism, future studies of duplicate genes may be essential to understanding survival and reproduction across the phylogeny. Genome annotation often masks highly repetitive sequences prior to annotation to reduce the likelihood of identifying TEs as false gene sequences. This standard practice of masking repetitive sequences prior to annotation would miss many members of these important gene families relevant to the unique biology of freshwater Unionidae. Instead, a more focused, precise study of transposable elements with post-annotation filtering is able to sort out this adaptive variation, and offer a more complete description of evolutionary change in *M. nervosa*, an approach that should be noted in future genome annotation efforts.

Freshwater ecosystems have recently been threatened by widespread upheaval and human encroachment (Lake et al., 2000). In the modern industrial era, waterways have been dammed throughout the United States limiting dispersal (Ortmann, 1924). Alongside these changes to rivers, waters are compromised with pesticides, industrial pollution, and agricultural runoff (Woodside,
2004). One unintended consequence has been limited dispersal of aquatic life through waterways, leading to a marked reduction in species diversity and prevalence (Ortmann, 1924). TVA has monitored unionids in the Tennessee River for decades as an indicator of water quality and to track impacts of commercial mussel capture that was common until the 1990s, providing a rich historical record (Cahn, 1936; Isom et al., 1973). Improved environmental regulations and targeted conservation efforts have encouraged recovery, but the historic mark of these environmental challenges is not fully known. The addition of whole-genome sequence analysis to this rich historical record will enhance conservation and population monitoring in this focal location that is widely recognized for its high diversity of Unionidae.

These genome sequences can further help target conservation efforts where they are needed most. Unionidae with the lowest sequence diversity can be marked as first priority for conservation. Monitoring whole-genome genetic variation can help identify a baseline so that genetic diversity is preserved as wildlife management efforts survey freshwater Unionidae populations. In the future, even low-level whole-genome sequencing to identify nuclear genetic variation is likely to facilitate phylogenetic and evolutionary analysis in Unionidae and offer a more complete account of genetic diversity in a clade where so many species are threatened. *M. nervosa* populations have remained hardy so far in the face of many ecological challenges. They offer a benchmark that can be used for comparison in species whose populations are dwindling. For low diversity species, adaptation to newly arising challenges will be increasingly difficult with a lesser substrate for adaptation. In these cases, early intervention may be warranted so that their modest diversity remains preserved. Additionally, when evaluating which efforts are most likely to succeed, species that retain moderate to high diversity may have the greatest chances of surviving the modern anthropogenic challenges. Similar genetic studies in the future will be increasingly essential for evolutionary theory and conservation in Unionidae.

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**AUTHOR CONTRIBUTIONS**

Rebekah L. Rogers, Sagar Patel, James E. Titus-McQuillan, Cathy C. Moore and John P. Wares designed experiments and analyses. Jeffrey T. Garner collected specimens from wild populations. Rebekah L. Rogers, Stephanie L. Grizzard, Katherine Bockrath and Cathy C. Moore performed experiments. Rebekah L. Rogers, Sagar Patel, James E. Titus-McQuillan and Stephanie L. Grizzard performed analyses. Rebekah L. Rogers, John P. Wares, Cathy C. Moore, James E. Titus-McQuillan and Jeffrey T. Garner wrote and edited the manuscript.

**DATA AVAILABILITY STATEMENT**

Venustaconcha ellipsiformis transcriptome data are available at SRR6279374, and genomic sequence data were taken from SRR6689532 and SRR6689533. Megalonaias nervosa genomic sequence data are available in PRJNA646917, and transcriptome data are available at SRA PRJNA646778. The Megalonaias nervosa genome assembly is available at PRJNA681519. Elliptio crassidens transcriptomes are deposited under SRA PRJNA647326. Elliptio hopotonensis genomic reads are deposited at SRA PRJNA647325. Full annotations, de novo assembled transcriptomes and interproscan results are available in Supporting Information, currently on Dryad (https://doi.org/10.5061/dryad.bzh1897b).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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