ABSTRACT

Resolving the Evolutionary History of Multiple Groups
of Freshwater Mussels (Bivalvia: Unionidae)

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Freshwater mussels are a group of aquatic invertebrates comprised of approximately one thousand species worldwide, and the greatest diversity of freshwater mussels lies within North America with at least three hundred species. The high level of biodiversity in this group is strongly shaped by a life history strategy that includes an obligate parasitic larval stage. Alarmingly, anthropogenic alterations to freshwater ecosystems have disproportionately impacted mussels as a group, resulting in freshwater mussels being one of the most imperiled groups of organisms on Earth, and global collaboration is needed to understand the factors contributing to their demise. Although considerable progress has been made in understanding ecology and evolution of freshwater mussels, the biology of many species is poorly understood and there remains a critical need for robust phylogenetic evaluation to understand the evolutionary history of many freshwater mussels. Genetic techniques have emerged as one of the most promising tools in understanding of the basic biological processes and dynamics of species; however, it is evident that integrating molecular data with multiple lines of evidence

should be used to resolve evolutionary relationships. My dissertation research focuses on integrating robust phylogenetic evaluations with independent data types to resolve the evolutionary history and systematic relationships in multiple groups of North American freshwater mussels. My approach helped resolve numerous enigmatic questions pertaining to multiple groups of mussels, including accurately defining systematic placement, resolving species boundaries, and identifying functional traits that have driven lineage diversification. My findings have profound implications on the understanding of evolution and taxonomy, as well as illustrating the importance in incorporating multiple lines of evidence into phylogenetic assessments of freshwater mussels. As the scientific community continues to resolve the ecology and evolution of freshwater mussels globally, a firm understanding of species-specific traits will be critical toward determining conservation priorities and predicting species-specific responses in these highly imperiled organisms.

Resolving the Evolutionary History of Multiple Groups
of Freshwater Mussels (Bivalvia: Unionidae)

by

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DEDICATION

To My Wife, Melisa, and My Parents, Stephen and Cami

ATTRIBUTIONS

For chapter two titled “Integrative taxonomy reveals a new species of freshwater mussel, *Potamilus streckersoni* sp. nov. (Bivalvia: Unionidae): Implications for conservation and management”, author contributions were as follows: conceptualization, C.S. and N.J.; methodology, C.R., C.S., K.I., and N.J.; original draft preparation, C.S.; review and editing, C.R., K.I., N.J., and R.D.; supervision, N.J.; funding acquisition, N.J. For chapter three titled “Comparative phylogenomics reveal complex evolution of life history strategies in a clade of bivalves with parasitic larvae (Bivalvia: Unionoida: Ambleminae)”, author contributions were as follows: conceptualization, C.S., J.P., and N.J.; methodology, C.S. and J.P.; original draft preparation, C.S.; review and editing, J.P. and N.J.; supervision, N.J.; funding acquisition, N.J. For chapter four titled “Resolving species boundaries in the critically imperiled freshwater mussel species, *Fusconaia mitchelli* (Bivalvia: Unionidae)”, author contributions were as follows: conceptualization, C.R., C.S., and N.J.; methodology, C.R., C.S., K.H., and N.J.; original draft preparation, C.S. and K.H.; review and editing, C.R., C.S., N.J., and R.D.; supervision, C.R., N.J., and R.D.; funding acquisition, C.R. and N.J. For chapter fiv: “A comparative phylogeographic approach to facilitate recovery of an imperiled freshwater mussel (Bivalvia: Unionida: *Potamilus inflatus*)”, author contributions were as follows: conceptualization, C.S. and N.J.; methodology, C.S. and N.J.; original draft preparation, C.S.; review and editing, N.J.; supervision, N.J.; funding acquisition, N.J.

CHAPTER ONE

Introduction

Freshwater mussels (Bivalvia: Unionoida) are a group of aquatic invertebrates comprised of approximately one thousand species worldwide (Graf & Cummings, 2007; Lopes-Lima et al., 2018), and the greatest diversity of freshwater mussels lies within North America with at least three hundred native species in the family Unionidae alone (Graf & Cummings, 2007; Williams et al., 2017). This high level of biodiversity is largely explained by their peculiar life history. Nearly all mussels are obligate parasites that require temporary larval attachment to freshwater vertebrates (primarily fishes) to complete metamorphosis to a free-living juvenile (Barnhart et al., 2008). Selective pressures toward successful parasitism has led to many species evolving specialized patterns of host use, including reliance on one or more host fishes to complete their life cycle, and the radiation of the group has been influenced in part by the partitioning of a diverse, sympatric host fish community resource (Haag, 2012).

Mussels contribute significant ecological benefits to freshwater ecosystems, including biofiltration, integrating the fluvial food web, nutrient sequestration, and providing and stabilizing benthic habitat (Haag & Williams, 2014; Vaughn, 2018; Vaughn et al., 2008). Due to these intrinsic traits, freshwater mussels are often considered bioindicators of the health of aquatic ecosystems (Williams et al., 1993). Alarmingly, anthropogenic alterations to freshwater ecosystems have disproportionately impacted mussels as a group, leading to widespread extirpation and reduction in density of nearly

all species (Haag & Williams, 2014; Vaughn & Taylor, 1999). These declines stem from the inherent biological characters of mussels that are susceptible to systematic habitat alteration, including limited locomotive capabilities, reliance on host fish for dispersal, and extreme sensitivity to organic and inorganic pollutants (Bringolf et al., 2007; Haag, 2012; Wang et al., 2017). Additionally, some mussel species, particularly those considered imperiled, tend to have life history traits more characteristic of K-strategists (i.e., long-lived, low maturation rates, low fecundity, slow growth rates) making evolutionary response to rapidly changing environments less likely (Haag & Williams, 2014; Lighten et al., 2016; Martin & Palumbi, 1993). As a result, freshwater mussels are one the most imperiled groups of organisms globally (Lopes-Lima et al., 2018) and the most imperiled in North America (Strayer et al., 2004) with approximately 70% of species considered either threatened, endangered, or extinct (Haag & Williams, 2014; Williams et al., 1993).

Genetic techniques have emerged as promising tools to understand of the basic biological processes and dynamics of species (Allendorf et al., 2013; Ekblom & Galindo, 2011; McMahon et al., 2014). In freshwater mussels, molecular studies have been integral in inference of important biological characteristics (e.g., host use, reproductive traits, habitat preference), ensuring the taxonomic validity of protected species or those being considered for protection (Johnson et al., 2018; Pfeiffer, Johnson, Randklev, Howells, & Williams, 2016; Smith, Johnson, Pfeiffer, & Gangloff, 2018), and establishing effective conservation strategies (Smith et al., 2018, 2019). Although considerable progress has been made in understanding ecology (Dudding, Hart, Khan, Robertson, & Lopez, 2019; Hart, Haag, Bringolf, & Stoeckel, 2018; Johnson, McLeod,

Holcomb, Rowe, & Williams, 2016; Sietman, Hove, & Davis, 2018) and evolution (Inoue, Harris, Robertson, Johnson, & Randklev, 2019; Lopes-Lima et al., 2017; Pfeiffer et al., 2019; Pfeiffer, Breinholt, & Page, 2019; Smith, Johnson, Inoue, Doyle, & Randklev, 2019) of freshwater mussels globally, the basic biology of many species still remain poorly understood (Haag, 2012; Lopes-Lima et al., 2018). Thus, there remains a critical need for robust phylogenetic evaluation to understand the evolutionary history of many freshwater mussel groups.

As modern taxonomic studies are beginning to improve, it has become evident that integrating molecular data with multiple lines of evidence should be used to resolve evolutionary relationships (Dayrat, 2005; Edwards & Knowles, 2014; Knowles et al., 2007; Leaché et al., 2014; Padial et al., 2010; Schlick-Steiner et al., 2010; Will et al., 2005), including within freshwater mussels (Inoue et al., 2013, 2014, 2020; Johnson et al., 2018; Keogh & Simons, 2019; Smith et al., 2018, 2019). My dissertation research focuses on integrating robust phylogenetic evaluations with independent data types (e.g., ecological, geographic, life history, and morphological data) to resolve the evolutionary history and systematic relationships in multiple groups of North American freshwater mussels. Specifically, I set out to accomplish five objectives:

Objective 1. Resolve a Phylogeny of Lampsilini Using Multi-locus Sequence Data

Freshwater mussels of the subfamily Ambleminae and, in particular, the tribe Lampsilini have been the subject of many taxonomic studies considering the wide diversity of host infection strategies unique to the Unionidae (Barnhart et al., 2008; Graf, 2013; Zanatta & Murphy, 2006). However, many of these have focused on the species-rich genus *Lampsilis*, and supraspecific relationships between many genera remain

unresolved. For this objective, I will use robust taxon sampling paired with mitochondrial and nuclear sequence data to resolve supraspecific relationships between genera in Lampsilini.

Objective 2. Test Species Boundaries in Potamilus ohiensis Using an Integrative Approach

Potamilus ohiensis occurs throughout much of the Mississippi River basin including the Red, Sulfur, and Big Cypress rivers in northern Texas, as well as a disjunct population in the Brazos River drainage in Texas (Howells et al., 1996; Williams et al., 2008). This biogeographic pattern is unique within freshwater mussels, as no other unionid species is distributed only in the Mississippi and Brazos River drainages (Haag, 2010; Howells et al., 1996). However, specimens from the Brazos River drainage have atypical shell morphologies that resemble those of *P. amphichaenus*, a congener endemic to the Sabine, Neches, and Trinity Rivers in eastern Texas (Howells et al., 1996). This morphological similarity of *P. amphichaenus* and *P. ohiensis* from the Brazos River has led to speculation that *P. ohiensis* has been introduced into the Trinity River drainage (Howells et al., 1996), which is particularly troubling considering *P. amphichaenus* is petitioned for listing under the Endangered Species Act (USFWS, 2009). For this objective, I will integrate multi-locus sequence data and shell morphometrics to characterize the geographic distribution of *P. ohiensis* and test species boundaries between populations in the Mississippi and Brazos River drainages.

Objective 3. Resolve Life History Evolution in Aplodinotus grunniens Specialists

Life history traits in freshwater mussels are often phylogenetically conserved and useful in identifying clades with distinct evolutionary trajectories (Graf & Cummings,

2006; Hewitt et al., 2019; Pfeiffer, Breinholt, et al., 2019; Pfeiffer & Graf, 2015). One such clade is characterized by specialization on parasitizing *Aplodinotus grunniens*, a common molluscivorous fish distributed throughout Gulf of Mexico drainages (Haag, 2012; Page & Burr, 2011). This clade consists of the genera *Ellipsaria*, *Leptodea*, *Potamilus*, and *Truncilla* and appears to have evolved several distinct life history traits, including axe-head shaped glochidia, miniaturized glochidia, high fecundity, larval growth during encystment, and potential use of maternal sacrifice for host infection (Barnhart et al. 2008; Haag 2012). However, no study has recovered the monophyly of three life history adaptations: *A. grunniens* specialization, axe-head shaped glochidia, and miniaturized glochidia (Roe and Lydeard 1998; Campbell et al. 2005; Zanatta and Murphy 2006; Smith et al. 2019). The recovered non-monophyly of these traits suggests a complex pattern of life history evolution, emphasizing the need for robust phylogenetic evaluation. For this objective, I will reconstruct the origin and patterns of life history diversification within *A. grunniens* specialists using the freshwater mussel specific Anchored Hybrid Enrichment probe set Unioverse (Pfeiffer, Breinholt, et al., 2019) and ancestral character reconstruction.

Objective 4. Test Species Boundaries in Fusconaia mitchelli Using an Integrative Approach

Morphology driven taxonomic hypotheses in the freshwater mussel tribe Pleurobemini have been largely invalidated by molecular methods and resolving accurate phylogeny has been integral toward understanding the evolution of this group (Campbell & Lydeard, 2012b; Inoue et al., 2018). For members in the genus *Fusconaia* in Texas, there have been multiple systematic changes in recent years using molecular data and

some sympatric species are even morphologically indistinguishable (Campbell & Lydeard, 2012a; Pfeiffer et al., 2016; Pieri et al., 2018). One member of this genus, *Fusconaia mitchelli*, is endemic to the Brazos, Colorado, and Guadalupe drainages of central Texas (Howells et al., 1996). Recent molecular research revealed two distinct clades within *F. mitchelli* corresponding to the Brazos and Colorado drainages, and the Guadalupe drainage (Pfeiffer et al., 2016). Despite high levels of divergence between the two clades, recognizing two distinct species within *F. mitchelli* warranted increased taxon sampling, additional molecular markers, and morphological or life history data. Species boundaries in *F. mitchelli* remain a significant knowledge gap for natural resource managers, as conservation efforts based on current taxonomic hypotheses may lead to unsubstantiated conclusions about its status and bias management and recovery actions (TPWD, 2010; USFWS, 2009). The primary goal of this objective is to resolve species boundaries within *F. mitchelli* by incorporating multi-locus sequence and morphological data to better inform natural resource managers and facilitate conservation planning.

Objective 5. Use a Comparative Phylogeographic Approach to Facilitate Recovery of Potamilus inflatus

Potamilus inflatus is listed as threatened under the Endangered Species Act (ESA; USFWS, 1990) and was historically distributed throughout the Mobile, Pearl, and Lake Pontchartrain drainages (Jones et al., 2019; Williams et al., 2008). Systematic habitat destruction has extirpated the species from much of its historical range and extant populations are restricted to the Tombigbee and Black Warrior rivers in the Mobile Basin, and a 40 km-long stretch of the Amite River in the Lake Pontchartrain drainage (Brown & Daniel, 2014; Hartfield, 1988). One critical aspect of conservation biology is

delineating patterns of genetic diversity across geographic ranges of species (Allendorf et al., 2013). Comparative phylogeographic approaches offer options for resolving the effects of geological processes on observed genetic diversity in co-distributed taxa with similar life histories (Hickerson et al., 2010; Moritz & Faith, 1998). However, determining relationships among populations of imperiled species can be problematic when taxa have been extirpated from a significant portion of their historical range. The use of surrogate species is increasingly being used in conservation practices of rare species (Grantham et al., 2010), but this practice has not been explored in many freshwater taxa (Stewart et al., 2018), or to our knowledge, within a comparative phylogeographic framework. For this objective, I explore the use of comparative phylogeography for hypothesizing relationships among extant and extirpated populations of *P. inflatus* by characterizing genetic structure in the sympatric congeners *Potamilus fragilis* and *Potamilus purpuratus* using mitochondrial and nuclear sequence data to facilitate ongoing conservation and recovery efforts.

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CHAPTER TWO

Integrative Taxonomy Reveals a New Species of Freshwater Mussel, *Potamilus streckersoni* sp. nov. (Bivalvia: Unionidae): Implications for Conservation and Management

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Abstract

Inaccurate systematics confound our ability to determine evolutionary processes that have led to the diversification of many taxa. The North American freshwater mussel tribe Lampsilini is one of the more well-studied groups in Unionidae; however, many supraspecific relationships between lampsiline genera remain unresolved. Two genera previously hypothesized to be non-monophyletic that have been largely overlooked are *Leptodea* and *Potamilus*. We set out to resolve supraspecific relationships in Lampsilini and test the monophyly of *Leptodea* and *Potamilus* by integrating molecular, morphological, and life history data. Our molecular matrix consisted of four loci: *cytochrome c oxidase subunit 1* (CO1), *NADH dehydrogenase subunit 1* (ND1), *internal transcribed spacer 1* (ITS1), and *28S ribosomal RNA*. Secondly, we performed both traditional and Fourier shape morphometric analyses to evaluate morphological differences and finally, we compared our results with available life history data. Molecular data supported the paraphyly of both *Leptodea* and *Potamilus*, but nodal support was insufficient to make any conclusions regarding generic level assignments at this time. In contrast, inference from our integrative taxonomic assessment depicts

significant support for the recognition of a new species, *Potamilus streckersoni* sp. nov., the Brazos Heelsplitter. Our data show clear separation of three taxonomic entities in the *P. ohiensis* species complex: *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni* sp. nov.; all molecularly, geographically, and morphologically diagnosable. Our findings have profound implications for unionid taxonomy and will aid stakeholders in establishing effective conservation and management strategies.

Introduction

Inaccurate systematics continue to be a fundamental problem that confounds our ability to determine evolutionary processes that have led to the diversification of many taxa (Johnson et al., 2018; Perkins, Johnson, & Gangloff, 2017; Pfeiffer, Johnson, Randklev, Howells, & Williams, 2016; Satler, Carstens, & Hedin, 2013; Smith, Johnson, Pfeiffer, & Gangloff, 2018). Unionid bivalves (Bivalvia: Unionidae) represent the most species-rich taxonomic group in the order Unionida, with over 650 recognized species (Graf & Cummings, 2007; Lopes-Lima et al., 2018). The unique life cycle of unionids, which involves parasitic larvae (glochidia) that must attach to vertebrate hosts prior to becoming sessile adults, has likely contributed significantly to the rampant diversification of this group (e.g., Barnhart, Haag, & Roston, 2008). This complex life cycle creates a unique coevolutionary system, as freshwater mussels continually adapt to successfully infect their hosts.

Taxonomy in Unionidae has been particularly unstable and recent studies using molecular data have revealed cases of convergent evolution, cryptic diversity, inaccurate supraspecific relationships, and overestimated diversity at the species level (Inoue, Hayes, Harris, & Christian, 2013; Johnson et al., 2018; Perkins et al., 2017; Pfeiffer et al.,

2016; Smith et al., 2018; Williams et al., 2017). The freshwater mussel tribe Lampsilini Ihering, 1901 exhibits a wide diversity of host infection strategies unique to the Unionidae (Barnhart et al., 2008; Graf, 2013; Zanatta & Murphy, 2006) and has been the subject of many taxonomic studies. These previous studies primarily focused on the species-rich genus *Lampsilis* Rafinesque, 1820 and supraspecific relationships between many lampsiline genera remain unresolved. Two genera that have been largely overlooked are *Leptodea* Rafinesque, 1820 and *Potamilus* Rafinesque, 1818 which consist of 10 species endemic to the United States and Canada including several imperiled taxa (Williams et al., 2017). *Leptodea* and *Potamilus* have been considered closely related due to similar adult morphology, larval hosts, and habitat preference (Barnhart et al., 2008; Haag, 2012; Hoggarth, 1999; Sietman, Hove, & Davis, 2018); however, *Leptodea* and *Potamilus* have been classified as distinct genera based on differing glochidial morphologies (Barnhart et al., 2008; Hoggarth, 1999; Watters, Hoggarth, & Stansbery, 2009; Williams et al., 2017). Considering the strong selective pressures against parasitism, glochidial morphology is thought to be highly conserved and considered one of the most useful morphological characters in reconstructing the evolutionary history of freshwater mussels (Barnhart et al., 2008; Graf & Cummings, 2006; Haag, 2012; Hoggarth, 1999; Hoggarth & Gaunt, 1988; Williams, Butler, Warren, & Johnson, 2014). However, a previous phylogenetic assessment showed polyphyly between *Leptodea* and *Potamilus*, indicating that glochidial morphology may not be diagnostic for the two genera (Roe & Lydeard, 1998).

Concomitant to questionable monophyly at the generic-level, species in the genus *Potamilus* depict disjunct distributional patterns and high levels of intraspecific variation

in shell morphology. For instance, *P. ohiensis* (Rafinesque, 1820) occurs throughout much of the Mississippi River Basin including the Red, Sulfur, and Big Cypress rivers in northern Texas, as well as a disjunct population in the Brazos River drainage in Texas (Howells, Neck, & Murray, 1996; Williams, Bogan, & Garner, 2008). This biogeographic pattern is unique within freshwater mussels, as no other unionid species is distributed only in the Mississippi and Brazos River drainages (Haag, 2010; Howells et al., 1996). High levels of intraspecific variation in shell morphology are also present in *P. ohiensis* with individuals from the Brazos River resembling *P. amphichaenus* (Frierson, 1898), a congener endemic to the Sabine, Neches, and Trinity River drainages in eastern Texas (Howells et al., 1996). Morphological convergence of *P. amphichaenus* and *P. ohiensis* from the Brazos River has led to the hypothesized introduction of *P. ohiensis* in the Trinity River drainage (Howells et al., 1996); however, no specimens have been validated using molecular techniques. The possibility of a syntopic form of *P. ohiensis* with *P. amphichaenus* is troubling, especially considering *P. amphichaenus* is petitioned for listing under the Endangered Species Act (USFWS, 2009) and a recent phylogenetic study revealed multiple morphologically cryptic sympatric species of *Fusconaia* Simpson, 1900 in the Trinity River (Pieri et al., 2018).

Previous studies evaluating phylogenetic relationships between *Leptodea* and *Potamilus* implemented a single locus coupled with limited sample sizes and incomplete taxon sampling (Roe & Lydeard, 1998). Although phylogenetic reconstruction based off a single locus has been implemented in recent freshwater mussels studies (Inoue et al., 2018), this methodology has been criticized due to the significant increase in accuracy when analyzing loci from both nuclear and mitochondrial genomes (Fujita, Leaché,

Burbrink, McGuire, & Moritz, 2012; Yang & Rannala, 2010; Zhang, Zhang, Zhu, & Yang, 2011). Phylogenetic inference from limited sampling has also been well-documented to greatly increase phylogenetic estimation error (Hillis, Pollock, McGuire, & Zwickl, 2003; Pollock, Zwickl, McGuire, & Hillis, 2002; Zwickl & Hillis, 2002), thus proper sampling should be implemented before taxonomic recommendations are warranted. In this study, we present a robust multi-locus approach based on extensive taxonomic sampling to investigate supraspecific relationships between the genera *Leptodea* and *Potamilus*. We also investigate species-level diversity in *Potamilus* and implement an integrative taxonomic approach to resolve species boundaries and distributional patterns in the *P. ohiensis* species complex (*P. amphichaenus*, *P. ohiensis* from the Brazos River, and *P. ohiensis* from the Mississippi River Basin). We collect and analyze multiple independent lines of evidence, all of which support the recognition of three evolutionarily divergent groups within the *P. ohiensis* species complex: *P. amphichaenus* (Sabine, Neches, and Trinity rivers), *P. ohiensis* (Mississippi River Basin), and *P. ohiensis* endemic to the Brazos River. Below we present significant molecular, morphological, and biogeographic evidence that species-level diversity in this group was previously underestimated and we formally describe *Potamilus streckersoni* sp. nov., which is endemic to the Brazos River in Texas.

Materials and Methods

Taxon Sampling and Molecular Data Generation

To test the phylogenetic placement of *Leptodea* and *Potamilus*, we sampled material for North American genera in the tribes Lampsilini, Amblemini, Rafinesque,

1820, and additional material from Ambleminae *incertae sedis* (Williams et al., 2017). We focused our sampling on type species of each genus and type locality (APPENDIX; Table S2.1). We selected *Quadrula quadrula* (Rafinesque, 1820) to root our phylogeny following findings of tribe relationships in a previous study (Lopes-Lima et al., 2017). We sequenced two mitochondrial genes and two nuclear loci: a partial portion of *cytochrome c oxidase subunit 1* (CO1), *NADH dehydrogenase subunit 1* (ND1), the nuclear-encoded *ribosomal internal transcribed spacer 1* (ITS1), and a portion of the large ribosomal subunit *28S*. Mantle tissue samples were taken for DNA extraction either directly after specimens were euthanized or from samples preserved in 95% ethanol. Genomic DNA was extracted using the PureGene DNA extraction kit with the standard extraction protocol (Gentra Systems, Inc., Minneapolis, MN, USA). Primers used for polymerase chain reaction (PCR) and sequencing were: CO1 5'-GTTCCACAAATCATAAGGATATTGG-3' and 5'-TACACCTCAGGGTGACCAAAAAACCA-3' (Campbell et al., 2005); ND1 5'-TGGCAGAAAAGTGCATCAGATTAAAGC-3' and 5'-CCTGCTTGGAAGGCAAGTGTACT-3' (Serb, Buhay, & Lydeard, 2003); ITS1 5'-AAAAAGCTTCCGTAGGTGAACCTGCG-3' and 5'-AGCTTGCTGCGTTCTTCATCG-3' (King, Eackles, Gjetvaj, & Hoeh, 1999); 28S 5'-GGGACTACCCCCTGAATTTAAGCAT-3' and 5'-CCAGCTATCCTGAGGGAAACTTCG-3' (Park & Foighil, 2000). Thermal cycling conditions for CO1 followed Johnson et al., (2018), while all other conditions followed the publication of origin (King et al., 1999; Park & Foighil, 2000; Serb et al., 2003). PCR plate amplifications were conducted using a 12.5 µl mixture of the following: molecular

grade water (4.25 µl), MyTaq^{TM} Red Mix (6.25 µl) (Bioline), primers (0.5 µl each) and DNA template (50 ng). PCR product was sent to the Molecular Cloning Laboratories (MCLAB, South San Francisco, CA, USA) for bi-directional sequencing on an ABI 3730. All ITS1 sequences were readable without cloning, similar to recent studies in unionids (Johnson et al., 2018; Pfeiffer et al., 2016; Pieri et al., 2018; Smith et al., 2018). Geneious v 10.2.3 was used to assemble contigs and edit chromatograms (Kearse et al., 2012) and sequences were aligned in Mesquite v 3.31 (Maddison & Maddison, 2017) using MAFFT v 7.311 (Katoh & Standley, 2013). The protein coding genes (CO1 and ND1) were aligned using the L-INS-i method in MAFFT and translated into amino acids to ensure absence of stop codons and gaps. The ITS1 and 28S sequences were aligned using the E-INS-i method in MAFFT to better account for indels.

Phylogenetic Reconstruction

We created a 4-locus concatenated dataset of CO1, ND1, ITS1, and 28S to estimate a phylogeny of Lampsilini using both Maximum Likelihood (ML) and Bayesian Inference (BI). Before phylogenetic inference was performed, we tested for nucleotide saturation in the three codon positions for protein coding mitochondrial markers (i.e., CO1 and ND1) using the Xia test in Dambe v 7.0.35 (Xia, 2018; Xia, Xie, Salemi, Chen, & Wang, 2003). ML and BI analyses were subsequently performed in IQ-TREE v 1.6.6 (Chernomor, von Haeseler, & Minh, 2016; Nguyen, Schmidt, von Haeseler, & Minh, 2015) and MrBayes v 3.2.6 (Ronquist et al., 2012), respectively. We used ModelFinder (Kalyaanamoorthy, Minh, Wong, von Haeseler, & Jermiin, 2017) to select appropriate partitions and substitution models before conducting 10 independent IQ-TREE runs of an initial tree search and 10,000 ultrafast bootstrap replicates (BS) for nodal support (Hoang,

Chernomor, von Haeseler, Quang Minh, & Sy Vinh, 2018). Partitions and substitution models available for use in MrBayes were determined by PartitionFinder v 2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) using BIC. MrBayes analyses executed 2 runs of 8 chains for 10^7 MCMC generations sampling every 1000 trees. Log likelihood scores for each sampling point were analyzed using Tracer v 1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018) to determine an appropriate burn-in value. Chains were considered stationary when the log likelihood values reached a plateau. Convergence of the two independent runs was monitored using the Potential Scale Reduction Factor (PSRF) of each parameter and the average standard deviation of split frequencies. Strongly supported nodes were represented by BS and PP values greater than 95.

To test for significant differences between BI and ML reconstructions, we implemented an Approximately Unbiased (AU) Test (Shimodaira, 2002) in IQ-TREE using 10,000 RELL replicates (Kishino, Miyata, & Hasegawa, 1990). We chose to implement an AU test in IQ-TREE rather than CONSEL (Shimodaira & Hasegawa, 2001) as it is more appropriate for partitioned analyses considering CONSEL is not partition-aware. Mesquite was used to move branches in the ML phylogenetic construction to match the topology resolved by MrBayes. A significance level of $\alpha=0.05$ was assumed when assessing the statistical significance between topologies.

Genetic Diversity and Phylogeographic Analyses

To get estimates of genetic diversity, we used DnaSP v 6.12.0 (Rozas et al., 2017) to estimate unique haplotypes (h), haplotype diversity (Hd), mean number of nucleotide differences (k) and mean nucleotide diversity (π) at CO1 and ND1 independently for five

groups in the *P. ohiensis* species complex: *P. ohiensis*, *P. streckersoni* sp. nov., and three geographic groupings for *P. amphichaenus* (Sabine, Neches, and Trinity drainages). DNA sequence divergence was calculated within and between groups using uncorrected pairwise genetic distances in MEGA7 (Kumar, Stecher, & Tamura, 2016) for CO1 and ND1 independently. Model-based distances have been shown to inflate genetic distance values (Collins & Cruickshank, 2012; Lefébure, Douady, Gouy, & Gibert, 2006; Ratnasingham & Hebert, 2013); therefore, we chose to use uncorrected p-distances to remove biases from nucleotide substitution model assumptions. Partial deletion was used to handle missing data in MEGA7 calculations. To further compare genetic divergence between *P. amphichaenus* and *P. streckersoni* sp. nov., we created histograms of intraspecific and interspecific distance values in the R package ggplot2 (Wickham, 2016). To visualize genetic structuring with respect to geographic distribution, we generated TCS haplotype networks (Clement, Posada, & Crandall, 2000) from CO1 and ND1 independently using PopART 1.7 (Leigh & Bryant, 2015) for groups in the *P. ohiensis* species complex. Missing data were handled using complete deletion, as PopArt does not support partial deletion.

Species Delimitation Analyses

We implemented the coalescent species delimitation models STACEY v 1.2.4 (Jones, 2017) and *BEAST2 (Ogilvie, Bouckaert, & Drummond, 2017) in BEAST v 2.4.8 (Bouckaert et al., 2014) on a concatenated alignment of CO1 and ND1 for all individuals representing *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni* sp. nov. Partitions and substitutions models for the STACEY analysis were reevaluated using PartitionFinder (Lanfear et al., 2016) similar to phylogenetic analyses, except allowing

for all possible nucleotide evolution models. STACEY infers species boundaries without *a priori* species designations; therefore, we allowed the model to consider all individuals as minimum clusters and freely assign individuals to appropriate clusters. A strict molecular clock was set at 1.0 for the 1st position of CO1 and remaining partitions were estimated by STACEY. Our STACEY analyses consisted of 8 independent runs executing 10^8 generations and logged every 5000 trees with an initial 10% burn-in. We used LogCombiner v 2.4.8 (Bouckaert et al., 2014) to combine trace logs and species trees from individual runs. We used Tracer to evaluate the combined trace log to ensure convergence of all parameters (ESS > 200). The most likely number of species clusters was calculated by SpeciesDelimitationAnalyser (SpeciesDA) v 1.8.0 (Jones, 2017) using the combined species trees from the 8 individual STACEY runs (144,000 trees). SpeciesDA implemented a collapse height of 0.0001 and a 1.0 simcutoff.

For *BEAST2 analyses, we allowed the most likely species clusters recovered by STACEY to guide our species models. Three species models were implemented to test the log likelihood of clustering scenarios: 1 – *P. amphichaenus* from the Sabine and Neches rivers, *P. amphichaenus* from the Trinity River, *P. ohiensis*, and *P. streckersoni* sp. nov.; 2 – *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni* sp. nov.; and 3 – *P. amphichaenus* from the Sabine and Trinity rivers, *P. amphichaenus* from the Neches River, *P. ohiensis*, and *P. streckersoni* sp. nov. We used the partitions and substitution models appropriate for the STACEY analysis in *BEAST2 analyses, except the substitution model for ND1 1st codon position (K81/TPM1 not available for *BEAST2) which was reevaluated. *BEAST2 analyses executed $1.5x10^7$ generations logging every 5000 trees to reconstruct species tree for each scenario. Like STACEY analyses, a strict

molecular clock was set at 1.0 for the 1st position of CO1 and remaining partitions were estimated by *BEAST2. The population model was set to linear with a constant root and the Yule model was the species tree prior. Marginal likelihood of each model was estimated using a path sampling executing 100 path steps with a chain length of $1.5x10^6$ and a 25% burn-in (Baele, Li, Drummond, Suchard, & Lemey, 2012; Lartillot & Philippe, 2006). Bayes factors delimitation (BFD) was used to reject species models, using twice the difference of -*ln* likelihood (2*ln*BF) and 2*ln*BF > 10 depicting significant support (Grummer, Bryson, & Reeder, 2014; Kass & Raftery, 1995).

Morphometrics Analyses

Traditional and Fourier shape morphometrics were used to compare shell shapes within members of the *P. ohiensis* species complex. Specimens were binned into three groups: *P. amphichaenus* (Sabine, Neches, Trinity; n = 24), *P. ohiensis* (Mississippi; n = 7), and *P. streckersoni* sp. nov. (Brazos; n = 40; APPENDIX; Table S2.2); and specimens showing obvious damage of shells were excluded. For traditional morphometrics, we took four shell measurements: maximum length (anterior to posterior), height 1 (posterior dorsal wing to ventral), height 2 (umbo to ventral), and max width (right to left valve) to the nearest 0.01 mm for all specimens using digital calipers (APPENDIX; Fig. S2.1). To characterize shell shape, we calculated six ratios: height 1/length (elongation), height 2/length (elongation), height 2/height 1 (wing height), weight/length (inflation), width/height 1 (inflation), and width/height 2 (inflation). Ratios were normalized using an arcsine-transformation. For Fourier shape morphometrics, we used the right valve of each specimen and took digital photographs with a Canon EOS7D SLR camera. The outline of the shell was extracted for each photo by cropping the image using Adobe

Photoshop CC v2015.0.0 (Adobe System) (APPENDIX; Fig. S2.1). Using the cropped shell image, the shell outline was described by 20 Fourier coefficients using SHAPE v 1.3 (Iwata & Ukai, 2002).

Morphological variation within and among putative species were described through a principal component analysis (PCA) and canonical variate analysis (CVA). Additionally, a multivariate analysis of variance (MANOVA) and discriminant function analysis (DFA) were used to determined how frequently principal component (PC) scores correctly distinguished between groups. Confusion matrices were calculated based on the DFA for each morphometric analysis, where percentages of correct group assignments were calculated. Statistical analyses were performed using the software PAST (Hammer, Harper, & Ryan, 2001) and SHAPE. A significance level of $\alpha=0.05$ was assumed when assessing the statistical significance of all tested hypotheses.

Range Map

We complied distribution data for freshwater mussel surveys conducted in the Brazos River basin to provide information critical for the conservation status assessment of *P. streckersoni* sp. nov. Sources of the distribution data were as follows: Baylor University Mayborn Museum, Fort Worth Museum of Science and History, Texas Parks and Wildlife Department, Joseph Britton Freshwater Mollusk Collection, Texas A&M Natural Resources Institute, Texas Department of Transportation, University of Florida Museum of Natural History, University of Michigan Museum of Zoology, and U.S. Fish and Wildlife Service. We assumed all historical records of *P. ohiensis* and specimens misidentified as *P. amphichaenus* from the Brazos River were *P. streckersoni* sp. nov. We used these distribution data (APPENDIX; Table S2.3) to develop a conservation

status assessment map using ArcMap 10.3 (ESRI) following the protocol produced by Georgia Department of Natural Resources (2018) and modified approach of Johnson et al. (2016). The spatiotemporal distribution of *P. streckersoni* sp. nov. was illustrated at the Hydrological Unit Code (HUC) 10-level and all known survey locations were included to illustrate both the presence or absence of *P. streckersoni* sp. nov. from 1900-2018.

Results

Taxon Sampling

All novel DNA sequences were made available on Genbank (MK036068-MK036232; MK044901-MK045202) and Sciencebase (https://doi.org/10.5066/P92CV9QZ), and all accession numbers used in this study can be found in Table S2.1 (APPENDIX). We included representatives of all genera in Lampsilini except for *Dromus,* Simpson, 1900, which has been shown in previous phylogenetic studies to be closely related to the genus *Cyprogenia* Agassiz, 1852 (Campbell et al., 2005; Zanatta & Murphy, 2006) (APPENDIX; Table S2.1). All genera were represented by the type species except *Obovaria*, Rafinesque, 1819. All currently recognized species in *Ellipsaria* Rafinesque, 1820, *Leptodea*, *Potamilus*, and *Truncilla*, Rafinesque, 1819, were represented in phylogenetic analyses (Williams et al., 2017). In addition to our data matrix for phylogenetic reconstructions, we sequenced a total of 78 individuals from the *P. ohiensis* species complex for CO1 and ND1: *P. amphichaenus* (n = 29), *P. ohiensis* (n = 19), *P. streckersoni* sp. nov. (n = 30; Fig. 2.1; APPENDIX; Table S2.1). Both CO1 and ND1 alignments did not contain indels or stop codons.

Phylogenetic Reconstruction

Xia's saturation test indicated little saturation at all codon positions for CO1 and ND1; therefore, all codon positions were retained in phylogenetic analyses. Nucleotide substitution models were determined for eight partitions by ModelFinder for IQ-TREE analyses: CO1 1st position- TN+F+I+G4, CO1 2nd position- TPM3+F, CO1 3rd position- TVM+F+G4; ND1 1st position- TIM2e+I+G4, ND1 2nd position- TIM2+F+I+G4, ND1 3rd position- TIM+F+G4, ITS1- TIM2e+I+G4, and 28S- TN+F+I+G4. For BI analyses, nucleotide substitution models were determined for seven partitions by PartitionFinder: CO1 1st position and ND1 2nd position- HKY+I+G, CO1 2nd position- F81+I, CO1 3rd position- GTR+G, ND1 1st position- SYM+I+G, ND1 3rd position- GTR+G. ITS1- K80+I+G, and 28S- HKY+I+G. Convergence of the two MrBayes runs was supported by the PSRF value for each parameter equal to 1.0 and the mean of the standard deviation of split frequencies (0.001288). A 25% burn- was deemed appropriate for each MrBayes run by Tracer and was implemented before optimal log likelihood and trees were reported. Both ML and BI topologies resolve a monophyletic grouping of *Ellipsaria*, *Leptodea*, *Potamilus*, and *Truncilla* (Figs. 2.2 & S2.2; APPENDIX); however, supraspecific relationships between these genera were not be resolved with strong nodal support. Topologies depict four strongly supported clades (PP/BS = 100): *Ellipsaria* and *Truncilla*; *L. fragilis* and *L. leptodon*; *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni* sp. nov.; and *P. alatus*, *P. metnecktayi*, and *P. purpuratus*. Topologies strongly support *P. streckersoni* sp. nov. sister to *P. amphichaenus* rather than *P. ohiensis*, with significant divergence from both species. Phylogenetic placement of *L. ochracea*, *P. capax*, and *P. inflatus* were inconsistent between ML and BI analyses (Figs. 2.2 & S2.2; APPENDIX).

To test these inconsistencies, we implemented an AU test but no significant difference between BI and ML topologies was recovered ($\alpha = 0.5018$).

Phylogeographic Analyses

Genetic diversity statistics generated by DnaSP are reported in Table 2.1 for members of the *P. ohiensis* species complex. High levels of genetic diversity were depicted in *P. ohiensis* and the Trinity River population of *P. amphichaenus*, while *P. streckersoni* sp. nov. depicted excessive haplotype sharing and limited nucleotide diversity. Mean pairwise genetic distance values for within and between groups at CO1 and ND1 are reported in Table 2.2. Distance values for CO1 and ND1 depicted *P. ohiensis* largely divergent from both *P. amphichaenus* and *P. streckersoni* sp. nov. (Table 2.2). Genetic distance between *P. streckersoni* sp. nov. and all populations of *P. amphichaenus* at CO1 and ND1 ranged from 1.81-2.29% and 1.59-2.15%, respectively (Table 2.2). Histograms of intra- and interspecific uncorrected p-distance values for *P. amphichaenus* and *P. streckersoni* sp. nov. depicted clear separation between intraspecific variation and interspecific divergence (Figs. S2.3.1 & S2.3.2). TCS haplotype networks also showed clear divergence at mtDNA markers between *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni* sp. nov.; and depicted limited divergence within *P. amphichaenus* with respect to drainage of capture at ND1 (Fig. 2.3.2). Similar to genetic diversity statistics, haplotype networks depicted excessive haplotype sharing in *P. streckersoni* sp. nov. at both mtDNA markers.

Species Delimitation Analyses

The molecular matrix used in the STACEY and *BEAST2 analyses was aligned to 1558 bp and included all individuals in the *P. ohiensis* species complex. Five partitions and substitution models were selected for STACEY and *BEAST2 by PartitionFinder: CO1 1st position- HKY, CO1 and ND1 2nd position- HKY, CO1 3rd position- HKY, ND1 1st position- TPM1, and ND1 3rd position- TrN. TPM1 is not available in *BEAST2; therefore, we implemented K80, the most-appropriate substitution model available for the analysis. Convergence of the STACEY and *BEAST2 analyses was indicated by all ESS values > 200. STACEY resolved three species models with probabilities greater than 5%, but not with high probabilities: Species Model 1 (27.2%) - *P. amphichaenus* from the Sabine and Neches drainages, *P. amphichaenus* from the Trinity drainage, *P. ohiensis*, and *P. streckersoni* sp. nov.; Species Model 2 (21.2%) - *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni* sp. nov.; and Species Model 3 (12.5%) - *P. amphichaenus* from the Sabine and Trinity drainages, *P. amphichaenus* from the Neches drainage, *P. ohiensis*, and *P. streckersoni* sp. nov. (Fig. 2.4; Table 2.3). *BEAST2 analyses resolved Species Model 1 as the most likely, and 2*ln*BF rejected Species Model 2 but could not reject Species Model 3 (Table 2.3).

Morphometric Analyses

For traditional morphometrics, the PCA yielded three distinct eigenvalues that described > 99% of the total variation among individuals, with the first two PCs describing 90.69% of the total variation (Fig. 2.5). The PCA and CVA plots showed differentiation among species, where a small portion of the cluster of *P. amphichaenus* overlapped with the cluster of *P. streckersoni* sp. nov. (Figs. 2.5.1 & 2.5.2). The

MANOVA depicted that shell morphologies were significantly different among species (Wilk's $\Lambda = 0.1298$; $F_{12,126} = 18.65$; $\alpha < 0.001$; Table 2.4). On average, the DFA assigned 85.9% of individuals to the correct group (Table 2.4).

For Fourier shape morphometrics, the PCA yielded six distinct eigenvalues and described >90% of the total variation among individuals (Fig. 2.5). The PCA and CVA plots showed similar clustering patterns to the traditional morphometrics (Figs. 2.5.3 & 2.5.4), with divergence between species and limited overlap between *P. amphichaenus* and *P. streckersoni* sp. nov. The MANOVA depicted significant differences in shell morphologies between species (Wilk's $\Lambda = 0.1756$; $F_{12,126} = 14.56$; $\alpha < 0.001$; Table 2.4). Fourier morphometrics had a slightly better assignment rate, with 90.1% of individuals assigned to the correct group (Table 2.4).

Range Map

During our searches of museum records and available field observations, we located collection information for 2,049 freshwater mussel surveys conducted from 1900-2018 in the Brazos River basin. Shells (fresh dead or recently dead) or live individuals of *P. streckersoni* sp. nov. were reported during 213 surveys conducted from 1934-2018 (APPENDIX; Table S2.3), including a total of 231 live individuals. *Potamilus streckersoni* sp. nov. records were distributed across 27 HUC units in the Brazos River basin (Fig. 2.6). The status of the species in each HUC unit was categorized as follows: 13 HUCs with shell only; 3 with historical records (prior to 1995); 2 with recent records (1995-2010); and 9 with current records (2011 to present).

Taxonomic Accounts

Potamilus streckersoni sp. nov.

Brazos Heelsplitter

HOLOTYPE: UF439497, length 128 mm, Brazos River upstream of FM 485 bridge (30.86586°N; -96.69575°W), Milam/Robertson Counties, TX, 10 Nov. 2017 (Fig. 2.7).

PARATYPES: UF439478, 4 wet specimens, length 93-117 mm, Brazos River upstream of FM 485 bridge (30.86586°N; -96.69575°W), Milam/Robertson Counties, TX, 10 Nov. 2017.

UF441294, 4 wet specimens, length 76-105 mm, Brazos River about 1 mile downstream of FM1093, about 2.7 miles ENE of Wallis, TX (29.650845°N; -96.026521°W), Austin/Fort Bend Counties, TX, 24 Oct. 2012.

ETYMOLOGY: The specific epithet *streckersoni* is in honor of John K. Strecker and Lorraine L. Frierson. John K. Strecker, former curator of the Baylor University Museum (Waco, TX, USA), authored one of the first publication regarding distribution and biodiversity of Texas unionids (Strecker, 1931), which provided the foundation for freshwater mussel conservation in Texas. He had a strong relationship with esteemed malacologist Mr. Lorraine L. Frierson, who corresponded nearly 20 years with Mr. Strecker regarding mussel taxonomy and identification. Between Strecker and Frierson, 2277 unionid specimens were collected and donated to the Mayborn Museum at Baylor University.

DIAGNOSIS: *Potamilus streckersoni* sp. nov. is significantly different from *P. ohiensis* using both molecular and morphological characters (Figs 2.3, 2.4 & 2.5; Tables 2.2 & 2.4). Of the 30 *P. streckersoni* sp. nov. and 19 *P. ohiensis* individuals we examined, the

two taxa were diagnosable at 25 of 658 sites examined at CO1 and 66 of 900 sites examined at ND1. *Potamilus streckersoni* sp. nov. is also morphologically divergent, with individuals more elongate and less alate than specimens of *P. ohiensis* (Fig. 2.5; Table 2.4); however, future work evaluating additional material from throughout the range of *P. ohiensis* is encouraged to better assess the wide range of morphological variation in this species.

Potamilus streckersoni sp. nov. can be diagnosed from other similar sympatric freshwater mussels in the Brazos River using conchological characters including periostracum color, lack of sculpturing, reduced umbo, and absence or weak posterior ridge. *Potamilus streckersoni* sp. nov. may be confused with *Cyrtonaias tampicoensis* (Lea, 1838) or *P. purpuratus*; however, *P. streckersoni* sp. nov. is generally more elongate than both species. The pseudocardinal teeth of *P. streckersoni* sp. nov. are less developed and only one tooth is present in the left valve, while *C. tampicoensis* and *P. purpuratus* have two well-developed pseudocardinal teeth in the left valve. *Potamilus streckersoni* sp. nov. may also be confused with *L. fragilis*. Larger specimens of *P. streckersoni* sp. nov. are typically less elongate than similar sized *L. fragilis*, and the dark brown periostracum is easily distinguishable from the horn yellow periostracum of *L. fragilis*. In smaller individuals where periostracum color may not be diagnostic, *P. streckersoni* sp. nov. can be distinguished from *L. fragilis* by presence of an anterior dorsal wing, which is absent in *L. fragilis*.

DESCRIPTION: Maximum shell length to 144 mm (JBFMC26.1). Shell thin to moderately thick and compressed. General outline of the shell is oval; however, may be triangular in smaller individuals when posterior dorsal wing has not been eroded or

broken; posterior and anterior margins rounded. Dorsal margin with weak wing posterior to umbo, which is typically more prominent in smaller individuals. Small triangular dorsal wing anterior to umbo in smaller specimens, usually eroded away in larger individuals. Ventral margin straight to convex, posterior ridge absent or very low, posterior slope flattened to slightly concave, merging with the posterior dorsal wing. Umbo low, broad, and barely extends above the hinge line, with limited sculpturing. Periostracum shiny, greenish to yellowish in smaller specimens, becoming chestnut brown in larger individuals. Pseudocardinal teeth compressed and delicate, one in each valve with an accessory denticle usually present in right valve. Lateral teeth moderately long, slightly curved, two in left valve and one in right. Interdentum moderately long, narrow; umbo cavity wide but shallow. Nacre deep pink or purple.

DISTRIBUTION: *Potamilus streckersoni* sp. nov. is endemic to the Brazos River drainage in Texas.

REMARKS: Based off of systematic placement, it is likely that *P. streckersoni* sp. nov. is a host fish specialist, with glochidia transforming on *A. grunniens*. *Potamilus streckersoni* sp. nov. is likely a long-term brooder and gravid females have been collected in May (UF439481), October (UF441294), and November (UF439478). Observational studies of natural fish infection and additional surveys are necessary to determine detailed host fish use and brooding characteristics.

Historical records indicate *P. streckersoni* sp. nov**.** occurred throughout the mainstem Brazos River and most of its tributaries; however, recent survey efforts depict that it is likely extirpated from much of its historical range (Fig. 2.6). Two isolated populations may still be extant north of current impoundments coinciding with river segments

between Lake Granbury and Lake Whitney, and north of Possum Kingdom Reservoir. Additional mussel surveys in these areas, along with evaluation with fine-scale genomic markers (e.g., microsatellites, GBS, etc.), are needed to determine population dynamics and possible designation of these populations as ESUs for future conservation and management efforts.

Discussion

Supraspecific Relationships in Lampsilini

Our data support that evolutionary relationships in Lampsilini have largely been shaped by life history characters, as we see a strong correlation between host fish use, host infection strategies, and phylogenetic placement. More specifically, our analyses resolved a monophyletic group consisting of *Ellipsaria*, *Leptodea*, *Potamilus*, and *Truncilla*. In general, these four genera are linked by two synapomorphic characters unique to Lampsilini: being host specialists, with glochidia only transforming on freshwater drum, *Aplodinotus grunniens* Rafinesque, 1819; and the growth of glochidia during encapsulation (i.e., while attached to host) (Barnhart et al., 2008; Roe, Simons, & Hartfield, 1997; Sietman et al., 2018; Williams et al., 2008). Despite strong behavioral and morphological characters supporting the monophyly of this group, BI and ML reconstructions depict incongruence regarding relationships between species in these genera, primarily regarding the placement of species in *Leptodea* and *Potamilus* (Figs. 2.1 & S2.2; APPENDIX). More specifically, the phylogenetic placement of *L. ochracea*, *P. capax*, and *P. inflatus* is incongruent between the BI and ML phylogenies. The generic placement of *L. ochracea* has been questioned due to significant morphological

divergence from remaining species of *Leptodea* (Davis & Fuller, 1981; Johnson, 1970; Smith, 2000; Stiven & Alderman, 1992); and furthermore, the use of *A. grunniens* as a host is not possible considering their ranges do not overlap (Johnson, 1970; Page & Burr, 2011). In the BI topology, *L. ochracea* was resolved sister to *Ellipsaria* and *Truncilla* with relatively low posterior support, while ML resolved *L. ochracea* sister to *Potamilus* and the remaining species in *Leptodea*. We see similar patterns of incongruence in *Potamilus*, with *P. inflatus* resolved basal to a monophyletic clade of *L. fragilis*, *L. leptodon*, and remaining members of *Potamilus*, while *P. capax* is resolved sister to a monophyletic clade comprised of *L. fragilis* and *L. leptodon* in our ML reconstruction. However, the position of two species switch in BI topologies with *P. capax* resolved basal and *P. inflatus* resolved sister to *L. fragilis* and *L. leptodon*. To test these incongruences, we implemented an AU test and results indicated no significant differences between BI and ML reconstructions ($\alpha = 0.4831$), likely due to weak nodal support (i.e., BS/PP) for phylogenetic relationships between *Leptodea* and *Potamilus* species.

Our study represents the first robust phylogenetic evaluation of *Leptodea* and *Potamilus* with comprehensive taxon sampling and evaluation of both mtDNA and nDNA loci. Despite employing multiple independently evolving markers used in recent freshwater mussel phylogenetic studies (Johnson et al., 2018; Lopes-Lima et al., 2017; Perkins et al., 2017; Pfeiffer et al., 2016; Pfeiffer, Sharpe, Johnson, Emery, & Page, 2018; Pieri et al., 2018; Smith et al., 2018), we could not resolve topologies that strongly support phylogenetic relationships between *Leptodea* and *Potamilus*. Therefore, we take a precautionary approach by not making any conclusions regarding generic-level

assignments at this time. However, our evaluation and comprehensive taxon sampling provides a baseline for future hypotheses regarding phylogenetic relationships of lampsiline genera. We believe that future investigations focusing on glochidial morphology and next-generation sequencing technologies targeting conserved but phylogenetically informative loci (Faircloth et al., 2012; Lemmon, Emme, & Lemmon, 2012) will be necessary to elucidate supra-specific relationships and move forward with any generic-level taxonomic revisions.

Species Boundaries in the Potamilus ohiensis Species Complex

Based on previous taxonomic accounts, *P. ohiensis* is assumed to occur in the Mississippi River drainage with disjunct populations in the Brazos River (Howells et al., 1996). This distributional pattern is thought to be a result of historical stream capture events, as seen in other freshwater fish and mussel species (Haag, Warren, Wright, & Shaffer, 2002; Hubbs, Edwards, & Garrett, 1991; Smith et al., 2018). However, the results of our phylogenetic and phylogeographic analyses resolve *P. streckersoni* sp. nov. closely related to *P. amphichaenus*, rather than a conspecific of *P. ohiensis* from the Interior Basin. Results also depict clear genetic separation between *P. amphichaenus* and *P. streckersoni* sp. nov., and no evidence for the two species existing in sympatry in the Trinity River drainage. These findings are similar to other faunal relationships in the western Gulf of Mexico drainages, given the high levels of endemism across these drainages (Haag & Williams, 2014; Howells et al., 1996; Hubbs, 1957; Hubbs et al., 1991; Strecker, 1931).

Allopatry is known as the driving force in many speciation processes (Mayr, 1942, 1963) and many riverine speciation events are indicative of extended periods of

genetic isolation (Jordan, 1905; Mayr, 1959), including diversification of freshwater mussels (Inoue, McQueen, Harris, & Berg, 2014; Johnson et al., 2018; Smith et al., 2018). However, resolving speciation processes from patterns of genetic drift via metapopulation structure continues to confound modern systematic research (De Queiroz, 2007; Leaché, Zhu, Rannala, & Yang, 2019; Sukumaran & Knowles, 2017). In the case of *P. streckersoni* sp. nov., if allopatric population structure was responsible for divergence, we would expect to see similar patterns of divergence between populations of *P. amphichaenus* (i.e., Sabine, Neches, and Trinity drainages). However, we see limited levels of divergence in *P. amphichaenus* populations and haplotype sharing in peripheral populations (Table 2.2; Figs. 2.3.1 & 2.3.2). Phylogeographic analyses suggest an extended period of allopatry of *P. streckersoni* sp. nov. from all populations of *P. amphichaenus*. Genetic distances between the two entities are similar to or greater than patterns of species-level diversity in other unionids (Inoue et al., 2014a; Jones, Neves, Ahlstedt, & Hallerman, 2006; Pfeiffer et al., 2016; Pieri et al., 2018; Roe & Lydeard, 1998), and haplotype networks depicting clear molecular separation between *P. streckersoni* sp. nov. and *P. amphichaenus* with no haplotype sharing at either mtDNA markers (Figs. 2.3.1 & 2.3.2). We also see a clear gap between intra- and interspecific genetic distance (APPENDIX; Figs. S2.3.1 & S2.3.2), indicative of a long period of genetic isolation.

To further investigate species boundaries in the *P. ohiensis* species complex, we employed two coalescent-based species delimitation models: STACEY and *BEAST2. STACEY resolved four strongly supported species clusters without *a priori* designation as the most likely species model: *P. amphichaenus* from the Sabine and Neches

drainages, *P. amphichaenus* from the Trinity drainage, *P. ohiensis*, and *P. streckersoni* sp. nov. (Fig. 2.4). However, there was not decisive support based on the probability of the model; therefore, we implemented *BEAST2 to test the marginal likelihood of the three most likely species scenarios identified by STACEY. *BEAST2 analyses depicted significant support for the recognition of four species clusters in the *P. ohiensis* species complex; however, models could not find significant support for a consensus designation of the two clusters recognized within *P. amphichaenus* (Table 2.3). Species Model 1 recognized *P. amphichaenus* from the Sabine and Neches, and *P. amphichaenus* from the Trinity as distinct species, which reconstructs a similar biogeographic pattern recovered in a recent assessment of species-level diversity in another group of unionids (Pieri et al., 2018). Despite this congruence with a previous study, Species Model 1 was only found marginally better than Species Model 3 (Table 2.3), which groups peripheral populations of *P. amphichaenus* as a species cluster (Sabine and Trinity). These results are likely due to haplotype sharing and lack of monophyly between the peripheral populations of *P. amphichaenus* (i.e., Sabine and Trinity drainages) at CO1, indicative of limited divergence time and the possibility of ongoing gene flow (Fig. 2.3.1). Furthermore, coalescent-based approaches have been repeatably criticized for delimiting population structure rather than species (Leaché et al., 2019; Sukumaran & Knowles, 2017), and have been shown to inflate estimates of biodiversity in freshwater mussels (Pfeiffer et al., 2016; Smith et al., 2018). We believe that STACEY and *BEAST analyses overestimate the biodiversity in *P. amphichaenus* and agree with previous research that when used alone, coalescent-based species delimitation models may be insufficient for taxonomic evaluations (Fujita et al., 2012; Leaché et al., 2019).

Similar to molecular evidence, we see strong morphological divergence between members of the *P. ohiensis* species complex. MANOVAs of traditional and Fourier shape morphometrics depicted significant divergence between *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni* sp. nov. (Table 2.4). We did observe slight overlap between *P. amphichaenus* and *P. streckersoni* sp. nov.; however, DFAs for both traditional and Fourier shape morphometrics were able to assign *P. streckersoni* sp. nov. correctly from other members of *P. ohiensis* species complex 90% and 92.5% of the time, respectively. These values are similar to or higher than studies utilizing similar morphological analyses to resolve species boundaries in freshwater mussels (Gangloff, Williams, & Feminella, 2006; Inoue et al., 2014; Johnson et al., 2018; Pieri et al., 2018), indicative of significant morphological divergence of *P. streckersoni* sp. nov. from *P. amphichaenus*. However, our morphological dataset does have several weaknesses. Morphological characteristics, especially external shell morphology in unionids, can be the result of environmental variables (Eagar, 1950; Ortmann, 1920). Furthermore, our sample sizes are low when compared to other species-delimitation studies incorporating morphological data (Inoue et al., 2014; Johnson et al., 2018; Pieri et al., 2018; Smith et al., 2018); especially for *P. ohiensis*, a wide-ranging species that likely depicts high levels of morphological plasticity throughout its range. Despite this, molecular data clearly depicts that *P. ohiensis* is divergent from other members of the species complex; therefore, we focused interpretation of our morphological assessment on species delimitation between *P. amphichaenus* and *P. streckersoni* sp. nov.

Inference from our integrative taxonomic assessment provides significant support for the recognition of a new species, *P. streckersoni* sp. nov. and we see clear separation

of three well-supported taxonomic entities in the *P. ohiensis* species complex: *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni* sp. nov. These three lineages exhibit clear divergence at mtDNA markers (Table 2.2; Figs. 2.3.1 & 2.3.2), depict significant differences in shell shape (Table 2.4; Fig. 2.5), and are geographically diagnosable. Considering the congruence across molecular, morphological, and geographic data, we have formally described *P. streckersoni* sp. nov.

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Figures

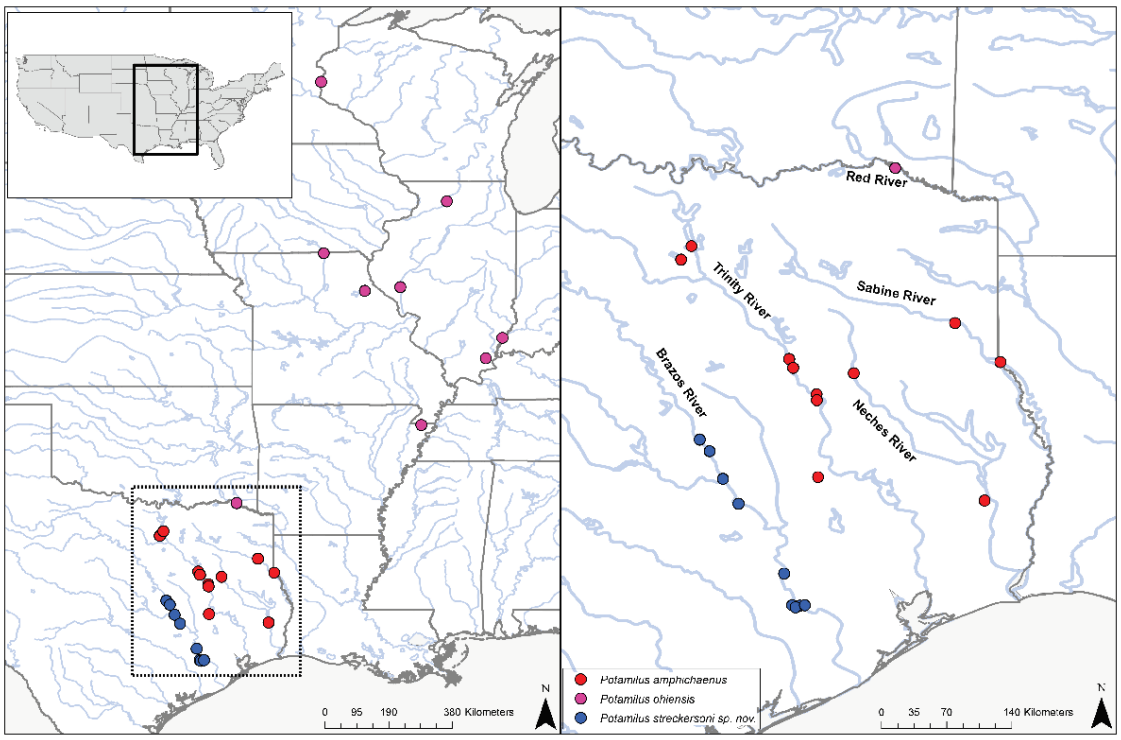

Figure 2.1. Collection localities for specimens in the *Potamilus ohiensis* species complex used in this study. Colors correspond to the species in the complex: *P. amphichaenus* (Sabine, Neches, and Trinity River drainages), *P. ohiensis* (Mississippi River drainage), and *P. streckersoni* sp. nov (Brazos River drainage).

Figure 2.2. Bayesian inference topology reconstructed using MrBayes on a concatenated molecular matrix (CO1, ND1, ITS1, 28S). Values above branches represent posterior probabilities (PP). Strongly supported nodes (i.e., PP ≥ 95) are indicated by asterisks.

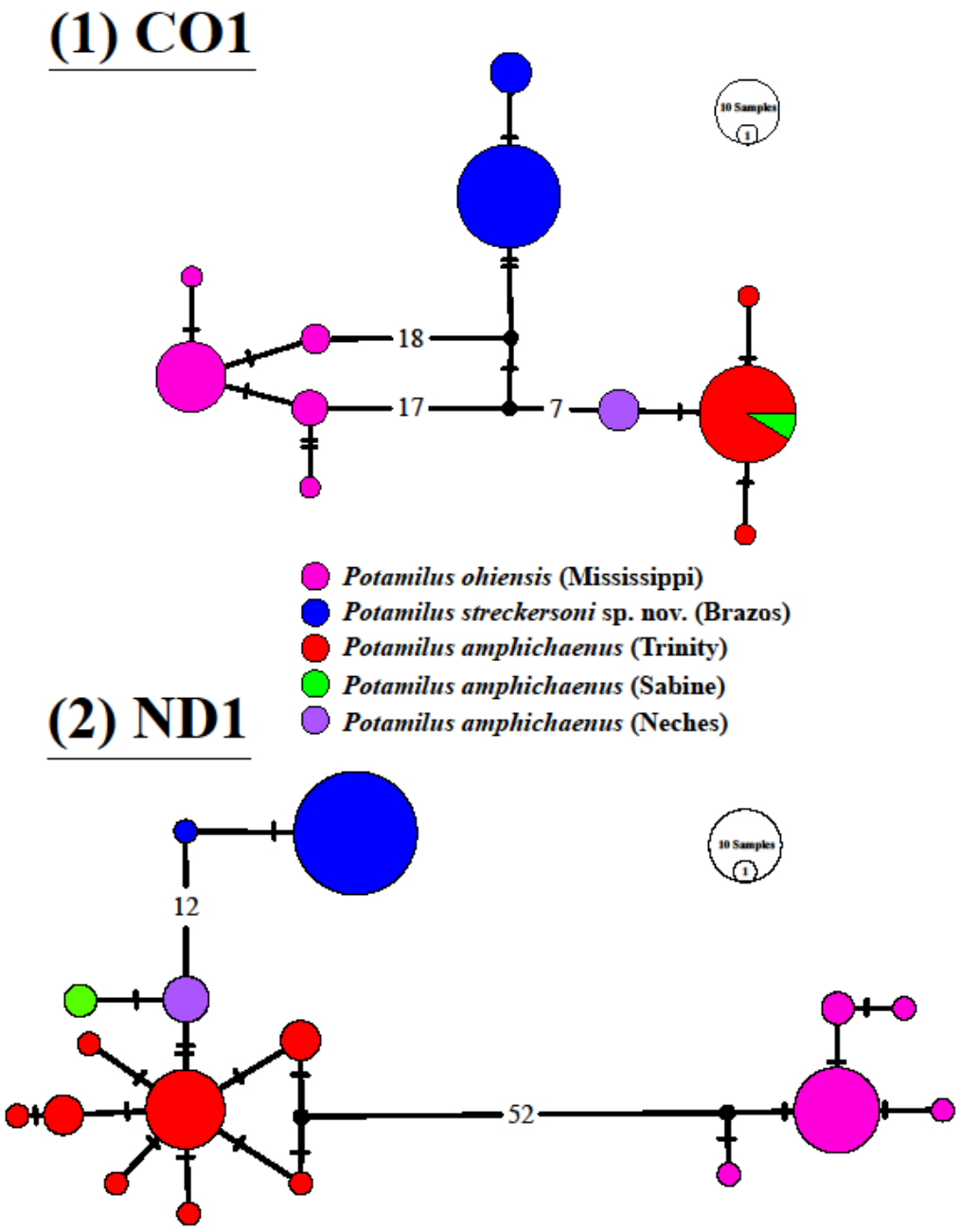

Figure 2.3. Haplotype networks based on CO1 (2.3.1) and ND1 (2.3.2) from individuals in the *Potamilus ohiensis* species complex. Each circle represents a unique haplotype with size relative to the number of individuals with each haplotype. Black circles represent unsampled haplotypes and individual tick marks or numbers indicate nucleotide substitutions between haplotypes.

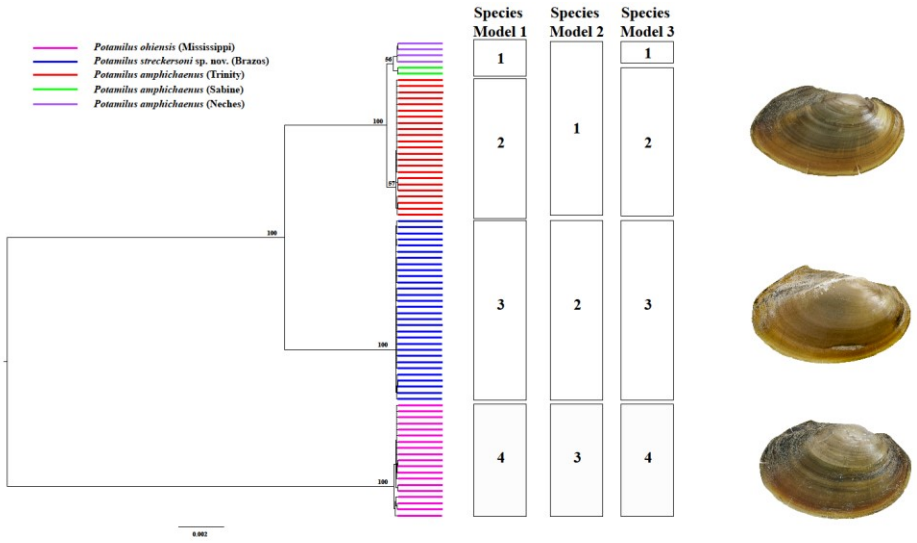

Figure 2.4. Inference from coalescent-based species delimitation models. The phylogeny represents the topology resolved by STACEY with posterior probabilities (PP) presented above nodes for each clade of interest. Each line represents an individual sampled and colors correspond to species and drainage of capture. Species models implemented in *BEAST2 are shown to the right, along with photographs of *Potamilus amphichaenus*, *Potamilus ohiensis*, and *Potamilus streckersoni* sp. nov.

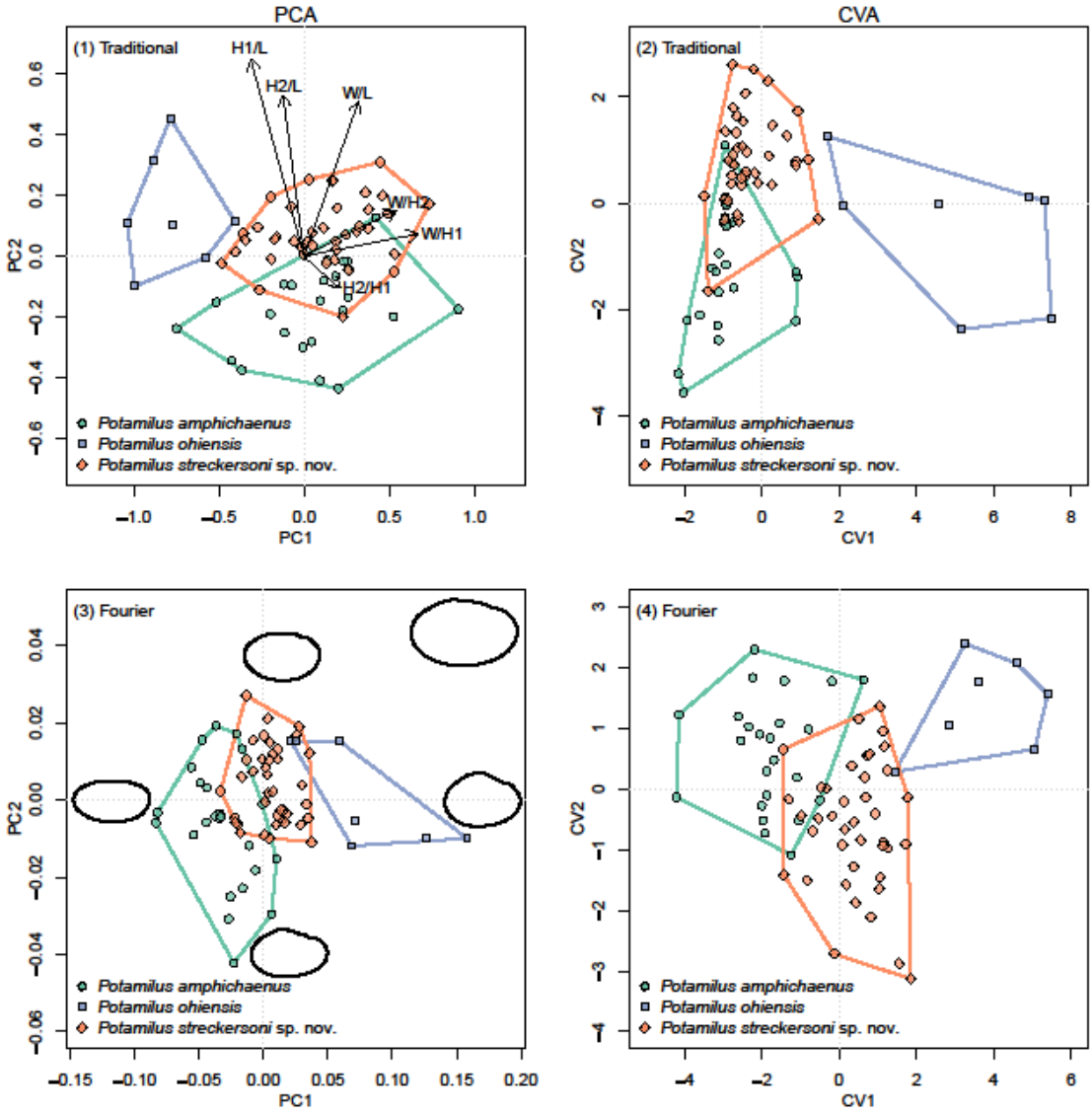

Figure 2.5. Scatter plots from principal component analysis (PCA) and canonical variate analysis (CVA) of traditional (2.5.1, 2.5.2) and Fourier (2.5.3, 2.5.4) morphometrics. Colors and shapes of points correspond to putative species (green = *Potamilus amphichaenus*, blue = *Potamilus ohiensis*, orange = *Potamilus streckersoni* sp. nov.). Polygons enclose convex hulls of each species. Biplots of variables from traditional morphometrics (2.5.1) are shown in arrows. Outlined shell shapes from Fourier morphometrics (2.5.3) represent a mean shape (top-right) and ±2 × SD on PC1 and PC2 axes.

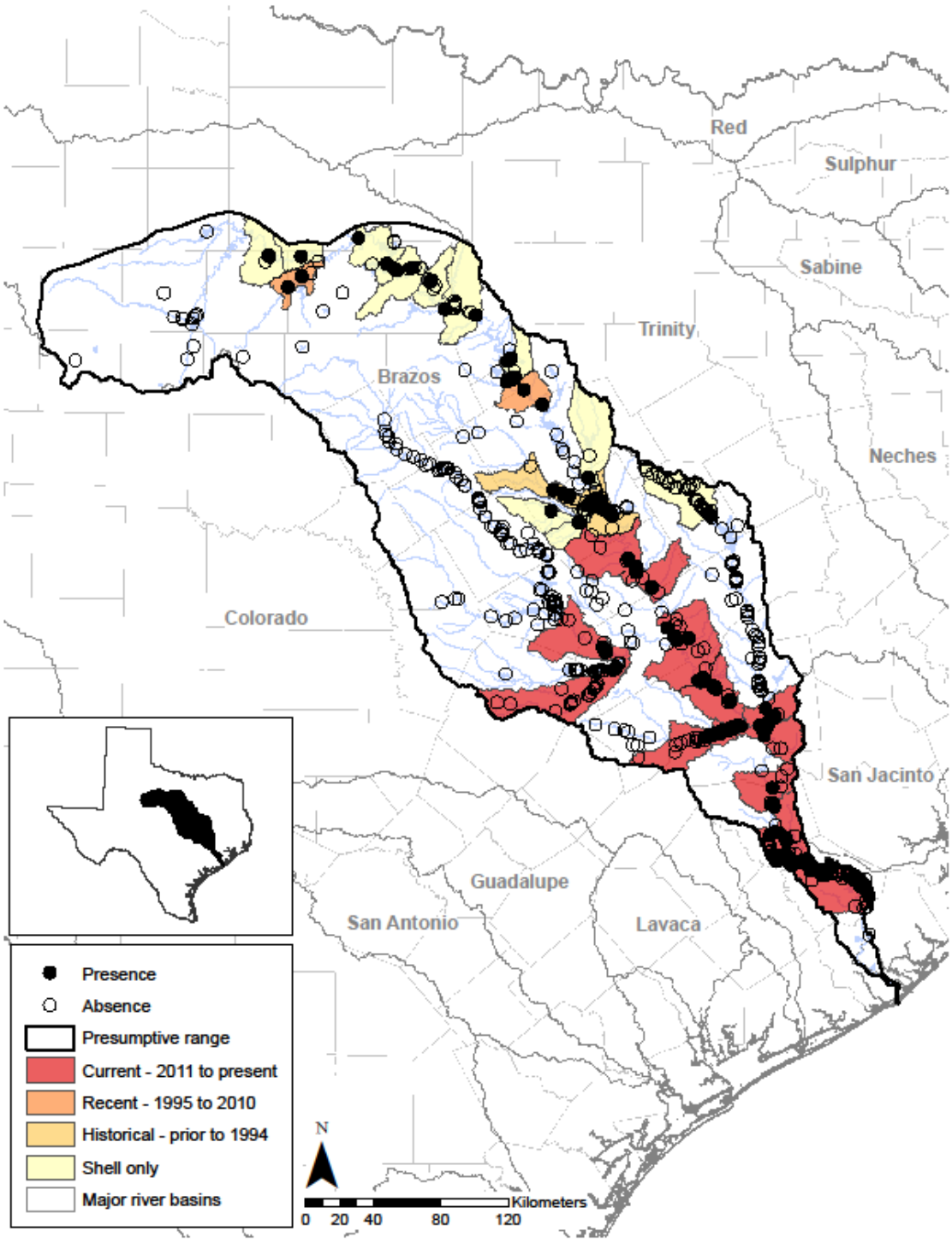

Figure 2.6. Conservation status map for *Potamilus streckersoni* sp. nov. (Brazos Heelsplitter). Shaded circles denote presence and unshaded circles indicate absence. Hydrologic Unit Codes (HUC) 10-level are colored based on live versus shell. For the former, HUCs are further shaded by when a live specimen of *P. streckersoni* sp. nov. was collected. Solid black line denotes the presumptive range.

Figure 2.7. *Potamilus streckersoni* sp. nov. holotype (UF439497).

Tables

Table 2.1. Summary statistics for genetic diversity within *Potamilus amphichaenus*, *Potamilus ohiensis*, and *Potamilus streckersoni* sp. nov., including number of unique haplotypes (h), haplotype diversity (Hd), average number of nucleotide differences (k), and nucleotide diversity (π) for CO1 and ND1.

Taxa (Drainage; Sample Size)	CO1				ND1			
	h	Hd	k	π	h	Hd	k	π
P. amphichaenus (Sabine; n = 2)	1	0	0	0	1	0	0	0
P. amphichaenus (Neches; n = 4)	1	0	0	0	1	0	0	0
P. amphichaenus (Trinity; n = 23)	3	0.17	0.17391	0.00027	8	0.715	0.97233	0.00110
P. ohiensis (Mississippi; n = 19)	5	0.591	0.86550	0.00166	5	0.462	0.70175	0.00084
P. streckersoni sp. nov. (Brazos; n = 30)	2	0.239	0.23908	0.00043	2	0.067	0.06667	0.00008

Table 2.2. Mean intra- and interspecific genetic uncorrected p-distance values for *Potamilus amphichaenus*, *Potamilus ohiensis*, and *Potamilus streckersoni* sp. nov. CO1 values are represented in the lower triangle and ND1 in the upper triangle.

Taxa (Drainage; Sample Size)	1	2	3	4	5	Within Group CO1	Within Group ND1
1. *P. amphichaenus* Sabine; n = 2)		0.11	0.50	7.33	1.88	0	0
2. *P. amphichaenus* (Neches; n = 4)	0.16		0.40	7.17	1.74	0	0
3. *P. amphichaenus* (Trinity; n = 23)	0.02	0.17		7.12	1.93	0.03	0.11
4. *P. ohiensis* (Mississippi; n = 19)	5.08	4.87	5.04		7.34	0.13	0.09
5. *P. streckersoni* sp. nov. (Brazos; n = 30)	2.03	1.87	2.02	4.10		0.04	0.01

Table 2.3. Species models implemented in *BEAST2 following results from most likely species clusters in STACEY analyses. Values in bold font represent Bayes factors that are significantly worse than the best model.

Species Model	STACEY Probability	*BEAST2 *ln*	2*ln*BF	Reject
1	27.2%	-2898.01	-	-
2	21.2%	-2915.19	34.37	Yes
3	12.5%	-2899.09	2.17	No

Table 2.4. Significance values (α) for pairwise comparisons of morphometric analyses with traditional morphometric values represented in the lower triangle and Fourier shape morphometrics represented in the upper triangle, along with the percentage of individuals binned accurately by discriminant function analyses (DFA) for traditional and Fourier shape morphometrics.

Taxa	1	2	3	Traditional DFA	Fourier DFA
1. P. amphichaenus		2.08E-08	1.71E-09	83.3%	87.5%
2. P. ohiensis	4.73E-09		9.15E-08	71%	85.7%
3. P. streckersoni sp. nov.	3.00E-07	4.29E-12		90.0%	92.5%

CHAPTER THREE

Comparative Phylogenomics Reveal Complex Evolution of Life History Strategies in a Clade of Bivalves with Parasitic Larvae (Bivalvia: Unionoida: Ambleminae)

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Abstract

Freshwater mussels are a species-rich group with biodiversity patterns strongly shaped by a life history strategy that includes an obligate parasitic larval stage. In this study, we set out to reconstruct the life history evolution and systematics in a clade of mussels adapted to parasitizing a molluscivorous host fish. Anchored hybrid enrichment and ancestral character reconstruction revealed a complex pattern of life history evolution with host switching and multiple instances of convergence, including reduction in size of larvae, increased fecundity, and growth during encapsulation to increase survival post-metamorphosis. Our phylogenomic analyses also recovered non-monophyly of taxa exhibiting multiple traits used as the basis for previous taxonomic hypotheses. Taxa with axe-head shaped glochidia were resolved as paraphyletic, but our results strongly suggest the complex morphology is an adaptation to reduce size, with larval reduction further accentuated in taxa previously assigned to *Leptodea*. To more accurately reflect the evolutionary history of this group, we make multiple systematic changes, including the description of a new genus, *Atlanticoncha* gen. nov., and the synonymy of the genus *Leptodea* under *Potamilus*. Our findings contribute to the growing body of

literature showing that morphological characters, including larval morphology, can be misleading for cladistics in mussels.

Introduction

Understanding processes that cause shifts in life history strategies are of the upmost importance in evolutionary biology as they directly impact fitness and promote biological diversification. Life histories are shaped by ecological interactions (Stearns, 2000), and coevolutionary processes that drive specialized life history adaptations may be amplified in parasites and their hosts (Buckling and Rainey, 2002; Thompson, 2005; Scanlan *et al.*, 2015; Laanto *et al.*, 2017). Phylogenetic methods have emerged as powerful tools to better understand life history evolution (e.g., Martinsen *et al.*, 2008; Li *et al.*, 2018), and of particular promise for deeper-level phylogenetic studies are hybrid enrichment methods, which can produce hundreds to thousands of orthologous markers with relative ease (Faircloth *et al.*, 2012; Lemmon *et al.*, 2012; Lemmon and Lemmon, 2013). Using robust phylogenomic methods, we set out to reconstruct the evolution of life history traits and refine taxonomy in a clade of bivalves that have a highly specialized suite of characters ostensibly adapted to infecting its molluscivorous host.

Freshwater mussels (hereafter mussels) of the subfamily Ambleminae represent the most diverse subfamily in the bivalve order Unionoida with over 340 species (Graf and Cummings, 2007). Like nearly all mussels, amblemines are obligate parasites that require temporary larval attachment to freshwater vertebrates (primarily fishes) to complete metamorphosis to a free-living juvenile (Barnhart *et al.*, 2008). Many amblemines have evolved narrow, specialized patterns of host use, including reliance on one or more host fishes to complete their life cycle, and the radiation of the group has

been influenced in part by the partitioning of a diverse, sympatric host fish community resource (Haag, 2012). Highly specific patterns of host use are often associated with equally specialized host infection strategies, including elaborate conglutinates and mantle margins that mimic host prey items (e.g., insect larvae, fish, worms, snails, crayfish), denticular shell margins to capture hosts, and maternal sacrifice, where brooding females are hypothesized to behave in a manner that increases predation or attempted predation by molluscivorous fishes (Barnhart *et al.*, 2008; Haag, 2012). Morphological and behavioral adaptations for specialized parasitism have largely shaped the diversity of Ambleminae, and robust evaluations of life history characteristics have been integral to understanding the ecology and evolution of this group (Haag and Warren, 1999; Haag and Staton, 2003; Campbell *et al.*, 2005; Graf and Cummings, 2006; Zanatta and Murphy, 2006; Sietman *et al.*, 2012, 2018; Haag, 2013; Hewitt *et al.*, 2019; Pfeiffer *et al.*, 2019a, 2019b; Smith *et al.*, 2019).

Many life history traits of mussels are phylogenetically conserved and therefore useful in identifying clades with distinct evolutionary trajectories (Graf and Cummings, 2006; Pfeiffer and Graf, 2015; Hewitt *et al.*, 2019; Pfeiffer *et al.*, 2019b). One such clade is characterized by specialization on parasitizing *Aplodinotus grunniens*, a common molluscivorous fish distributed throughout Gulf of Mexico drainages (Page and Burr, 2011; Haag, 2012). This clade consists of the genera *Ellipsaria*, *Leptodea*, *Potamilus*, and *Truncilla* (collectively called the *A. grunniens* specialists) and appears to have evolved several distinct life history traits that are unlike most other representatives of the Ambleminae, including axe-head shaped glochidia (*Potamilus*), miniaturized glochidia (<100 µm; *Leptodea* and *Truncilla*), high fecundity (>500,000; *Leptodea*, *Potamilus*, and

Truncilla), larval growth during encystment (*Leptodea*, *Potamilus,* and *Truncilla*), and potential use of maternal sacrifice for host infection (*Leptodea* and *Truncilla*; reviewed by Barnhart et al. 2008; Haag 2012).

Life history strategies in this group are generally well studied, but many questions regarding the evolution of these taxa and traits remain unanswered. Previous phylogenetic reconstructions have consistently failed to recover the monophyly of taxa with specialized parasitization of *A. grunniens* (Campbell *et al.*, 2005; Zanatta and Murphy, 2006; Pfeiffer *et al.*, 2018; Smith *et al.*, 2019). Specifically, the Atlantic coast endemic *L. ochracea*, which does not use *A. grunniens* as a host, was nested within a clade otherwise restricted to *A. grunniens* host use (Johnson, 1970; Kneeland and Rhymer, 2008; Smith *et al.*, 2019). Furthermore, no study has recovered the monophyly of two striking life history adaptations despite their morphological cohesiveness: axe-head shaped glochidia and miniaturized glochidia (Roe and Lydeard 1998; Campbell et al. 2005; Zanatta and Murphy 2006; Smith et al. 2019). The recovered non-monophyly of these traits (i.e., host use, axe-head shaped glochidia, miniaturized glochidia) suggests a complex pattern of life history evolution, possibly including trait reversal, adaptive convergence, and host switching, emphasizing the need for robust phylogenetic evaluation.

We reconstructed the origin and patterns of life history diversification within *A. grunniens* specialists using a recently developed mussel-specific anchored hybrid enrichment (AHE) probe set (Pfeiffer *et al.*, 2019b) and ancestral character reconstruction (ACR). Specifically, we set out to accomplish the following: 1) estimate a phylogeny of Ambleminae with a focus on *A. grunniens* specialists using multiple AHE datasets; 2)

reconstruct the evolution of *A. grunniens* specialization and associated life history traits; 3) identify genome-wide signatures of selection associated with the diversification of life history traits; and 4) make taxonomic revisions to accurately reflect the evolutionary history of *A. grunniens* specialists.

Materials and Methods

Sampling Design and AHE Data Generation

We sampled representative individuals from all members of the genera *Ellipsaria*, *Leptodea*, *Potamilus*, and *Truncilla* (Table 3.1). We also used available AHE data for other members of Ambleminae to ensure the monophyly of ingroup genera and comparative analysis of life history traits. *Quadrula quadrula* was used to root the phylogeny of the Ambleminae following findings of previous research (Pfeiffer *et al.*, 2019b). Tissue samples were collected from live individuals or museum specimens, and information regarding taxon sampling, including catalog numbers and SRA accessions can be found in Table 3.1.

Genomic DNA was extracted using the PureGene DNA extraction kit following the standard extraction protocol (Gentra Systems, Inc., Minneapolis, MN, USA). High molecular weight was ensured by visualizing isolations on a 1% agarose gel stained with GelRed nucleic acid stain (Biotium, Hayward, CA, USA), and each isolation was quantified using PicoGreen®. After assurance of high molecular weight, we used the Unioverse probe set (Pfeiffer *et al.*, 2019b) to capture phylogenetically informative nuclear protein-coding loci. Sequencing libraries, capture, and Illumina sequencing were carried out at RAPiD Genomics (Gainesville, FL). Libraries were constructed by shearing

DNA to an average length of 400 bp followed by an end-repair reaction and ligation of anadenine residue to the 3'-end of each blunt-end fragment. Barcoded adapters were ligated to the library followed by PCR amplification of the libraries. Libraries were pooled into groups of up to 16 samples and the SureSelectxt Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library protocol was followed for solution-based target enrichment. The probes were synthesized as Custom SureSelect probes from AgilentTechnologies (Santa Clara, CA, USA). An Illumina HiSeq 3000 (San Diego, CA) was used to generate 150-bp, paired-end reads.

To clean the raw sequencing reads and assemble loci we used the AHE processing pipeline developed in Breinholt *et al.*, (2018). We used TRIM GALORE! v0.4.0 (www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and FastQC v 0.74 (www.bioinformatics.babraham.ac.uk/projects/fastqc/) in Galaxy (www.usegalaxy.org) to clean and ensure quality of reads. In TRIM GALORE!, Illumina data were filtered to a minimum read size of 30 nt and reads were quality trimmed (Phred score < 20). Individual loci were then assembled using an iterative bait assembly (IBA.py - Breinholt *et al.*, 2018), and the Unioverse reference sequences were used as baits (Pfeiffer *et al.*, 2019b). Briefly, the iterative bait assembly used USEARCH v 10.0.240 (Edgar, 2010) to select raw reads with high similarity to the probe region from the reference taxa alignment. The selected raw reads were then built into de novo assembled isoforms with Bridger v2014-12-01 (Chang *et al.*, 2015).

After de novo assembly, sequences were added to the Unioverse reference taxon alignment and were subsequently aligned using MAFFT v 7.245 (Katoh and Standley, 2013) with the options "-addlong" and "adjustdirectionaccurately." To separate exonic

and hypervariable flanking regions, individual loci were split into three parts using the script extract_probe_region.py (Breinholt *et al.*, 2018). The probe region (exonic region) was identified using sequences from reference taxa in the alignment, and the reads anterior and posterior to the probe region were split (head and tail region, respectively).

To ensure gene orthology, we used the ortholog_filter.py (Breinholt *et al.*, 2018) to select single hit sequences that mapped to the same location on the *Bathymodiolus platifrons* genome and reference sequence. Individual alignments for each locus were created using split.py (Breinholt *et al.*, 2018) and subsequently aligned with MAFFT. FASconCAT-G v 1.04 (Kück and Longo, 2014) was used to turn isoforms created by Bridger into a single consensus sequence for each independent locus. We used the script remove_duplicates.py (Breinholt *et al.*, 2018) to discard loci for each taxon that had more than one sequence.

AHE Datasets

We created four molecular supermatrices to reconstruct phylogenetic relationships:

Dataset 1- Probe Region

All sequences that passed the AHE pipeline and full-length assemblies (i.e., header, probe, and tail) were collected for each locus and subsequently aligned to reference sequences in MAFFT. Individual loci were split using extract_probe_region.py and only the probe region was retained. Probe region sequences were realigned using MACSE v 2.03 (Ranwez and Douzery, 2018) to better account for frame shifts. Individual locus alignments were visually inspected in AliView v 1.25 (Larsson, 2014) to ensure open reading frame and incomplete codons at each terminal end were removed. If stop codons

were present, alignments were trimmed to ensure open reading frames. We included loci that had a minimum of 70% AHE gene occupancy across our molecular matrix and were parsimony-informative. All probe regions were concatenated into a supermatrix using FASconCAT-G.

Dataset 2- Flanking Regions Only

Aligning and analyzing hypervariable flanking regions can be challenging due to the presence of transposable elements, abundance of indels, and variable locations of exons across genomes. We followed Breinholt *et al*., (2018) to remove areas in the flanking regions that were problematic. First, reads that passed the pipeline were aligned in MAFFT with the commands "–allowshift –unalignlevel 0.8 –reorder –leavegappyregion" to produce a global alignment. We used alignment_DE_trim.py and flank_dropper.py (Breinholt *et al.*, 2018) to trim and filter out problematic flanking sequences before splitting the loci into three parts using extract_probe_region.py: the header region, probe region, and tail region. Head and tail regions consisting of less than 30 nt or lacking parsimony informative sites were deleted before remaining head and tail regions were concatenated into a supermatrix using FASconCAT-G.

Dataset 3 and 4 - Probe + Flanking Regions

We created two datasets that used both probe and flanking regions to test whether there were significant differences between how loci were partitioned. For dataset 3, we combined the probe, header, and tail alignments into a supermatrix using FASconCAT-G. Loci with no parsimony-informative sites were removed, and coding probe and flanking regions were treated as separate partitions. For Dataset 4, we create a data matrix with both probe and flanking loci as one partition using the same 626 probe loci that passed

the pipeline. We followed the same methods as dataset 2 to trim problematic regions. Loci with no parsimony-informative sites were removed, and loci were then concatenated into a supermatrix using FASconCAT-G.

Phylogenomic Analyses

Phylogenomic analyses using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses were performed in PAUP* v 4.0a165 (Swofford, 2003), IQ-TREE v 1.6.11 (Nguyen *et al.*, 2015; Chernomor *et al.*, 2016), and MrBayes v 3.2.6 (Ronquist *et al.*, 2012), respectively. Partitions and substitution models for PAUP*, IQ-TREE, and MrBayes were determined by PartitionFinder v 2.1.1 (Lanfear *et al.*, 2016) using the rcluster search method (rcluster = 10%; rcluster-max = 100*Number of Loci) to find the best GTR+G partitioning scheme (Lanfear *et al.*, 2014; Stamatakis, 2014). PAUP* analyses were performed using heuristic searches with 100 random sequence addition replicates conducted with tree-bisection-reconnection branch-swapping, and 1000 bootstrap replicates (BS). IQ-TREE analyses conducted 10 independent runs of an initial tree search and 1000 ultrafast bootstrap replicates (ufBS) for nodal support (Hoang *et al.*, 2018). MrBayes analyses executed 4 runs of 4 chains for 10^7 MCMC generations sampling every 250 generations. Log likelihood scores for each sampling point were analyzed using Tracer v 1.7.1 (Rambaut *et al.*, 2018) to determine an appropriate burn-in value. Chains were considered stationary when the log likelihood values reached a plateau. Convergence of the four independent runs was monitored using the Potential Scale Reduction Factor (PSRF) of each parameter and the average standard deviation of split frequencies (ASDSF). Strongly supported nodes are represented by BS greater than or equal to 95, and ufBS and PP equal to 100.

We enforced multiple topological constraint analyses under MP, ML, and BI to test the monophyly of taxa exhibiting four traits: 1) *A. grunniens* specialization (*Ellipsaria*, *L. fragilis*, *L. leptodon*, *Potamilus*, and *Truncilla*), 2) axe-head shaped glochidia (*Potamilus*), 3) miniaturized glochidia (*L. fragilis*, *L. leptodon*, and *Truncilla*), and 4) larval growth during encystment (*L. fragilis*, *L. leptodon*, *Potamilus,* and *Truncilla*). Templeton (Templeton, 1983) and winning-sites tests (Prager and Wilson, 1988) were used to test if topological constraint topologies were significantly worse than the optimal in PAUP*. An AU test (Shimodaira, 2002) implementing 10,000 RELL (Kishino *et al.*, 1990) replicates was used to test if the topological constraint reconstructions in IQ-TREE significantly differed from the optimal topology. A significance level of α=0.05 was used when assessing the statistical significance between topologies. For BI analyses, we used Bayes factors to determine the marginal likelihood difference between the topology tests and the optimal reconstruction. Bayes factors were measured using twice the difference of $-ln$ likelihood from harmonic mean estimations in MrBayes with $2ln\text{BF} > 10$ depicting significant support (Kass and Raftery, 1995).

We also applied a coalescent-based species tree approach on each of our datasets using ASTRAL-III v 5.6.3 (Zhang *et al.*, 2018). We generated partitioned ML trees for each individual locus using IQ-TREE and measured nodal support with 1000 ufBS. ModelFinder (Kalyaanamoorthy *et al.*, 2017) was used to find the best available nucleotide substitution model for each locus. Bipartitions with <10 ufBS were removed using Newick Utilities v 1.6 (Junier and Zdobnov, 2010) prior to species tree estimation as it has been shown to improve species tree accuracy (Zhang *et al.*, 2018). We used the

option t -1 for nodal support values which is based on the percentage of trees that agree with a branch.

Tests for Positive Selection

We conducted tests for episodic selection using the FUBAR and aBSREL algorithms (Murrell *et al.*, 2013; Smith *et al.*, 2015) in HyPhy (Pond *et al.*, 2005) using the default parameters. The FUBAR algorithm was employed to detect loci and sites that depicted significant evidence of diversifying selection (Murrell *et al.*, 2013). We used FUBAR to identify sites within loci that showed significant evidence of positive selection and only implemented these loci in downstream analyses to reduce background noise and computational demand. To investigate selective pressures on specific branches within the phylogeny, we implemented the aBSREL algorithm. We tested for significant evidence of selective pressures on the branch and foreground coinciding with the origin of *A. grunniens* host fish specialization (i.e., MRCA of *Ellipsaria*, *Leptodea*, *Potamilus*, and *Truncilla*). For the aBSREL analysis, we used default parameters and the IQ-TREE phylogenetic reconstruction for dataset 1 as the topological prior. We used a likelihood ratio test to test for significant evidence of diversifying selection with a significance level of $\alpha=0.05$.

Scanning Electron Microscopy

Micrographs of glochidia for taxa in the *A. grunniens* specialists group were gathered from previous published literature (Cummings *et al.*, 1990; Hoggarth, 1999; Hove *et al.*, 2012; Sietman *et al.*, 2018) or generated herein. For micrographs generated in this study, glochidia were removed from the marsupia and preserved in 70% EtOH.

Glochidia were air dried and placed on a stub for examination on the SEM**.** After mounting, samples were sputter-coated with 20 nm of iridium and subsequently imaged using a Versa 3D SEM (FEI Company, Hillsboro, OR, USA) at 5 kV and spot size 3 under high vacuum. Individual glochidia from each species were measured and unique morphological features (e.g., size, shape, marginal appendages) were characterized. All visualization using SEM was performed at the Center for Microscopy and Imaging at Baylor University (Waco, TX).

Ancestral Character Reconstruction

We created a morphological data matrix of several life history characters for the taxa included in phylogenetic analyses by scoring three discrete characters and two continuous characters: host fish use, growth during encapsulation, axe-head shaped glochidia, larval surface area, and average annual fecundity. Information about these characters was extracted from micrographs generated in this study and from published literature (Surber, 1913; Howard, 1914; Howard and Anson, 1922; Howells *et al.*, 1996; Watters *et al.*, 1998; Kneeland and Rhymer, 2008; White *et al.*, 2008; Fritts *et al.*, 2012; Haag, 2012, 2013; Hart *et al.*, 2018; Sietman *et al.*, 2018). Life history information (i.e., host fish use and growth during encapsulation) was unavailable for *Pachynaias spheniopsis*, *P. streckersoni*, *Psoronaias semigranosa*, *T. cognata*, and *T. macrodon* and these taxa were not included in ACR. To compare size of glochidia, standardized measurements of surface area were performed on micrographs for available species using imageJ (Schneider *et al.*, 2012). To account for differences in shape, a scatter plot was created in the R package ggplot2 (Wickham, 2016) to show the distribution of larval surface area with respect to height. To augment fecundity data available in published

literature, we estimated fecundity for *Cyrtonaias tampicoensis* (n=2), *L. ochracea* (n=2), *P. alatus* (n=1), *P. inflatus* (n=3), and *P. streckersoni* (n=3) using a volumetric approach (Jones *et al.*, 2010; Ehlo and Layzer, 2014). Briefly, we estimated fecundity for *C. tampicoensis*, *L. ochracea*, *P. alatus*, and *P. inflatus* by suspending a homogenized solution of the entire larval contents of one marsupial gill in a total volume of 1 L and counting the number of glochidia in 10, 1-ml sub-samples. Methodologies were identical for *P. streckersoni* except 10 sub-samples of glochidia were counted from 50 µL aliquots. Fecundity was determined for all individuals by doubling the total number of glochidia estimated from the gill examined.

Ancestral character reconstructions (ACRs) were performed on the all five characters described above. A roughly time-calibrated tree was generated from the topological reconstruction for dataset 4 using the 'chronos' function in the package ape v 5.3 (Paradis and Schliep, 2018). We estimated the evolutionary history of host fish use, growth during encapsulation, and axe-head shaped glochidia using Bayesian stochastic character mapping (Huelsenbeck *et al.*, 2003; Bollback, 2006) and the ML re-rooting method (Yang *et al.*, 1995) in the package phytools v 0.6-60 (Revell, 2012). Bayesian stochastic character mapping was performed using the make.simmap command and used 1000 simulations. The ML re-rooting method was performed using the rerootingMethod command and the equal rate model. To estimate the evolutionary history of larval surface area and fecundity, we used the ML based contMap function in phytools. Taxa lacking larval samples and fecundity estimates were not included in the ACR analysis.

Results

AHE Datasets and Molecular Analyses

All novel AHE reads were made available on GenBank SRA database (BioProject PRJNA593235). Information regarding material and accession numbers used in this study can be found in Table 3.1. All tree files generated from phylogenetic analyses can be on ScienceBase (https://doi.org/10.5066/P9X3J54C). Datasets used in phylogenomic reconstructions consisted of 626 probe regions and 1,247 flanking regions. The number of loci, total concatenated length, percent missing data, average length per locus, and number of partitions used for phylogenetic analyses are reported in Table 3.2. Convergence of all unconstrained MrBayes analyses was supported by the average PSRF value of all parameters (1.0) and ASDSF (0). A 25% burn-in value was deemed appropriate for all analyses by Tracer. For each individual dataset, BI, ML, and MP topologies were completely concordant. BI, ML, and MP phylogenetic reconstructions for datasets 2-4, which incorporated flanking regions, showed strong support (PP/ufBS/BS = 100) for almost all supraspecific relationships and showed few differences based on partitioning scheme. The only minor topological difference between phylogenies produced using datasets 1-4 was the relationship between *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni*, and the sister group to *P. capax* and *P. inflatus*. In dataset 1, a clade consisting of *P. capax* and *P. inflatus* was resolved as sister to *L. fragilis*, *L. leptodon*, and the remainder of *Potamilus*, while in dataset 2-4 *P. capax* and *P. inflatus* were resolved as sister to a clade consisting of *P. amphichaenus*, *P. ohiensis,* and *P. streckersoni* (Fig. 3.1). *Aplodinotus grunniens* specialists (*Ellipsaria*, *L. fragilis*, *L. leptodon*, *Potamilus*, and *Truncilla*), and the taxa with axe-head shaped glochidia

(*Potamilus*), and miniaturized glochidia (*L. fragilis*, *L. leptodon*, and *Truncilla*) were recovered as non-monophyletic. All topological constraints forcing the monophyly of *A. grunniens* specialists, taxa bearing axe-head shaped and miniaturized glochidia, and taxa with larval growth during encystment resulted in topologies with likelihood values significantly worse than the optimal topology in BI, ML, and MP analyses for all datasets ($\alpha < 0.0001$; $2ln\text{BF} > 544$).

ASTRAL-III reconstructions showed complete concordance for almost all supraspecific relationships and showed little differentiation from BI, ML, and MP phylogenies. The ASTRAL-III reconstruction based on dataset 4 is presented in Figure 3.2. Dependent on the dataset used, minor topological differences were present due to phylogenetic relationships of *P. capax* and *P. inflatus*. ASTRAL-III analyses showed Datasets 1 and 4 resolved *P. capax* and *P. inflatus* as sister to *L. fragilis*, *L. leptodon*, and the remainder of *Potamilus*, concordant with BI, ML, and MP reconstructions on dataset 1 (Fig. 3.2). Datasets 2-3 did not resolve *P. capax* and *P. inflatus* as sister, but *P. capax* and *P. inflatus* were both independently resolved sister to *L. fragilis*, *L. leptodon*, and the remainder of *Potamilus*.

FUBAR identified 286 sites in 183 loci that showed significant evidence of diversifying selection and we used these loci to test for selective pressures on the branch and foreground coinciding to the MRCA of *A. grunniens* specialization using aBSREL. The aBSREL analysis depicted significant indication of selection ($\alpha < 0.05$) on nine branches that coincided to the following taxa: 1) *Ellipsaria*+*Truncilla*, 2) *Truncilla*, 3) *T. cognata*, 4) *L. ochracea*, 5) *L. fragilis*+*L. leptodon*+*Potamilus*, 6) *P. amphichaenus*+*P. ohiensis*+*P. streckersoni*, 7) *P. streckersoni*, 8) *L. fragilis*+*L. leptodon* and 9) *P.*

alatus+P. purpuratus (Fig. 3.3). The majority of branches identified by aBSREL were congruent with shifts in life history traits such as host use, fecundity, larval growth during encystment, and larval surface area (see below).

Larval Morphology

Glochidia were examined using SEM for all *A. grunniens* specialists except *P. metnecktayi, T. cognata*, and *T. macrodon*. We documented six distinct larval morphologies within *A. grunniens* specialists that are congruent with phylogenetic reconstructions: 1) large (height > 300 µm) fan-shaped glochidia (*E. lineolata*); 2) miniaturized (<100 µm) subelliptical glochidia (*L. fragilis*, *L. leptodon*, and *Truncilla*); 3) moderately large (250-300 µm) subelliptical glochidia (*L. ochracea*); 4) axe-head shaped glochidia (150-350 µm) with 2 lanceolate teeth on the ventral margin (*P. alatus*, *P. capax*, *P. purpuratus*); 5) small (100-150 µm) axe-head shaped glochidia with no lanceolate teeth (*P. amphichaenus*, *P. ohiensis*, and *P. streckersoni*); 6) moderately sized (200-250 µm) axe-head shaped glochidia with rows of 5-7 teeth along the ventral margin (*P. inflatus*; Fig. 3.3).

Ancestral Character Reconstruction

ACRs using Bayesian stochastic character mapping and ML re-rooting methodologies yielded congruent results regarding host fish use, growth during encapsulation, and larval morphology. ACR for host fish use in the sampled Ambleminae favored a single origin of *A. grunniens* specialization in the MRCA of *Ellipsaria+Leptodea+Potamilus+Truncilla* with a shift of host use in *L. ochracea* (PP>93; Fig. 3.4A). Growth during encapsulation was strongly supported to have evolved

three times in the sampled Ambleminae: MRCA of *L. fragilis*+*L. leptodon*+*Potamilus* (PP>99), *Quadrula* (PP = 100), and *Truncilla* (PP > 97; Fig. 3.4B). Axe-head shaped glochidia evolved once in the MRCA of *L. fragilis*+*L. leptodon*+*Potamilus* (PP>87) and was subsequently lost in *L. fragilis*+*L. leptodon* (Fig. 3.5). There were several branches within the *A. grunniens* specialists clade that showed reductions in larval surface area (Fig. 3.6A), and these reductions were largely concordant with the presence of larval growth (Fig. 3.4B). Larval surface area varied as a function of height and showed differentiation in axe-head shaped, miniature, and subelliptical glochidia (Fig. 3.7). Axe-head shaped glochidia, however, tended to have reduced surface area (48% reduction) when compared to subelliptical glochidia with similar heights. ACR showed multiple increases in fecundity in *A. grunniens* specialists, especially in *L. fragilis*+*L. leptodon* and *P. ohiensis*+*P. streckersoni* (Fig. 3.6B). Mean annual fecundity and ranges from fecundity estimates are provided in Table S3.1 and on ScienceBase (https://doi.org/10.5066/P9X3J54C).

Discussion

Our phylogenomic and ancestral state reconstructions recovered a complex pattern of life history diversification in a clade of parasitic bivalves that have specialized in *A. grunniens* host parasitization. These shifts in life history help us to better understand the evolutionary and ecological processes shaping the diversity of this clade. Additionally, several of these major trait transformations coincide with genome-wide signatures of positive selection, which further contributes to our understanding of how these lineages have evolved. We discuss these shifts in terms of their ecological (i.e.,

interactions with host fish), biogeographic (i.e., vicariance via stream capture), and systematic implications (i.e. description of a new genus, *Atlanticoncha* gen. nov., and the synonymy of *Leptodea* under *Potamilus*).

Origin of Aplodinotus grunniens Specialization and Host Switching

Mussel host use has a strong phylogenetic signal and is conserved in many clades (Hewitt *et al.*, 2019), however the taxa specializing in *A. grunniens* parasitism (i.e., *Ellipsaria*, *L. fragilis*, *L. leptodon*, *Potamilus*, and *Truncilla*) were not recovered as monophyletic, similar to previous multi-locus assessments (Campbell *et al.*, 2005; Zanatta and Murphy, 2006; Pfeiffer *et al.*, 2018; Smith *et al.*, 2019). *Aplodinotus grunniens* specialists were resolved as paraphyletic with respect to *L. ochracea* (Fig. 3.1), which primarily parasitizes *Morone americana* (Kneeland and Rhymer, 2008) and is not sympatric with *A. grunniens*. Explicit tests of host fish evolution using ACR clearly indicate that the paraphyly of *A. grunniens* specialists is a product of host switching in *L. ochracea*, which has transitioned to parasitizing a phylogenetically and ecologically divergent host (Fig. 3.4A).

Colonization of mussels to novel drainages are typically indicative of physical changes to their environments (e.g., stream capture, vicariance events, etc.) rather than by biological invasion (Graf, 1997; Graf *et al.*, 2015; Smith *et al.*, 2018). Therefore, a vicariance event, likely a stream capture, is a plausible explanation for the distribution of *L. ochracea* in Atlantic coast drainages and aligns with several other freshwater faunal exchanges between the Mississippian and Atlantic regions (*sensu* Haag, 2010; Ortmann, 1913; Johnson, 1970; Sepkoski Jr. and Rex, 1974; Schmidt, 1986; Berendzen *et al.*, 2003, 2008). However, an instantaneous transition in host use may be an unrealistic

assumption. Another plausible explanation is a gradual host switch may have occurred as *L. ochracea* migrated to the Atlantic region through the St. Lawrence drainage, which aligns with putative dispersal routes of several mussel species (van der Schalie, 1963; Clarke, 1973; Haag, 2012; Scott *et al.*, 2020). The only known native co-occurrence of *A. grunniens* and *M. americana* is within the St. Lawrence drainage (Page and Burr, 2011), and multiple *A. grunniens* specialists (i.e., *L. fragilis* and *P. alatus*) were historically distributed in the drainage (Strayer and Jirka, 1997). Thus, a gradual host transition to *M. americana* could have occurred within the St. Lawrence drainage before colonization of the Atlantic region. *Morone americana* is an anadromous species that primarily inhabits brackish tidal waters (Kraus and Secor, 2004; Kerr *et al.*, 2009), and the ability to disperse among river drainages while attached to its host helps to explain the broad geographic range of *L. ochracea* across the Atlantic region. Subsequent adaptation occurred in *L. ochracea* to inhabit lower reaches of streams toward tidal regions in response to the habitat preference of its host (Johnson, 1970), which is further supported by significant positive selection ($\alpha < 0.0001$; Fig. 3.3).

Larval Evolution in Aplodinotus grunniens Specialists

Although larval morphology is often strongly conserved in many mussel clades (Hoggarth, 1999; Pfeiffer and Graf, 2015; Pfeiffer *et al.*, 2019b), we observed atypical levels of larval variation and a complex pattern of larval diversification within *A. grunniens* specialists (Fig. 3.3; Fig. 3.6A). Specifically, phylogenomic analyses resolved taxa with miniaturized and axe-head shaped glochidia as non-monophyletic despite their apparent morphological cohesiveness (Fig. 3.1). Miniaturized glochidia have evolved multiple times in Unionoida (i.e., *L. fragilis* and *L. leptodon*; *Truncilla*; *Quadrula* and

Tritogonia; and Margaritiferidae), and both topological constraints and ACR support that miniaturization in *L. fragilis* and *L. leptodon*, and *Truncilla* is a product of convergent evolution rather than shared ancestry (Fig. 3.6A). Further, taxa bearing axe-head shaped glochidia were resolved as paraphyletic with *L. fragilis* and *L. leptodon* nested within *Potamilus* (Fig. 3.1; Fig. 3.5). Our plots of larval surface area and height, however, clearly depict the numerous forms of axe-head shaped glochidia as morphological adaptations for miniaturization, with axe-head shaped glochidia having a substantial reduction in surface area relative to subelliptical glochidia (Fig. 3.7). Our ACR of larval surface area also supported a single miniaturization event in the evolutionary history of *L. fragilis*+*L. leptodon*+*Potamilus* (Fig. 3.6A) and suggest the loss of axe-head shape larvae in *L. fragilis* and *L. leptodon* may be a result of further larval reduction rather than an independent origin of miniaturized glochidia. The adaptive significance of axe-head shaped and miniaturized glochidia is uncertain but possibly related to a functional trait for increasing fecundity (see below).

Repeated Evolution of Life History Traits in Aplodinotus grunniens Specialists

While similar phenotypic characters can arise independently by chance (Stayton, 2008), the probability of repeated evolution is more likely in closely related species since the chance of taxa sharing the same genetic mechanism is increased (Conte *et al.*, 2012; Ord and Summers, 2015). This is likely the case for *A. grunniens* specialists, as ACRs clearly depict multiple reductions in larval size, several increases in fecundity, and multiple origins of growth during encapsulation (Fig. 3.4B; Fig. 3.6), all of which were further supported by genome-wide signatures of selection on coinciding branches in the

phylogeny (i.e., *L. fragilis*+*L. leptodon*; *P. amphichaenus*+*P. ohiensis*+*P. streckersoni*; and *Truncilla*; Fig. 3.3).

Decreased size of glochidia has been hypothesized to be correlated with high levels of fecundity (Bauer, 1994; Barnhart *et al.*, 2008) and for *A. grunniens* specialists, our ACRs of larval surface area and fecundity largely support that hypothesis (Fig. 3.6). Considering this, multiple independent transitions to reduced larval size in *A. grunniens* specialists may be indicative of selection towards greater fecundity, which is also consistent with the hypothesized modes of infection in this clade (i.e., broadcasting and maternal sacrifice). The primary mode of host infection in this group remains unknown but for some taxa there is substantial evidence that it may include maternal sacrifice (Coker *et al.*, 1921; Howard and Anson, 1922; Barnhart *et al.*, 2008; Haag, 2012; Sietman *et al.*, 2018). However, adults of most taxa exceed the gape size of *A. grunniens* and the broadcast of free glochidia is likely the mode of infection (Haag 2012). Broadcast of free glochidia is an effective infection strategy when host populations are abundant, but only a small proportion of glochidia typically encyst (Jansen *et al.*, 2001). Therefore, given the low probability of broadcast glochidia encountering a host in the water column (Bauer, 1994), the mode of infection elucidates selection for, and multiple origins of, high fecundity in *A. grunniens* specialists.

In addition to decreased larval size and fecundity, ACR demonstrates multiple origins of larval growth during encystment in *A. grunniens* specialists (Fig. 3.4B). Growth during encapsulation is atypical in Unionidae, but the trait has evolved independently at least three times in Ambleminae (i.e., *L. fragilis*+*L. leptodon*+*Potamilus*, *Truncilla*, *Quadrula*+*Tritogonia*), including twice within *A.*

grunniens specialists. These independent origins appear to be lineages that also have miniaturized glochidia, which led Barnhart et al (2008) to hypothesize that the lower limit of juvenile size is likely linked to post encystment settlement, where very small juveniles may have difficulties settling from suspension in the water column. However, significant growth during encystment is also present in taxa bearing relatively large axe-shaped glochidia (Fig. 3.4B), suggesting growth during encapsulation in this group may be related to shape, specifically the inability to fully close at the lateral margins, rather than size limitations. The two independent origins of growth during encapsulation within *A. grunniens* specialists is likely indicative of selective pressures for high fecundity, as reducing larval size (both miniaturized and axe-head shaped) has also led to parasitic growth necessary for juvenile survival.

Systematics in Leptodea and Potamilus

Similar to previous phylogenetic investigations (Roe and Lydeard, 1998; Smith *et al.*, 2019), *Leptodea* and *Potamilus* were recovered as non-monophyletic. The monophyly of *Leptodea* has been questioned due to *L. ochracea* having morphological characteristics that are divergent from remaining species of *Leptodea*, including the type species *L. fragilis* (Johnson, 1970; Davis and Fuller, 1981; Stiven and Alderman, 1992; Smith, 2000; Smith *et al.*, 2019). Specifically, reproductive characters (e.g., brooding characters, lack of growth during encapsulation) and larval morphology of *L. ochracea* are more similar to other genera in Ambleminae than *A. grunniens* specialists (Reardon 1929; Hoggarth 1999; Smith 2000; Fig. 3.3). Given the non-monophyly of *Leptodea* (Fig. 3.1; Fig. 3.2) and the distinct larval morphology (Fig. 3.3), host use (Fig. 3.4A), and divergent anatomical characters in *L. ochracea* (Johnson, 1970; Smith, 2000), we formally describe

a new genus, *Atlanticoncha* gen. nov., to more accurately reflect the evolutionary history of *A. ochracea*.

The foundation of *Potamilus*, and therefore separation from *L. fragilis* and *L. leptodon*, has long been based on the unique axe-head larval morphology (Ortmann, 1912; Frierson, 1927). We resolved axe-head shaped glochidia as the ancestral state of *L. fragilis*, *L. leptodon*, and *Potamilus* (Fig. 3.5), however, our results suggest that the trait is an adaptation to reduce larval size and reduction has been further accentuated in *L. fragilis* and *L. leptodon* (Fig. 3.6A). Based on the phylogenetic relationships resolved in this study (Fig. 3.1; Fig. 3.2) and larval morphologies that reduce size (Fig. 3.3), along with congruence in anatomical characters, adult morphological characters, brooding morphology, brooding phenology, host attraction, and host use (Ortmann, 1912; Frierson, 1927; Williams *et al.*, 2008; Haag, 2012; Sietman *et al.*, 2018), we formally recognize *Leptodea* as a junior synonym of *Potamilus*. Our findings contribute to the growing body of literature showing that morphological characters, including larval morphology, can be unreliable for cladistics and systematics in mussels (Hoggarth, 1999; Watters *et al.*, 2009; Williams *et al.*, 2014; Pfeiffer and Graf, 2015; Pfeiffer *et al.*, 2016; Perkins *et al.*, 2017; Johnson *et al.*, 2018; Smith *et al.*, 2018).

Conclusion

The use of hybrid enrichment strategies clearly represents an improvement in the ability to reconstruct accurate phylogeny and advancing knowledge of mussel ecology and evolution. We resolve the phylogenetic relationships of *A. grunniens* specialists and systematics within the genera *Leptodea* and *Potamilus*, including the phylogenetically unstable *L. ochracea*, which advances knowledge of both mussel evolution and

functional traits that have driven lineage diversification. Our analyses also recovered a complex evolution of life history strategies, each of which produces larval morphologies that reduce size, increase fecundity, and require growth during encapsulation for juvenile survival. The multiple origins of these life history traits illustrate their functional significance toward successful parasitism of *A. grunniens*, and a firm understanding of these traits will be useful toward determining conservation priorities and predicting species-specific responses in these highly imperiled organisms.

Taxonomic Accounts

Atlanticoncha, gen. nov. Smith, Pfeiffer, & Johnson 2020

Family Unionidae Rafinesque, 1820

Tribe Lampsilini Ihering, 1901

TYPE SPECIES: *Unio ochraceus* Say, 1817

ETYMOLOGY: The name *Atlanticoncha* is to typify this freshwater mussel genus as endemic to the Atlantic coast drainages of central North America.

DESCRIPTION: General outline of the shell is oval; anterior margin rounded; posterior margin rounded but may be pointed in males. Dorsal margin typically straight or slightly curved ending with a blunt angle descending toward the posterior margin. Ventral margin straight or slightly curved but may be concave in females, posterior ridge rounded and poorly defined, posterior slope slightly convex. Umbo moderately swollen and extends above the hinge line. Umbo sculpture weakly double-looped. Shell thin and subinflated but strong, surface smooth, periostracum subshiny, brownish olive to yellow, with greenish rays typically found over the entire surface of the shell and more prominent in smaller individuals. Pseudocardinal teeth compressed and delicate, two in each valve.

Lateral teeth moderately long, slightly curved, two in left valve and one in right. Interdentum greatly reduced or absent; umbo cavity narrow and moderately shallow. Nacre white, bluish white, or pinkish. Glochidia outline subelliptical; length 241-246 μm; height 289-294 μm; marginal appendages absent (Fig. 3.3). Dorsal margin straight, ventral margin rounded, anterior and posterior margins straight becoming slightly convex ventrally, absent to slight lateral valve gape; ventral margin with vertical rows of lamellate micropoints.

SPECIES: *Atlanticoncha* is monotypic with *A. ochracea* being the only recognized species.

DISTRIBUTION: Atlantic Region from the Savannah River drainage, Georgia, USA; north to the River Hebert, Nova Scotia, Canada (Johnson, 1970).

DIAGNOSIS: *Atlanticoncha* can be diagnosed from *Potamilus* using a suite of life history and anatomical characters, as well as geography. *Atlanticoncha* has distinct life history characters, including specialized parasitism of *M. americana* (Fig. 3.4A), no larval growth during encapsulation (Fig. 3.4B), and distinct subelliptical glochidia (Fig. 3.3). Anatomical characters such as the presence of papillae along the mantle margin further distinguish *Atlanticoncha* from *Potamilus* (Smith, 2000; Williams *et al.*, 2008; Sietman *et al.*, 2018). Geographically, *Atlanticoncha* only occurs in the Atlantic Region whereas *Potamilus* is only found in the Mississippian Region (*sensu* Haag, 2010).

Atlanticoncha may resemble *Lampsilis* but *Atlanticoncha* has a thinner shell and is more likely to be rayed than similar sized specimens of *Lampsilis*. The pseudocardinal teeth in *Atlanticoncha* are lamellate and less developed than the pyramidal teeth in

similar sized specimens of *Lampsilis*. The interdentum is greatly reduced to nonexistent in *Atlanticoncha* but well-defined in *Lampsilis*.

Potamilus Rafinesque, 1818

Family Unionidae Rafinesque, 1820

Tribe Lampsilini Ihering, 1901

SYNONYMY:

= *Proptera* Rafinesque, 1919

= *Lastena* Rafinesque, 1820

= *Leptodea* Rafinesque, 1820

= *Metaptera* Rafinesque, 1820

= *Symphynota* Lea, 1829

= *Lasmonos* Rafinesque, 1831

= *Lymnadia* G.B. Sowerby II, 1839

= *Naidea* Swainson, 1840

= *Monelasmus* Agassiz, 1846

= *Paraptera* Ortmann, 1911

TYPE SPECIES: *Unio alatus* Say, 1817

DESCRIPTION: General outline of the shell oval to elliptical or subtriangular in immature individuals. Low to well-developed wing often present on dorsal margin posterior to umbo but straight to slightly curved in species without posteriodorsal wing. Ventral margin straight to slightly convex, posterior margin rounded, posterior ridge absent to rounded or poorly defined, posterior slope flattened to slightly concave, umbo cavity wide and shallow. Shell thin to moderately thick, compressed to inflated,

periostracum yellow to dark brown, young individuals likely to have green rays. Pseudocardinal teeth variable ranging from lamellate, compressed and delicate to more developed and triangular, one or two teeth in left valve and one in right valve. Lateral teeth short, delicate to moderately thick, 2 in left valve and 1 in right valve; interdentum long and narrow; nacre bluish, pink, or purple. Glochidia outline axe-head shaped (except *P. fragilis* and *P. leptodon* = subelliptical); length 68-230 μm; height 80-410 μm; variable marginal appendages from absent to 5-7 lanceolate hooks on lateral margin of ventral flange (Fig. 3.3). Dorsal margin straight, ventral margin rounded to convex, anterior and posterior margins convex or straight and becoming convex ventrally; slight to moderate valve gape; ventral margin with vertical rows of lamellate or rounded micropoints.

SPECIES: *Potamilus alatus*, *P. amphichaenus*, *P. capax*, *P. fragilis*, *P. inflatus*, *P. leptodon*, *P. metnecktayi*, *P. ohiensis*, *P. purpuratus*, and *P. streckersoni*

DISTRIBUTION: Mississippian Region from Canada south to LA, USA; Gulf of Mexico drainages from the Mobile Basin in Alabama, USA west to the Rio Grande drainage of western Texas, USA (Williams *et al.*, 2017).

DIAGNOSIS: *Potamilus* can be diagnosed from other *A. grunniens* specialists using anatomical, life history, and molecular characters. *Potamilus* lack papillae along the mantle margin, which are present in all other *A. grunniens* specialists (Smith, 2000; Williams *et al.*, 2008; Sietman *et al.*, 2018). Distinct life history characters also separate *Potamilus* from *Atlanticoncha* and *Ellipsaria*, including larval growth during encapsulation (Fig. 3.4B) and glochidia that are reduced in size (i.e., axe-head shaped or miniaturized; Fig. 3.3). Phylogenetic reconstructions clearly depict *Potamilus* molecularly diagnosable from *Atlanticoncha*, *Ellipsaria*, and *Truncilla* (Fig. 3.1).

Potamilus may resemble *Lampsilis* but *Potamilus* has a thinner shell and more likely to be rayed than similar sized specimens of *Lampsilis*. *Potamilus* may also resemble *Lasmigona*; however, *Potamilus* is unsculptured while most members of *Lasmigona* sympatric with *Potamilus* are heavily plicate. *Lasmigona* species can also be distinguished from *Potamilus* by a lack of well-developed lateral teeth and a white nacre. *Potamilus* may be confused with *Cyrtonaias*. *Potamilus* species are more elongate than similar sized specimens of *Cyrtonaias*, and lateral teeth in *Potamilus* are straight while lateral teeth in *Cyrtonaias* are moderately curved.

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Figures

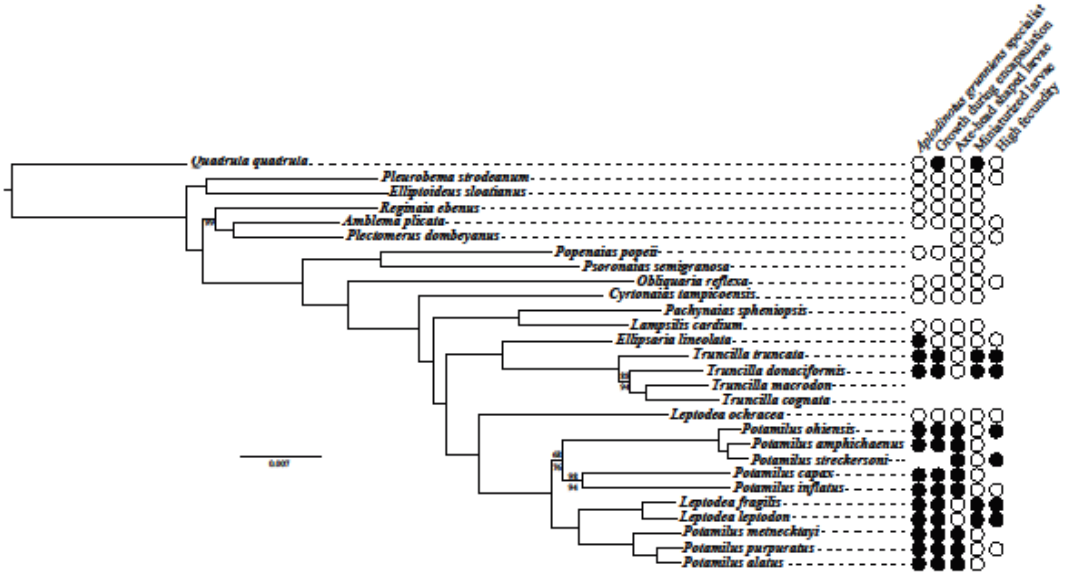

Figure 3.1. Phylogenomic reconstruction generated by the Bayesian inference analysis on Dataset 4 (concatenated probe and flanking regions) and all nodes were strongly supported (PP = 100). Maximum Likelihood and Maximum Parsimony recovered concordant topologies and only 4 nodes did not have full support (BS or ufBS < 100). For nodes without full support, ML ufBS values are denoted above and MP BS values below the branch. Circles denote the presence (filled circle) or absence of *Aplodintus grunniens* host specialization, growth during encapsulation, axe-head shaped glochidia, miniaturized glochidia, and high fecundity for each taxon. Missing data is represented by no circle.

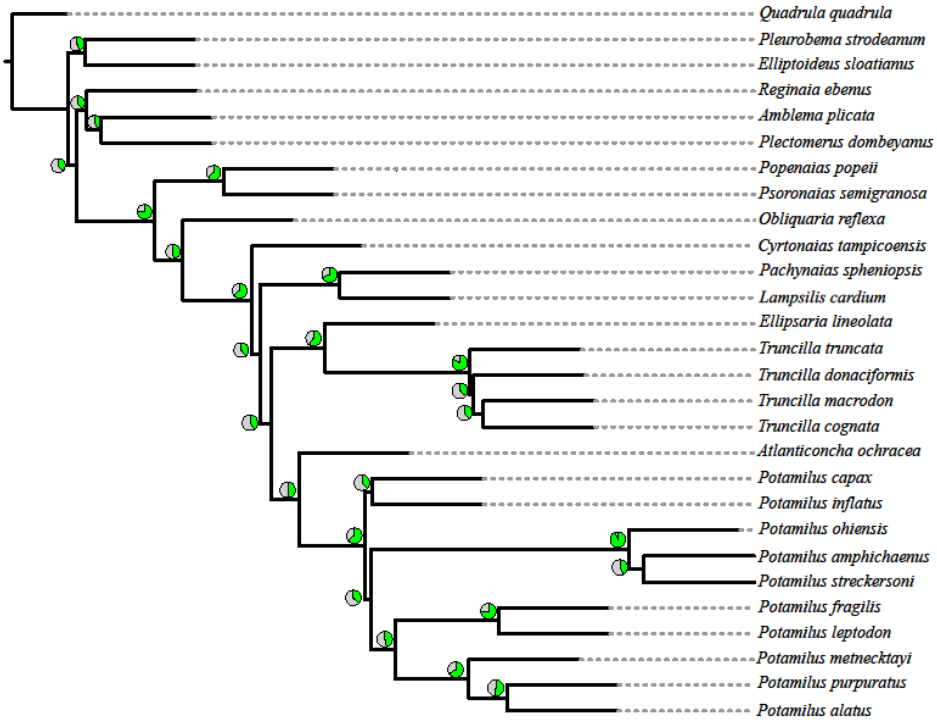

Figure 3.2. Phylogenomic reconstruction generated ASTRAL-III using Dataset 4 (Probe and Flanking regions). Green shading in each pie chart represents the proportion of loci supporting each node.

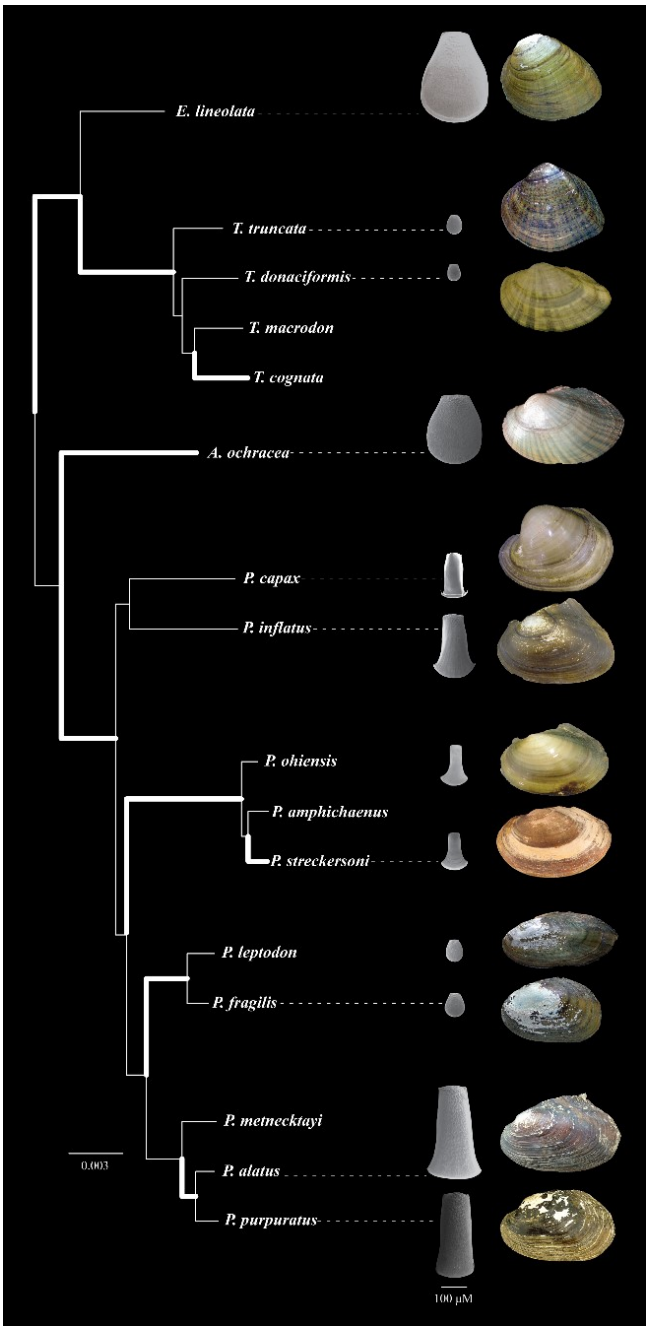

Figure 3.3. Phylogenomic reconstruction generated by IQ-TREE using Dataset 1 (probes regions only). Bolded branches indicate significant evidence of positive selection ($\alpha < 0.05$) as shown by aBSREL. Terminals marked with circles represent taxa with photographs of type specimens (courtesy of www.musselproject.uwsp.edu) and glochidia. From the top, type specimens and larval photos, respectively, are as followed: *Ellipsaria lineolata* (ANSP20242; OSUM:1984:14 - reformatted from Hoggarth, 1999), *Truncilla truncata* (ANSP20217; N/A - reformatted from Sietman et al., 2018), *T. donaciformis* (USNM84457; N/A - reformatted from Sietman et al., 2018), *Atlanticoncha ochracea* (MCZ178838; UF438217), *Potamilus capax* (USNM84892; INHS9180-2 - reformatted from Cummings et al. 1990), *P. inflatus* (USNM83909; UF439514), *P. ohiensis* (USNM83938; N/A - reformatted from Sietman et al., 2018), *P. streckersoni* (UF439497; UF439478), *P. leptodon* (ANSP20214; N/A - larval sample only), *P. fragilis* (ANSP20209; N/A - reformatted from Sietman et al., 2018), *P. alatus* (SMF4349; INHS44342), and *P. purpuratus* (USNM86108; UF439460).

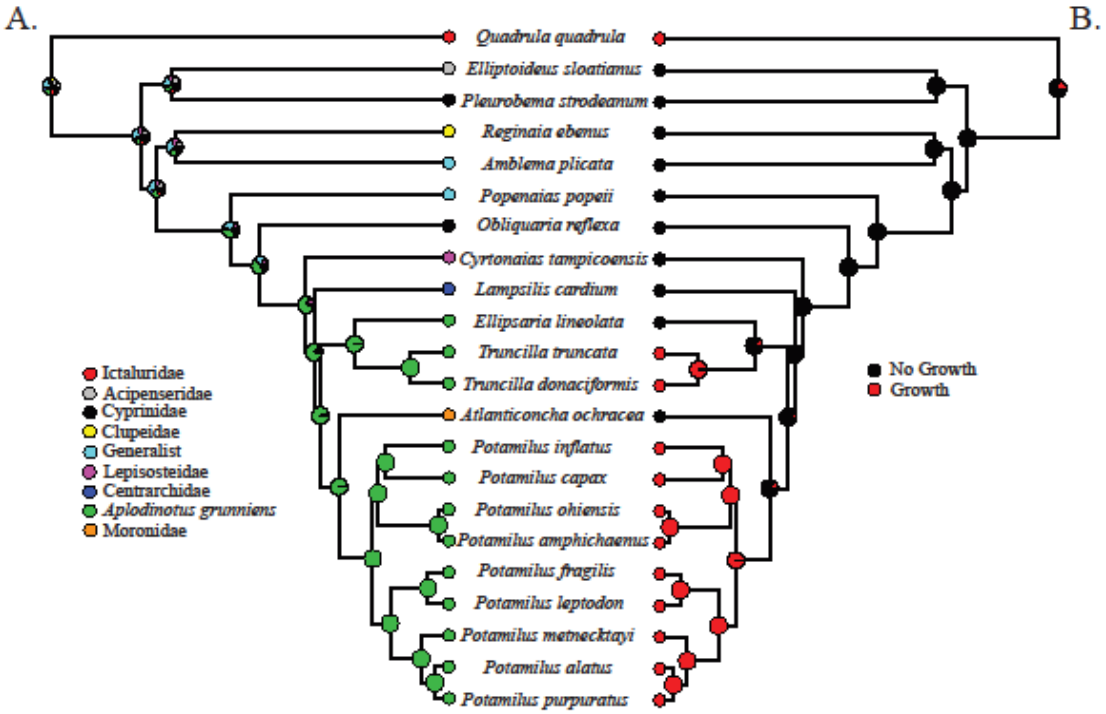

Figure 3.4. Bayesian stochastic character mapping of host fish use (Fig. 3.4A) and larval growth during encapsulation (Fig. 3.4B) using the Bayesian topology generated from Dataset 4 (concatenated probe and flanking regions). Pie charts represent posterior probability support for character states at each node.

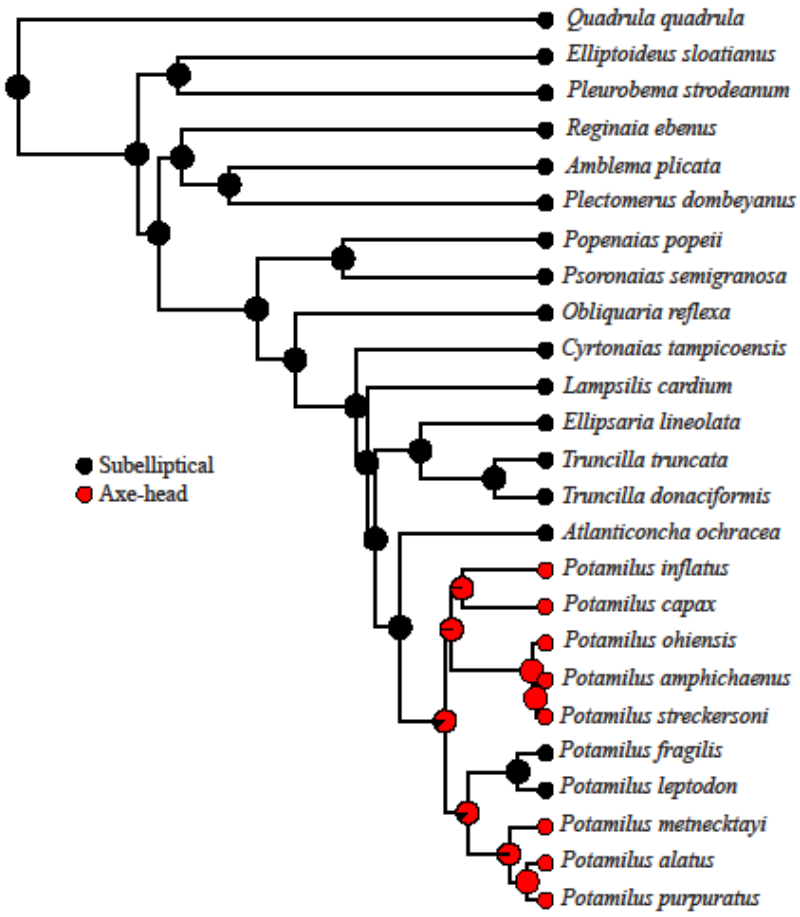

Figure 3.5. Bayesian stochastic character mapping using the Bayesian optimal topology generated from Dataset 4 (Probe + Flanking regions). Ancestral character reconstruction shows the evolutionary history of axe-head shaped glochidia. Pie charts represent posterior probability support for larval shape at each node.

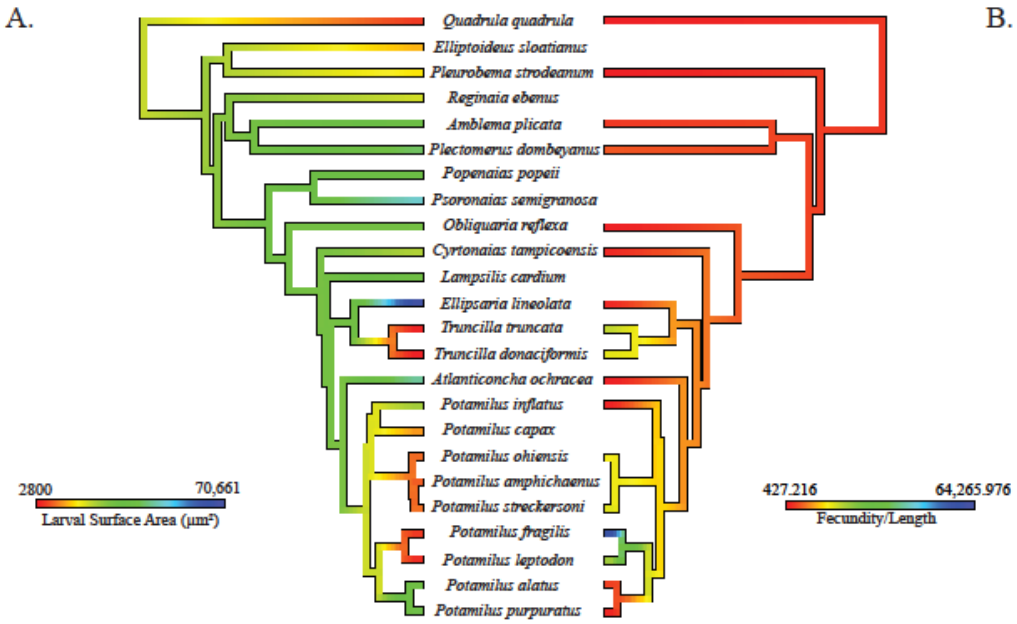

Figure 3.6. Ancestral character reconstructions of larval surface area (Fig. 3.6A) and fecundity divided by length (Fig. 3.6B) on the Bayesian topology generated from Dataset 4 (concatenated probe and flanking regions). Colors represent reconstructed trait values.

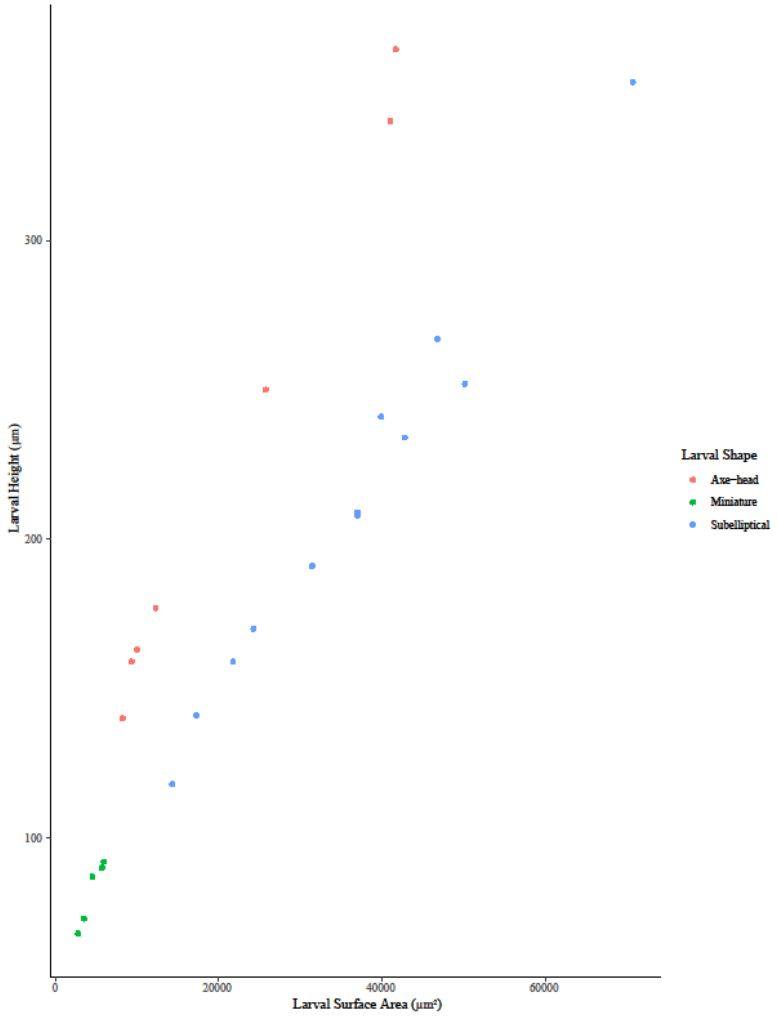

Figure 3.7. Scatter plot of the distribution of larval surface area with respect to height between axe-head shaped, miniature, and subelliptical larval morphologies.

Tables

Table 3.1. Samples used in anchored hybrid enrichment analyses. Museum abbreviations are as follows: ASU – Arkansas State University Museum; INHS – Illinois Natural History Survey; UF – Florida Museum of Natural History; UMMZ – University of Michigan Museum of Zoology.

Taxa	Catalog Number	Source	Accession
Amblema plicata	UF438572	Pfeiffer et al. 2019	SRR8473047
Atlanticoncha ochracea	UF438217	This Study	SAMN13473079
Cyrtonaias tampicoensis	UF438559	Pfeiffer et al. 2019	SRR8473040
Ellipsaria lineolata	UF439368	This Study	SAMN13473076
Elliptoideus sloatianus	UF440850	Pfeiffer et al. 2019	SRR8473067
Lampsilis cardium	UMMZ304654	Pfeiffer et al. 2019	SRR8473035
Obliquaria reflexa	UF438940	Pfeiffer et al. 2019	SRR8473034
Pachynaias spheniopsis	UF507900	Pfeiffer et al. 2019	SRR8473029
Plectomerus dombeyanus	UF438655	Pfeiffer et al. 2019	SRR8473056
Pleurobema strodeanum	UF441317	Pfeiffer et al. 2019	SRR8473051
Popenaias popeii	UF438742	Pfeiffer et al. 2019	SRR8473050
Potamilus alatus	UF438248	This Study	SAMN13473080
Potamilus amphichaenus	UF439483	This Study	SAMN13473081
Potamilus capax	N/A (swab only)	This Study	SAMN13473082
Potamilus fragilis	INHS39037	This Study	SAMN13473077
Potamilus inflatus	UF439131	This Study	SAMN13473083
Potamilus leptodon	INHS44305	This Study	SAMN13473078
Potamilus metnecktayi	UF438911	This Study	SAMN13473084
Potamilus ohiensis	INHS41658	This Study	SAMN13473086
Potamilus purpuratus	UF439453	This Study	SAMN13473087
Potamilus streckersoni	UF441294	This Study	SAMN13473085
Psoronaias semigranosa	UF507899	Pfeiffer et al. 2019	SRR8473037
Quadrula quadrula	UF441088	Pfeiffer et al. 2019	SRR8473075
Reginaia ebenus	UF438113	Pfeiffer et al. 2019	SRR8473078
Truncilla cognata	UF438552	This Study	SAMN13473088
Truncilla donaciformis	UF439324	This Study	SAMN13473089
Truncilla macrodon	UF440984	This Study	SAMN13473090
Truncilla truncata	ASU1253	This Study	SAMN13473091

Table 3.2. Number of loci, total length, the amount of missing data, and the average length per locus in each dataset as well as partitions used in Anchored Hybrid Enrichment analyses.

Dataset	Number of loci	Total length (nt)	Missing data	Average length per locus (nt)	Partitions
1 – Probe Only	626	118,083	11.11%	188.63	17
2 – Flank Only	1,247	155,949	16.79%	125.06	21
3 – Probe+Flank	1,873	274,032	14.34%	145.67	38
4 – Probe+Flank	626	278,557	13.68%	444.98	15

CHAPTER FOUR

Resolving species boundaries in the critically imperiled freshwater mussel species, *Fusconaia mitchelli* (Bivalvia: Unionidae)

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Abstract

Species are a fundamental unit of biology and defining accurate species boundaries is integral to effective conservation and management of imperiled taxa. Freshwater mussels (Bivalvia: Unionidae) are among the most imperiled groups of organisms in North America yet species boundaries remain uncertain for many taxa. The False Spike, *Fusconaia mitchelli* (Simpson in Dall, 1895), is a freshwater mussel considered to be endemic to central Texas (Brazos, Colorado, and Guadalupe drainages). Recent research revealed significant intraspecific genetic variation between geographically separated populations of *F. mitchelli*, which could be indicative of speciation; however, small sample sizes for several of the populations precluded formal taxonomic revision. Here, we increase taxon sampling and use multi-locus sequence data and traditional morphometrics to re-evaluate species boundaries in *F. mitchelli*. We sequenced three loci: the protein-coding mitochondrial DNA genes *cytochrome oxidase subunit 1* and *NADH dehydrogenase 1*, and the nuclear ribosomal *internal transcribed spacer 1*. Phylogenetic analyses depicted deep molecular divergence between *F. mitchelli* in the Guadalupe and those in the Brazos and Colorado drainages, which was further

supported by available biogeographic information. Morphometric analyses and coalescent-based species delimitation models integrating both molecular and morphological data provided strong support for the divergence observed between the two geographically isolated clades of *F. mitchelli*. Based on these results, we revise taxonomy accordingly by elevating the junior synonym *Fusconaia iheringi* (Wright, 1898) to represent the Brazos and Colorado populations and restrict the distribution of *F. mitchelli* to the Guadalupe River drainage. Our findings may impact pending management decisions to protect *F. mitchelli* under the U.S. Endangered Species Act.

Introduction

Species are a fundamental unit of biology and defining accurate species boundaries is integral to effective conservation and management of imperiled taxa. Freshwater mussels (Bivalvia: Unionida) are a group of aquatic invertebrates comprised of approximately one thousand species worldwide (Graf & Cummings, 2007; Lopes-Lima et al., 2018), and the greatest diversity of freshwater mussels lies within North America with at least three hundred native species in the family Unionidae alone (Graf & Cummings, 2007; Williams et al., 2017). Mussels contribute significant ecological benefits to freshwater ecosystems by integrating the fluvial food web and providing and stabilizing benthic habitat (Haag & Williams, 2014; Vaughn, 2018; Vaughn, Nichols, & Spooner, 2008). Freshwater mussels are also a compelling system in the study of evolutionary biology. This is due to the unionid life cycle which involves parasitic larvae (glochidia) that must attach to vertebrate hosts (primarily fish) prior to becoming adults (Barnhart, Haag, & Roston, 2008). These coevolutionary relationships have led to a

variety of life history strategies across species, resulting in subsequent biological diversification (Barnhart et al., 2008; Haag, 2012).

Anthropogenic alterations to freshwater ecosystems have disproportionately impacted mussels as a group, resulting in extinctions of some species, extirpation of populations of other species, and reduction in density of most mussel populations (Haag & Williams, 2014; Vaughn & Taylor, 1999). These declines stem from the inherent biological characters of mussels, including limited locomotive capabilities in many species, reliance on host fish for dispersal, and extreme sensitivity to organic and inorganic pollutants (Bringolf et al., 2007; Haag, 2012; Wang et al., 2017). Additionally, some mussel species, particularly those considered imperiled, tend to have life history traits more characteristic of K-strategists (i.e., long-lived, low maturation rates, low fecundity, slow growth rates) making evolutionary response to rapidly changing environments less likely (Haag & Williams, 2014; Lighten et al., 2016; Martin & Palumbi, 1993). As a result, freshwater mussels are one of the most imperiled groups of organisms on Earth, with approximately 70% of species in North America considered either threatened, endangered, or extinct (Haag & Williams, 2014; Lopes-Lima et al., 2018; Williams, Warren, Cummings, Harris, & Neves, 1993).

Molecular studies on freshwater mussels that address phylogenetic placement and species boundaries have been pivotal in inferring important biological characteristics of species (e.g., host use, reproductive traits, habitat preference) and ensuring the taxonomic validity of protected species or those being considered for protection (Johnson et al., 2018; Pfeiffer, Johnson, Randklev, Howells, & Williams, 2016; Smith, Johnson, Pfeiffer, & Gangloff, 2018). Although considerable progress has been made in understanding

ecology (Dudding, Hart, Khan, Robertson, & Lopez, 2019; Hart, Haag, Bringolf, & Stoeckel, 2018; Johnson, McLeod, Holcomb, Rowe, & Williams, 2016; Sietman, Hove, & Davis, 2018) and evolution (Inoue, Harris, Robertson, Johnson, & Randklev, 2019; Lopes-Lima et al., 2017; Pfeiffer et al., 2019; Pfeiffer, Breinholt, & Page, 2019; Smith, Johnson, Inoue, Doyle, & Randklev, 2019) of freshwater mussels in recent years, many species still remain poorly understood and species validity has not been confirmed using robust molecular methodologies (Haag, 2012; Lopes-Lima et al., 2018). This is certainly the case in the southwestern United States, where diversity, distribution, and ecology of many mussel species are still poorly understood (Ford & Oliver, 2015; Haag, 2012).

Morphology driven taxonomic hypotheses in the freshwater mussel tribe Pleurobemini Hannibal, 1912 have been largely invalidated by molecular methods and resolving accurate phylogeny has been integral toward understanding the evolution of this group (Campbell & Lydeard, 2012b; Inoue et al., 2018). For members in the genus *Fusconaia* Simpson, 1900 in Texas, there have been multiple systematic changes in recent years using molecular data and some sympatric species are even morphologically indistinguishable (Campbell & Lydeard, 2012a; Pfeiffer et al., 2016; Pieri et al., 2018). One member of this genus, *Fusconaia mitchelli* (Simpson in Dall, 1895) or the False Spike, is endemic to the Brazos, Colorado, and Guadalupe drainages of central Texas (Howells, Neck, & Murray, 1996). *Fusconaia mitchelli* was presumed extinct until its recent rediscovery in 2011 when several individuals were collected from the lower Guadalupe River (Randklev et al., 2012). At the time of rediscovery, the validity and systematic position of *F. mitchelli* was unknown. The taxon was assigned to the genus *Quadrula* Rafinesque, 1820, based on morphology and phylogenetic positioning of

assumed closely related species (Mabe & Kennedy, 2014; Randklev et al., 2012; Randklev et al., 2013); however, taxonomic placement remained an enigmatic issue toward understanding the basic biology and ecology of the species. Recent molecular research revealed that *F. mitchelli* belonged to the genus *Fusconaia* rather than *Quadrula*, and also reported two distinct clades within *F. mitchelli* corresponding to the Brazos and Colorado drainages, and the Guadalupe drainage (Pfeiffer et al., 2016). Despite high levels of divergence between the two clades, recognizing two distinct species within *F. mitchelli* warranted increased taxon sampling, additional molecular markers, and morphological or life history data.

Species boundaries in *F. mitchelli* remain a significant knowledge gap for natural resource managers, as conservation efforts based on current taxonomic hypotheses (TPWD, 2010; USFWS, 2009) may lead to unsubstantiated conclusions about its status and bias management and recovery actions. Given the importance of accurate taxonomy in conservation and management of imperiled taxa, the primary objective of this study was to resolve species boundaries within *F. mitchelli* by incorporating both molecular and morphological data. Specifically, we set out to accomplish the following objectives: 1) use multi-locus sequence data to resolve a phylogeny of Pleurobemini, with emphasis on *F. mitchelli*; 2) delineate species boundaries within *F. mitchelli* using molecular and morphometric data; and 3) discuss the implications of our findings toward effective conservation and management practices.

Materials and Methods

Taxon Sampling

We evaluated genetic relationships within *F. mitchelli* by sampling all extant populations, including individuals from the Brazos, Colorado, and Guadalupe river drainages. We also utilized published data on Genbank and added additional loci to the individuals used in a previous study by Pfeiffer et al. (2016). Individuals representing all type species from genera in the Pleurobemini were also included and *Quadrula quadrula* (Rafinesque, 1820) was selected to function as the root based off of previous molecular assessments of Ambleminae Rafinesque, 1820 (Pfeiffer et al., 2019).

Small mantle tissue clips from each specimen were preserved in 100% ethanol and stored at -80°C. DNA was isolated with the Qiagen PureGene DNA extraction kit following manufacturer's suggested protocols (Gentra Systems, Inc., Minneapolis, MN, USA). We used three loci in our investigation: a portion of the protein-coding mitochondrial genes *cytochrome c oxidase subunit 1* (CO1) and *NADH dehydrogenase subunit 1* (ND1), and the nuclear spacer gene ribosomal *internal transcribed spacer 1* (ITS1). The primer sequences used for PCR were: CO1 5'-GTTCCACAAATCATAAGGATATTGG-3' and 5'-TACACCTCAGGGTGACCAAAAAACCA-3' (Campbell et al., 2005); ND1 5'-TGGCAGAAAAGTGCATCAGATTAAAGC-3' and 5'-CCTGCTTGGAAGGCAAGTGTACT-3' (Serb, Buhay, & Lydeard, 2003); and ITS1 5'-AAAAAGCTTCCGTAGGTGAACCTGCG-3' and 5'-AGCTTGCTGCGTTCTTCATCG-3' (King, Eackles, Gjetvaj, & Hoeh, 1999). PCR was conducted using a mixture of molecular grade water (4.25 µl), MyTaq Red Mix (Bioline;

6.25 µl), primers (0.5 µl each), and DNA template (50 ng). PCR product was sent to Molecular Cloning Laboratories (MCLAB, South San Francisco, CA, USA) for bidirectional sequencing on an ABI3730. PCR product for ITS1 was more difficult to sequence than mtDNA markers considering the possibility of multiple copies at ITS1. All individuals were sent directly for sequencing, similar to recent studies in freshwater mussels that yielded sequences that were readable without cloning (Johnson et al., 2018; Pfeiffer, Sharpe, Johnson, Emery, & Page, 2018; Pieri et al., 2018; Smith et al., 2019, 2018). Reliable ITS1 sequences could not be obtained for some heterozygous individuals and these individuals were excluded from phylogenetic analyses.

Molecular Analyses

Sequences were aligned with MAFFT v 7.311 (Katoh & Standley, 2013) in Mesquite v 3.31 (Maddison & Maddison, 2017) using the L-INS-I method. The protein-coding mtDNA genes were translated into amino acids to ensure absence of stop codons and indels. Phylogenetic inference was performed using MrBayes v 3.2.6 (Ronquist et al., 2012). We utilized Partionfinder v 2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) to find the best partition schemes and substitution models for the MrBayes analysis. The Bayesian information criterion (BIC) was selected and branch lengths were linked. MrBayes analyses were conducted using 2 runs of 8 chains for $3 * 10^7$ generations sampling every 1000 trees. Tracer v 1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018) was used to determine the appropriate burn-in value and ensure convergence of all parameters (ESS > 200). In addition, convergence of the two runs was monitored using the Potential Scale Reduction Factor (PSRF) and the average standard deviation of split frequencies (ASDSF). PopART v 1.7 (Leigh & Bryant, 2015) was used to create

haplotype networks for mtDNA loci (i.e., CO1 and ND1) and ITS1 using the TCS Network (Clement, Posada, & Crandall, 2000), and samples were grouped by drainage of origin (i.e., Brazos, Colorado, or Guadalupe). Nucleotide positions with gaps or missing data were not considered during creation of the haplotype networks. To further explore relationships within *F. mitchelli*, we used MEGA-X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) to compute uncorrected p-distances. All codon positions were included, ambiguous sections were handled using partial deletion, and individuals were grouped based on drainage of capture. We used MEGA-X to identify diagnostic sites that distinguish major clades of *F. mitchelli* (i.e., Brazos and Colorado, Guadalupe) at CO1, ND1, and ITS1 independently.

To estimate of divergence times among well-supported clades, we employed the coalescent-based model *BEAST (Heled & Drummond, 2010) in BEAST v 2.5.1 (Bouckaert et al., 2014). We utilized a coalescent approach considering concatenation methods typically overestimate the divergence times across species trees (Arbogast, Edwards, Wakeley, Beerli, & Slowinski, 2002; Ogilvie, Heled, Xie, & Drummond, 2016). We followed similar methodologies as Pieri et al. (2018) and created a molecular matrix including *Fusconaia* species used in BI (Table 1) and included *Pleurobema clava* (Lamarck, 1819) as the outgroup. The molecular matrix was re-aligned, and substitution models were evaluated for each locus (i.e., CO1, ND1, ITS1) using Partitionfinder. A strict molecular clock was fit to each locus and we used the CO1 substitution rate of 2.56 × 10–9 ± 0.6 × 10–9 substitutions per site per year (Froufe et al., 2016) for the CO1 partition. Substitution rates were estimated for ND1 and ITS1 based on the CO1 partition. Yule process was used as the species tree prior paired with a piecewise linear and

constant root population size model. The analysis was run for 3×10^7 generations sampling every 5000 generations and a 10% burn-in. Effective sample size (ESS) was ensured using Tracer, and a maximum clade credibility tree was created using TreeAnnotator v 2.5 (Bouckaert et al., 2014).

Morphometric Analyses

We collected morphometric data on external shell dimensions for *F. mitchelli* specimens used in genetic analyses along with museum specimens from all focal drainages (i.e., Brazos, Colorado, and Guadalupe). Three measurements were taken to the nearest 0.01 mm using digital calipers for morphological analyses: maximum length, height, and width. Measurements were $\log_e$-transformed to produce a scale-invariant matrix while preserving information about allometry (Jolicoeur, 1963; Kowalewski et al., 1997; Strauss, 1985) and subsequently converted into three ratios: height/length, width/length, and width/height. Morphological variation was assessed using a principal component analysis (PCA) in the package ggbiplot (Vu, 2011) and a canonical variate analysis (CVA) in the package Morpho (Schlager, 2017) using R v 3.5.3. PCA analyses allowed for visual inspection of morphological groupings without *a priori* assignment to a specific group. Canonical variate scores were used for cross-validated discriminant analyses (DA) to assess the ability of morphological data to assign individuals to 1) drainage of capture (i.e., Brazos, Colorado, Guadalupe) and 2) groupings depicted by molecular data (Brazos+Colorado, Guadalupe). Additionally, we used a permutational multivariate analysis of variance (MANOVA) utilizing 1000 iterations in the vegan package (Oksanen et al., 2016) to test for significant morphological differences between

the Brazos+Colorado and Guadalupe. A significance level of α=0.05 was assumed when assessing the statistical significance.

Species Delimitation

We implemented the coalescent species delimitation model STACEY v 1.2.4 (Jones, 2017) via BEAST using all available molecular data (CO1, ND1, and ITS1) for *F. mitchelli*. We used Partitionfinder to re-evaluate the best partitions and substitutions models for the STACEY analyses. We allowed the model to consider all individuals as minimum clusters and assign individuals to appropriate clusters considering STACEY infers species boundaries without *a priori* species designations. A strict molecular clock was set at 1.0 for the 1st position of CO1 for both analyses and remaining partitions were estimated by STACEY. Analyses executed 2x10^{8} generations and logged every 5000th tree and a 10% burn-in. Tracer v 1.7.1 was used to ensure convergence of all parameters (ESS > 200). The most likely number of species clusters was calculated by SpeciesDelimitationAnalyser (SpeciesDA) v 1.8.0 (Jones, 2017) using a collapse height of 0.0001 and a simcutoff of 1.0.

We integrated molecular and morphological data into a species delimitation framework using the program iBPP v. 2.1.3 (Solís-Lemus, Knowles, & Ané, 2015). This method uses the Bayesian Phylogenetics & Phylogeography (BP&P) model for coalescent species delimitation (Yang & Rannala, 2010) and integrates a Brownian motion model of trait evolution (Solís-Lemus et al., 2015). The data matrix used for the iBPP analysis consisted of all available CO1, ND1, and ITS1 sequences for members for *F. mitchelli*, as well as the PC scores for the 3 PCs created from R to represent morphological differences. For the iBPP analysis, we set the species tree topology to the

most likely species cluster scenario as resolved by STACEY (Brazos+Colorado and Guadalupe). We followed the most stringent methodologies presented by Pfeiffer et al. (2016) by using the priors $\theta \sim \Gamma(1, 10)$ and $\tau_0 \sim \Gamma(1, 10)$ for sequence data. A non-informative prior of 0 was used for the control parameters ν and κQ. Algorithm 0 was used as the species delimitation prior with an $\varepsilon = 2$ and default fine-tuning parameters (Yang & Rannala, 2010). We implemented 500,000 reversible-jump Markov chain Monte Carlo (rjMCMC) generations sampling every 10th generation with an initial burn-in of 10%. ESS>200 for all parameters was ensured for adequate generation time and convergence.

Range Map

We compiled distributional data from freshwater mussel surveys conducted from 1898-2018 in the Brazos, Colorado, and Guadalupe drainages to assess both the contemporary and historical geographic distribution of *F. mitchelli*. Sources of the distribution data were as follows: Baylor University Mayborn Museum, Florida Museum of Natural History, Fort Worth Museum of Science and History, Houston Museum of Natural Science, Joseph Britton Freshwater Mollusk Collection, Smithsonian National Museum of Natural History, Texas A&M Natural Resources Institute, Texas Department of Transportation, Texas Parks and Wildlife Department, University of Michigan Museum of Zoology, and U.S. Fish and Wildlife Service. We used distribution data for *F. mitchelli* to develop a conservation status assessment map using ArcMap 10.3 (ESRI) following protocols used in previous publications (Johnson et al., 2016; Smith et al., 2019). The spatiotemporal distribution of *F. mitchelli* was illustrated at the Hydrological

Unit Code (HUC) 10-level and all known survey locations were included to illustrate presence or absence from 1900–2018.

Results

Molecular Analyses

Our aligned molecular matrix included 2132 bp (CO1 = 658 bp; ND1 = 900 bp; ITS1 = 574 bp) from a total of 49 *F. mitchelli*: Brazos (12), Colorado (15), and Guadalupe (22). Detailed information regarding individuals and alignments used in molecular analyses are available in Table 1, GenBank (novel accessions: MN649033-MN649180), and on ScienceBase (https://doi.org/10.5066/P9Y7K5CD). Reliable ITS1 sequences for five individuals of *F. mitchelli* could not be obtained due to substantial heterozygosity and these individuals were not included in the phylogenetic reconstruction. The best partitioning scheme and substitution models determined by PartitionFinder for the MrBayes analysis were: HKY+G for CO1 codon 3, SYM+I+G for CO1 codon 1 and ND1 codon 1, HKY+I for CO1 codon 2 and ND1 codon 2, HKY+G for ND1 codon 3, and JC+G for ITS1. The phylogenetic reconstruction resolved *Fusconaia* as monophyletic and depicted two monophyletic clades within *F. mitchelli*: 1) Brazos+Colorado, and 2) Guadalupe (Fig. 4.1). The TCS networks for mtDNA and ITS1 show clear separation between the Brazos+Colorado and Guadalupe groupings (Fig. 4.2). Intra- and inter-drainage uncorrected p-distances for *F. mitchelli* as well as maximum and minimum values are reported in Table 2. Intra-drainage values for mtDNA markers ranged from 0-1.0% and there was no divergence in ITS1 for average p-distance (Table 2). For every marker, inter-drainage values for Brazos-Colorado were lower than Brazos-

Guadalupe or Colorado-Guadalupe comparisons (Table 2). *Fusconaia mitchelli* from the Brazos+Colorado were molecularly diagnosable from the Guadalupe at all three markers: CO1 (5), ND1 (13), and ITS1 (2 nucleotides and 1 indel).

Our molecular matrix used for *BEAST consisted of 60 individuals aligned to 2086 bp (CO1 = 658 bp; ND1 = 900 bp; ITS1 = 528 bp). Substitution models for each locus were: CO1 – HKY+I, ND1 – HKY+G, and ITS1 – JC. Convergence of the *BEAST analysis was supported by all parameters having ESS values > 200. The *BEAST topology was generally congruent with BI and resolved two monophyletic clades within *F. mitchelli* (i.e., Brazos+Colorado, and Guadalupe) (Fig. 4.1; Fig. 4.3). The split of *F. mitchelli* and east Texas lineages (i.e., *F. askewi* and *F. chunii*) was estimated to have occurred in the late Miocene, ~6.60 Mya (95% CI 3.78–9.76 Mya; Fig. 4.3). Subsequent diversification between *F. mitchelli* from the Brazos+Colorado and Guadalupe was estimated to have occurred ~3.18 Mya (95% CI 1.75–4.92 Mya) in the Pliocene/Pleistocene epochs (Fig. 4.3). The split between *F. mitchelli* from the Brazos and Colorado drainages was estimated to be recent, ~0.82 Mya (95% CI 0.33–1.38 Mya), during the late Pleistocene epoch (Fig. 4.3).

Morphometric Analyses

We measured 114 individuals for *F. mitchelli* from focal drainages: Brazos (17), Colorado (22), and Guadalupe (75). Detailed information regarding individuals used in the morphological dataset are available in Table S4.1 (APPENDIX; https://doi.org/10.5066/P9Y7K5CD). PC1 (70.2%) and PC2 (29.7%) eigenvalues explained 99.9% of the total variability in PCA. The PCA depicted overlap between *F. mitchelli* from the Colorado and Guadalupe drainages, while *F. mitchelli* from the Brazos

was shown to be more inflated (Fig. 4.4). Cross-validated DA scores provided an overall classification accuracy of 58.8% by drainage of capture (Brazos = 82.4%, Colorado = 31.8%, Guadalupe = 61.3%) and 71.1% for groupings supported by molecular data (Brazos+Colorado = 43.6%, Guadalupe = 85.3%). The permutational MANOVA between $\log_e$-transformed variables (i.e., H, W, and L) identified significant morphological differentiation between the Brazos+Colorado, and Guadalupe ($\alpha < 0.001$).

Species Delimitation

The molecular matrix used in the STACEY and iBPP analysis was aligned to 2076 nt (CO1 = 658 nt; ND1 = 900 nt; ITS1 = 518 nt). Five partitions and substitution models were selected for STACEY by PartitionFinder: CO1 and ND1 1st position- K80, CO1 and ND1 2nd position- F81, CO1 3rd position- HKY, ND1 3rd position- HKY, and ITS1- JC. Convergence of the STACEY analysis was indicated by all ESS values > 200. STACEY resolved the most likely species model as two species clusters: 1) Brazos+Colorado drainages; and 2) Guadalupe drainage (Fig. 4.5). Convergence of the iBPP analysis was indicated by all ESS values > 200 and iBPP strongly supported (PP=100) the two clusters (i.e., Brazos+Colorado, and Guadalupe) as distinct species (Fig. 4.5).

Range Map

During our searches of museum records and available field observations, we located collection information for 6,365 freshwater mussel observations conducted from 1898–2018 in the Brazos, Colorado, and Guadalupe River basins. Of these observations 158 were *F. mitchelli* based on shells (recently dead to subfossil; n = 102) and live

individuals (fresh dead + live; n = 56). Date of collection ranged from 1898–2016 for all observations of *F. mitchelli* (Table S2.2; APPENDIX; https://doi.org/10.5066/P9Y7K5CD). *Fusconaia mitchelli* records that could be mapped (n = 106) were distributed across 25 HUC units (Brazos 6; Colorado 12; Guadalupe 7; Fig. 4.6; Fig. S4.1; https://doi.org/10.5066/P9Y7K5CD). The status of the species in each HUC unit was categorized as follows: 20 HUCs with shell only (Brazos 4; Colorado 10; Guadalupe 6); 3 with historical records (fresh dead + live; prior to 1995; Colorado 3); and 9 with current records (fresh dead + live; 2011 to present; Brazos 3; Colorado 3; Guadalupe 3).

Taxonomic Accounts

Fusconaia mitchelli (Simpson in Dall, 1895)

COMMON NAME: False Spike

SYNONYMY:

Unio mitchelli Simpson in Dall, 1895: 5-6 [Guadelupe River, Victoria County, Texas, Hon. J. D. Mitchell; Rio Salado, near New Leon, Mexico]. Lectotype USNM128364 inadvertently selected by Johnson (1975: 15) as the "figured holotype".

Unio (sec. *Elliptio*) *mitchelli var. elongatus* Simpson, 1914: 623 [Guadalupe River, Kerr County, Texas]. Lectotype USNM251917 selected by Johnson (1975: 12).

Quadrula (*Quincuncina*) *guadalupensis* Wurtz, 1950: 2, figs. 1-5 [Guadalupe River above Seguin between Routes 123, and 90, Guadalupe County, Texas]. Holotype ANSP185974 designated by Wurtz (1950: 2) based on a single collected specimen.

The authority for *F. mitchelli* has been incorrectly referenced as Simpson in Dall, 1896 or Simpson, 1896 by numerous authors (e.g., Frierson, 1927; Howells et al., 1996; Pfeiffer et al., 2016; Simpson, 1914). The most recent assessment of North American unionid diversity (Williams et al., 2017) listed the authority for *F. mitchelli* as Simpson, 1895 which accurately reflects the date of description; however, Dall, not Simpson, is the author of the work containing the original description of *F. mitchelli*. Therefore, by recommendation 51E of the International Commission on Zoological Nomenclature (ICZN, 1999), we formally update the authority to Simpson in Dall, 1895 for *F. mitchelli*. This authority was also used by Johnson (1999).

We recognize *Unio* (sec. *Elliptio*) *mitchelli var. elongatus* and *Quadrula* (*Quincuncina*) *guadalupensis* as the only synonyms of *F. mitchelli* based on morphological characters, overlapping geographical distribution, and Principle of Priority (ICZN, 1999). Various authors have included *Sphenonaias taumilapana* (Conrad, 1855) as a synonym of *F. mitchelli* (Frierson, 1927; Howells et al., 1996; Johnson, 1999; Strecker, 1931) based on the assumption that the range of *F. mitchelli* extends west to the Rio Grande drainage. However, we agree with recent treatments that consider *S. taumilapana* a valid species endemic to the Rio Grande drainage (Graf & Cummings, 2007; Pfeiffer et al., 2016) and therefore not a synonym of *F. mitchelli*. Further, we do not include *F. iheringi* as a synonym of *F. mitchelli*, and formally elevate the taxon from synonymy.

TYPE MATERIAL: Lectotype hereby designated as USNM128364. Specimen incorrectly designated as the figured holotype by Johnson (1975: 15); however, the measurements in the original description (Simpson in Dall, 1895) match USNM128364.

Syntype USNM128364a elevated to paralectotype following Recommendation 74F of the ICZN (ICZN, 1999). Other possible paralectotypes include BV134 and MCZ165695, but the exact date and collection location of specimens cannot be confirmed at this time.

TYPE LOCALITY: Guadalupe River, Victoria County, Texas

The type locality in the original description (Simpson in Dall, 1895) was "Guadelupe River, Victoria County, Texas, Hon J.D. Mitchell; Rio Salado, near New Leon, Mexico." and the distribution of *F. mitchelli* was designated to span from "Southern Texas to New Leon, Mexico" (Simpson, 1900b). However, measurements of the type in the original description match the specimen collected from the Guadalupe River, Victoria, Texas by J.D. Mitchell. Additionally, Pfeiffer et al. (2016) considered the distribution of *F. mitchelli* restricted to the Brazos, Colorado, and Guadalupe drainages in Texas based on the assumption that specimens identified as *F. mitchelli* in New Leon, Mexico represent *S. taumilapana*. Accordingly, we restrict the type locality for *F. mitchelli* to Guadalupe River, Victoria County, Texas as specimens from the Rio Salado, near New Leon, Mexico are no longer considered *F. mitchelli* and the lectotype collected by J.D. Mitchell is from the Guadalupe River in Texas.

DISTIBUTION: *Fusconaia mitchelli* is endemic to the Guadalupe River drainage in Texas.

SHELL DESCRIPTION: Maximum length at least 68 mm (BV134). Shell moderately thick and moderately inflated. General outline of shell rhomboidal, anterior margin rounded, posterior margin truncate to bluntly pointed. Dorsal margin rounded, ventral margin straight to convex, posterior ridge moderately sharp dorsally to slightly rounded posterioventrally, posterior slope slightly concave. Umbo broad and slightly elevated

above the hinge line. Periostracum shiny, light brown to dark brown. Pseudocardinal teeth moderately thick with two in left valve and one in right valve. Lateral teeth short and well-developed, slightly curved, two in left valve and one in right valve. Interdentum short and narrow. Umbo cavity wide moderately deep. Nacre white, usually iridescent.

COMPARATIVE DIAGNOSIS: *Fusconaia mitchelli* resembles *F. iheringi* but is not syntopic with the species. *Fusconaia mitchelli* was found to be more compressed than *F. iheringi*; however, there was overlap in this character between *F. mitchelli* and *F. iheringi* from the Colorado (Fig. 4.4). *Fusconaia mitchelli* usually has a rounder posterior ridge and less shiny periostrocum when compared to *F. iheringi*. *Fusconaia mitchelli* can be distinguished from *F. iheringi* in our alignments by 5 diagnostic nucleotides at CO1 (284:C, 295:G, 313:A, 406:T, 479:C), 13 diagnostic nucleotides at ND1 (33:G, 93:G, 348:C, 403:A, 540:A, 588:T, 636:G, 643:G, 645:T, 720:C, 771:C, 801:T, 868:T), and 3 diagnostic loci at ITS1 (58:A, 90:G, 325-327:CAA/AAA).

MATERIAL EXAMINED:

Guadalupe River, Victoria County, Texas: BV134 (1), USNM128364 (1)

Geronimo Creek, Guadalupe Country, Texas: HMNS32346 (1)

Guadalupe River, Comal County, Texas: BV133 (1), BV135 (1)

Guadalupe River, DeWitt County, Texas: JBFMC8188 (9), JBFMC8233 (2), JBFMC9594 (54), UF438139 (5), UF438549 (2)

Guadalupe River, Gonzalez County, Texas: UF441081 (1), UF441082 (1), swabbed individuals (6)

Guadalupe River, Kendall County, Texas: BV144 (1), BV5287 (1)

Fusconaia iheringi (Wright, 1898)

COMMON NAME: Balcones Spike

SYNONYMY:

Unio iheringi Wright, 1898: 93 [San Saba River, Menard County, Texas]. Holotype USNM152171.

TYPE MATERIAL: Holotype USNM152171 fixed by monotypy (ICZN, 1999; Art. 73.1). Original description based on a single specimen, referred to as "type in National Museum." The same specimen was figured as the type by Simpson, 1900a: 79, pl. 4, fig. 5 and refigured and regarded as the holotype by Johnson, 1967: 7.

TYPE LOCALITY: San Saba River, Menard County, Texas

SHELL DESCRIPTION: Maximum length at least 96 mm (JBFMC8065.1). Shell moderately thick and compressed to moderately inflated. General outline of shell sub-quadrate, anterior margin rounded, posterior margin truncate to bluntly pointed. Dorsal margin straight to slightly rounded, ventral margin straight to convex, posterior ridge moderately sharp dorsally to slightly rounded posterioventrally, posterior slope slightly concave and sub-plicate to the posteriodorsal margin. Umbo narrow to broad, prominent, and slightly elevated above the hinge line. Periostracum yellowish green to brown and usually covered with coarse faint green rays. Pseudocardinal teeth moderately thick with two in left valve and one in right valve. Lateral teeth moderately short, slightly curved, two in left valve and one in right valve. Interdentum short and narrow. Umbo cavity wide and moderately deep. Nacre white, usually iridescent.

COMPARATIVE DIAGNOSIS: *Fusconaia iheringi* resembles *F. mitchelli* but is not syntopic with the species. *Fusconaia iheringi* was found to be more inflated than *F. mitchelli*; however, there was overlap in this character between *F. iheringi* from the

Colorado and *F. mitchelli* (Fig. 4.4). *Fusconaia iheringi* usually has a sharper posterior ridge and shinier periostrocum when compared to *F. mitchelli. Fusconaia iheringi* can be distinguished from *F. mitchelli* in our alignments by 5 diagnostic nucleotides at CO1 (284:T, 295:A, 313:G, 406:T, 479:C), 13 diagnostic nucleotides at ND1 (33:A, 93:G, 348:T, 403:G, 540:C, 588:C, 636:A, 643:A, 645:C, 720:T, 771:T, 801:A, 868:C), and 3 diagnostic loci at ITS1 (58:C, 90:T, 325-327:---).

DISTRIBUTION: *Fusconaia iheringi* is endemic to the Brazos and Colorado River drainages in Texas, USA. *Fusconaia iheringi* appears to be restricted to streams along the Blackland Prairie and Edwards Plateau (Fig. 4.6), including the Llano and San Saba rivers in the Colorado drainage; and Brushy Creek, San Gabriel River, and Little River in the Brazos drainage.

MATERIAL EXAMINED:

San Saba River, Menard County, Texas: BV127 (1), BV128 (1), BV129 (1), BV130 (1)

Colorado River, Travis County, Texas: BV2501 (1)

Leon River, Coryell County, Texas: BV131 (1), BV132 (1), BV5286 (1), BV6064 (1), BV6065 (1)

Llano River, Mason County, Texas: BV187 (1), BV188 (1), BV189 (1), BV190 (1), BV3552 (1), BV3553 (1), BV3554 (1), BV3555 (1), BV3556 (1), BV3557 (1), JBFMC8089 (1), JBFMC8502 (10), UF438155 (1), UF438745 (1)

Leon/Little River, Bell County, Texas: BV1544 (1), BV1545 (1)

Little River, Milam County, Texas: JBFMC8102 (3), UF439060 (4)

San Saba River, San Saba County, Texas: UF441083 (1), UF438010 (1)

San Gabriel River, Williamson County, Texas: JBFMC8065 (2), UF438156 (4)

Discussion

An integrative species concept using multiple independent lines of evidence is a powerful approach to species delimitation (De Queiroz, 2007), and this approach has been utilized with success in resolving taxonomic issues for freshwater mussels (Inoue, McQueen, Harris, & Berg, 2014; Johnson et al., 2018; Keogh & Simons, 2019; Lopes-Lima et al., 2018; Smith et al., 2019, 2018). In this study, we utilized multiple data types to re-evaluate species boundaries in *F. mitchelli*. Below, we describe how our holistic approach strongly supports the elevation of the binomial *Fusconaia iheringi* (Wright, 1898) to represent what was formerly referred to as *F. mitchelli* from the Brazos and Colorado drainages.

Species Delimitation in Fusconaia iheringi and Fusconaia mitchelli

A previous molecular assessment (Pfeiffer et al., 2016) identified two distinct clades within *F. mitchelli*, and similar to that study, our phylogenetic analyses and distance-based approaches strongly support *F. iheringi* and *F. mitchelli* as distinct species. *Fusconaia iheringi* and *F. mitchelli* were resolved as mutually exclusive based on multi-locus sequence data (Fig. 4.1), depicted a clear signal for genetic separation at both mtDNA and nDNA markers using uncorrected p-distances (Table 2), were molecularly diagnosable at all three markers, and did not share haplotypes at mtDNA or nDNA markers (Fig. 4.2). Furthermore, genetic divergence at mtDNA markers between the two species (Table 2) was greater than between congeners *F. burkei* (Walker in Ortmann & Walker, 1922) and *F. escambia* Clench & Turner, 1956 (Pfeiffer et al., 2016), and *F. askewi* (Marsh, 1896) and *F. chunii* (Lea, 1862) (Pieri et al., 2018). Despite nDNA having a slower mutation rate compared to mtDNA (Moore, 1995), *F. iheringi* and *F.*

mitchelli did not share haplotypes and were also diagnosable at ITS1 (Fig. 4.1), while *F. askewi*, *F. chunii*, and *F. flava*; and *F. burkei* and *F. escambia* independently shared ITS1 haplotypes (Pfeiffer et al., 2016; Pieri et al., 2018).

Biogeography is a critical component to species distribution and genetic divergence in freshwater mussels. Specifically, the host-parasite relationship between mussels and their host fish links their geographical distribution (Haag, 2010; Watters, 1992). Furthermore, dispersal is generally reliant on host fish, which are typically restricted by both terrestrial and marine barriers (Haag, 2012). In the case of *F. iheringi* and *F. mitchelli*, the species are specialized to parasitize freshwater fishes in the family Cyprinidae (Dudding et al., 2019), which are intolerant of marine environments (Matthews & Hill, 1977; Ostrand & Wilde, 2001) making ongoing gene flow between river drainages unlikely. If *F. iheringi* and *F. mitchelli* were conspecifics, populations in the three drainages (i.e., Brazos, Colorado, and Guadalupe) would be expected to be resolved as monophyletic with similar patterns of molecular divergence. However, phylogenetic and phylogeographic analyses using mtDNA and nDNA resolve two strongly supported groups corresponding to *F. iheringi* (Brazos+Colorado) and *F. mitchelli* (Guadalupe) differing from expected patterns based solely on intraspecific genetic drift. These biogeographic patterns mirror those of other freshwater mussel species endemic to the Edwards Plateau, including two newly described species from the Guadalupe drainage *Cyclonaias necki* Burlakova, Karatayev, Lopes-Lima, & Bogan, 2018 in Burlakova et al. 2018 and *Lampsilis bergmanni* Inoue & Randklev, 2019 in Inoue et al., 2019, further emphasizing the high levels of endemism in the Guadalupe drainage (Inoue et al., 2019; Johnson et al., 2018).

Geological processes have shaped patterns of molecular divergence in many freshwater mussels (Haag, 2010; Inoue et al., 2020; Inoue, Lang, & Berg, 2015; Smith et al., 2018) and account for the observed inconsistencies between geographic and molecular divergence in *F. iheringi* and *F. mitchelli*. Isolation of the western Gulf of Mexico drainages peaked in the late Miocene and early Pliocene (Galloway, Whiteaker, & Ganey-Curry, 2011), and subsequent climatic changes connected drainage fragments to create two 'mega-drainages': 1) Mega-Brazos (Brazos, Calcasieu, Sabine, and Trinity rivers), and 2) Mega-Colorado (Colorado and Guadalupe rivers; Blum & Hattier-Womack, 2009). The ancestral Mega-Colorado separated from the Mega-Brazos during the late Miocene, which led to the separation of lineages from central Texas (i.e., *F. iheringi* and *F. mitchelli*) and east Texas lineages (i.e., *F. askewi* and *F. chunii*; Fig. 4.3). Subsequently, the modern fluvial systems of western Gulf of Mexico drainages began to form in the Pliocene–Pleistocene epochs (Galloway et al., 2011), leading to the allopatry of *F. iheringi* and *F. mitchelli* lineages (Fig. 4.3). However, there may have been a more recent stream capture that introduced *F. iheringi* to the Brazos drainage, hence the close genetic relationship and incomplete lineage sorting between the Brazos and Colorado populations (Fig. 4.1). An equally plausible explanation is that during the last glacial low stand the Brazos and Colorado drainages were merged (Blum & Hattier-Womack, 2009), which could be the source of introduction or gene flow into the adjacent drainage. However, the lack of fossil records makes the exact pattern of biological invasion uncertain. Available museum records and contemporary distribution support that *F. iheringi* was not distributed throughout the Brazos drainage and historically occurred in streams flowing along the Blackland Prairie and Edwards Plateau (Fig. 4.6). Recent

distributional information supports a stream capture along the Edwards Plateau is likely the source of *F. iheringi* in the Brazos drainage rather than a merger of the two rivers during a lower sea level stand, which would theoretically lead to a wide-ranging distribution in the drainage. This biogeographic pattern is rare in aquatic taxa, but is also found in *Notropis amabilis* (Girard 1856), a small cyprinid with a distribution restricted to the Edwards Plateau in Texas drainages (Colorado, Guadalupe, Nueces, and Rio Grande), and a disjunct population in the San Gabriel River (Brazos drainage) along the Edwards Plateau and Blackland Prairie (Craig, Littrell, & Bonner, 2017; Hubbs, Edwards, & Garrett, 1991).

In recognizing *F. iheringi* and *F. mitchelli* we have gone beyond molecular characters and examined other lines of evidence (i.e., life history and morphological characters); however, many of these characteristics are uninformative in resolving species-level relationships in freshwater mussels. Specifically, host use and associated life history characteristics (e.g., brooding morphology, larval morphology, mode of infection) are conserved in freshwater mussels and typically only useful in the reconstruction of supra-specific relationships (Barnhart et al., 2008; Graf & Cummings, 2006; Haag, 2012; Hewitt, Wood, & Ó Foighil, 2019; Pfeiffer et al., 2019; Smith et al., 2019). This is certainly the case in *Fusconaia*, as primary host use is limited to cyprinid fishes and life history traits appear to be highly conserved across the genus (Bruenderman & Neves, 1993; Dudding et al., 2019; Haag & Warren, 2003; Neves, 1991; Ortmann, 1912, 1921; Simpson, 1914; White, Blalock-Herod, & Stewart, 2008).

External morphology has long been used by taxonomists to delineate freshwater mussels (Frierson, 1927; Simpson, 1914) and has also been integrated with molecular

data to assess species boundaries in previous studies (Inoue et al., 2019, 2014; Johnson et al., 2018; Keogh & Simons, 2019; Pieri et al., 2018; Smith et al., 2019, 2018). However, reliance on conchological characteristics have been particularly problematic within the Pleurobemini, where both generic- and species-level taxonomic hypotheses have been largely invalidated by molecular methods (Campbell & Lydeard, 2012b, 2012a; Campbell et al., 2005; Inoue et al., 2018; Pfeiffer et al., 2016; Pieri et al., 2018). Furthermore, misidentification in Pleurobemini is problematic due to high levels of interspecific morphological convergence and intraspecific variation (Williams et al., 2017; Williams, Bogan, & Garner, 2008). For example, two sympatric species in east Texas are morphologically indistinguishable (i.e. *F. chunii* and *F. flava* in the Trinity River) further emphasizing the limited morphological divergence present between *Fusconaia* spp. (Pieri et al., 2018). Aligning with these issues, our morphological analyses indicate clear overlap between groups in PCA (Fig. 4.5) and DAs had poor overall accuracy primarily due to the morphological overlap between *F. iheringi* from the Colorado and *F. mitchelli*. Although our ability to distinguish individuals among these drainages using morphometrics was limited, *F. iheringi* from the Brazos was found to be more inflated than both *F. iheringi* from the Colorado and *F. mitchelli*. This morphological divergence likely caused the strong statistical evidence for differences between *F. iheringi* and *F. mitchelli*; however, our data also suggests that morphological variation may be indicative of phenotypic plasticity rather than the presence of diagnostic morphological characters, a common phenomenon in freshwater mussels (Eagar, 1950; Ortmann, 1920). Our morphological results are similar to those in previous studies involving closely related *Fusconaia* spp. (i.e., *F. askewi*, *F. chunii*, and *F. flava*), where

there was significant overlap in shell characters yet significant statistical support for differences in shell shape (Pieri et al., 2018). The lack of morphological signal in our dataset may also be due to the scarcity of material available of *F. iheringi*, which limits a robust assessment of morphological diversity in this species.

Although morphological evidence alone was compelling, there were numerous issues with our dataset making reliance on this type of data alone problematic. We addressed these issues by integrating inference from both molecular and morphological evidence using the coalescent-based model iBPP (Solís-Lemus et al., 2015). Coalescent approaches are promising in species delimitation studies; however, the reliance on user-defined guide trees can lead to these models over-splitting species (Knowles et al., 2007; Leaché & Fujita, 2010; Olave, Solà, & Knowles, 2014; Sukumaran & Knowles, 2017; Yang & Rannala, 2010, 2014). In our analyses, we addressed this issue by employing STACEY before iBPP, which strongly supported two species clusters without *a priori* designation (i.e., *F. iheringi*, and *F. mitchelli*) similar to our other molecular approaches (Fig. 4.5). Considering the significant effects of demographic parameters on coalescent-based models (Yang & Rannala, 2010, 2014; Yang, 2015), we also utilized the most conservative priors for species delimitation presented by Pfeiffer et al. (2016). Despite conservative priors, our analyses unified the strong patterns of molecular divergence with significant morphological signal and provided decisive support (i.e., PP = 100) for the recognition of *F. iheringi* and *F. mitchelli* as distinct species. Given the results from our holistic approach for delineating species boundaries, we formally elevate the binomial *F. iheringi*.

Implications on Conservation and Management

Species conservation is largely dependent on the ability to distinguish one species from another (e.g., Inoue et al., 2019; Johnson et al., 2018; Keogh & Simons, 2019; Smith et al., 2019, 2018). Results of this study indicate the Brazos+Colorado (*F. iheringi*) and Guadalupe (*F. mitchelli*) groupings correspond to two distinct species, which has important conservation implications. First, the geographic range of *F. mitchelli* is now restricted to the Guadalupe basin. To date, stronghold subpopulations for this species occur primarily in the lower Guadalupe downstream of Gonzales, Texas (Randklev et al., 2013) and no live records of *F. mitchelli* in the upper Guadalupe have been reported (Fig. 4.6; Fig. S4.1). Second, historical records indicate *F. iheringi* has always been restricted to streams in the Brazos and Colorado river drainages flowing along the Blackland Prairie and Edwards Plateau, and not those in the coastal plain (Fig. 4.6). Based on this, the historical distribution of the species is much narrower than previously thought (Howells et al., 1996). Extant populations of *F. iheringi* are known from the Llano and San Saba rivers within the Colorado drainage; and Brushy Creek, San Gabriel River, and Little River in the Brazos drainage (Randklev et al., 2013; Randklev et al. 2017). One long-dead shell was found on the coastal plain; however, the lone record likely represents shell material transported downstream from waterways along the Blackland Prairie (Fig. 4.6). The distribution and abundance of *F. iheringi* within the Brazos and Colorado drainages is limited and stronghold subpopulations have not been identified for this species despite a significant amount of survey effort (Randklev et al., 2017, 2018). The exact causes for the rarity of *F. iheringi* are unknown but likely stem from changes in hydrology due to anthropogenic impacts such as groundwater pumping and increased

severity of droughts and floods brought about by ongoing climate change (Randklev et al., 2018).

The dependency on host fish exacerbates conservation concerns in all freshwater mussels, as they are threatened by actions directly impacting both mussels and host fish populations (Haag, 2012). *Cyprinella lutrensis* and *C. venusta* were identified as putative host fish for *F. mitchelli* (Dudding et al., 2019); however, multiple enigmatic questions remain regarding the early life history for both *F. iheringi* and *F. mitchelli*. Primarily, host use has not been confirmed for *F. iheringi* and is critical toward understanding the basic biology of the species. Additionally, ecological hosts (i.e., natural infections) have not been confirmed for *F. mitchelli* and many sympatric minnow species have not been tested for host suitability (e.g., *Notropis* spp.). Until thorough information is available for *F. iheringi* and *F. mitchelli*, it is uncertain if the status of host fish populations is contributing to imperilment.

The geographic distribution of mussels is largely shaped by host specificity and the movement of host fish during larval encystment; therefore, barriers preventing the movement of the host fish also disrupts the dispersal of mussels (Barnhart et al., 2008; Haag, 2012; Hoffman, Willoughby, Swanson, Pangle, & Zanatta, 2017; Strayer, 2008; Watters, 1992). This is certainly the case for *F. iheringi* and *F. mitchelli*, as the species are both presumably host specialists with glochidia exclusively transforming on cyprinids (Dudding et al., 2019). Typically, cyprinids have a small home range and limited dispersal capabilities (Chase, Caldwell, Carleton, Gould, & Hobbs, 2015; Johnston, 2000), making ongoing gene flow between suitable habitat patches in anthropogenically affected systems unlikely. These factors make both *F. iheringi* and *F. mitchelli*

susceptible to localized extirpation and it is likely that population recovery will only be possible through reintroduction using captive propagation or other human-mediated recovery efforts. Before these types of recovery actions are performed, comprehensive genetic management plans should be developed to ensure population viability and sustainability (McMurray & Roe, 2017). Our molecular data does not show significant evidence of intra-drainage population structuring (Fig. 4.2); however, more rapidly evolving nuclear markers (i.e., genotype-by-sequencing, microsatellites, whole-genome resequencing) will facilitate further evaluation of population structure, connectivity, genetic diversity, and viability of extant populations.

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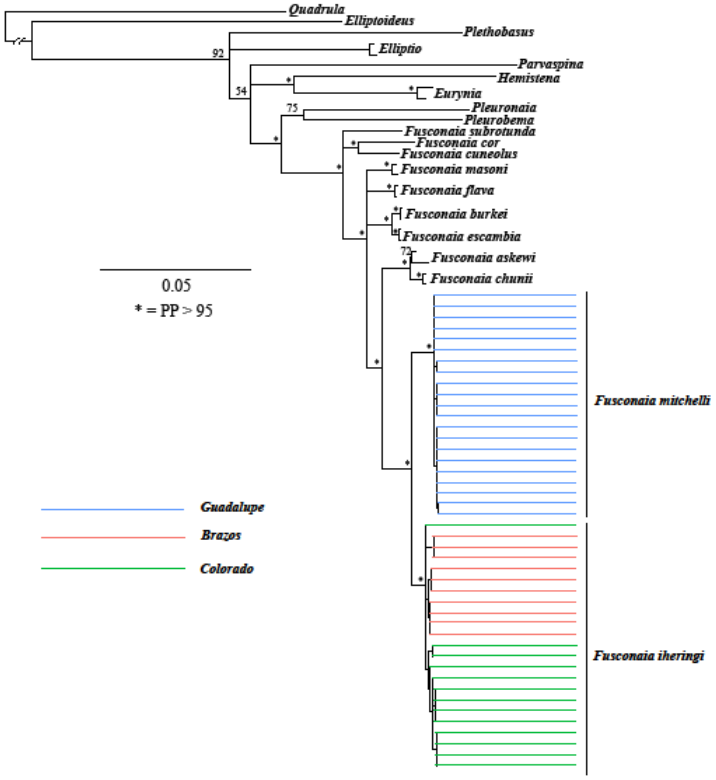

Figure 4.1. Bayesian inference optimal topology generated using MrBayes on a concatenated molecular matrix. Node labels indicate posterior probability (PP) and significant support represented by PP>95. Each line represents an individual of *Fusconaia iheringi* or *Fusconaia mitchelli* sampled and colors correspond to drainage of capture: red (*Fusconaia iheringi* - Brazos), green (*Fusconaia iheringi* - Colorado), and blue (*Fusconaia mitchelli* - Guadalupe).

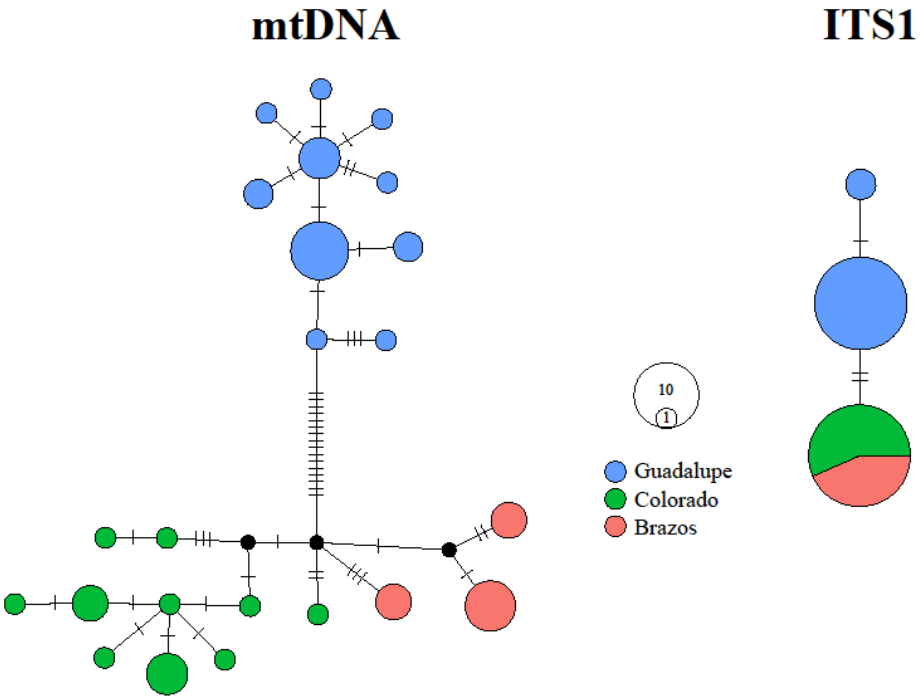

Figure 4.2. Haplotype network generated from mitochondrial DNA (CO1 and ND1), and ITS1 for *Fusconaia iheringi* and *Fusconaia mitchelli*. Dashes represent the number of substitutions between haplotypes, black circles indicate an unsampled haplotype, and colored circles represents a unique haplotype with size relative to the number of individuals with each haplotype. Colors indicate drainage of capture: red (*Fusconaia iheringi* - Brazos), green (*Fusconaia iheringi* - Colorado), and blue (*Fusconaia mitchelli* - Guadalupe).

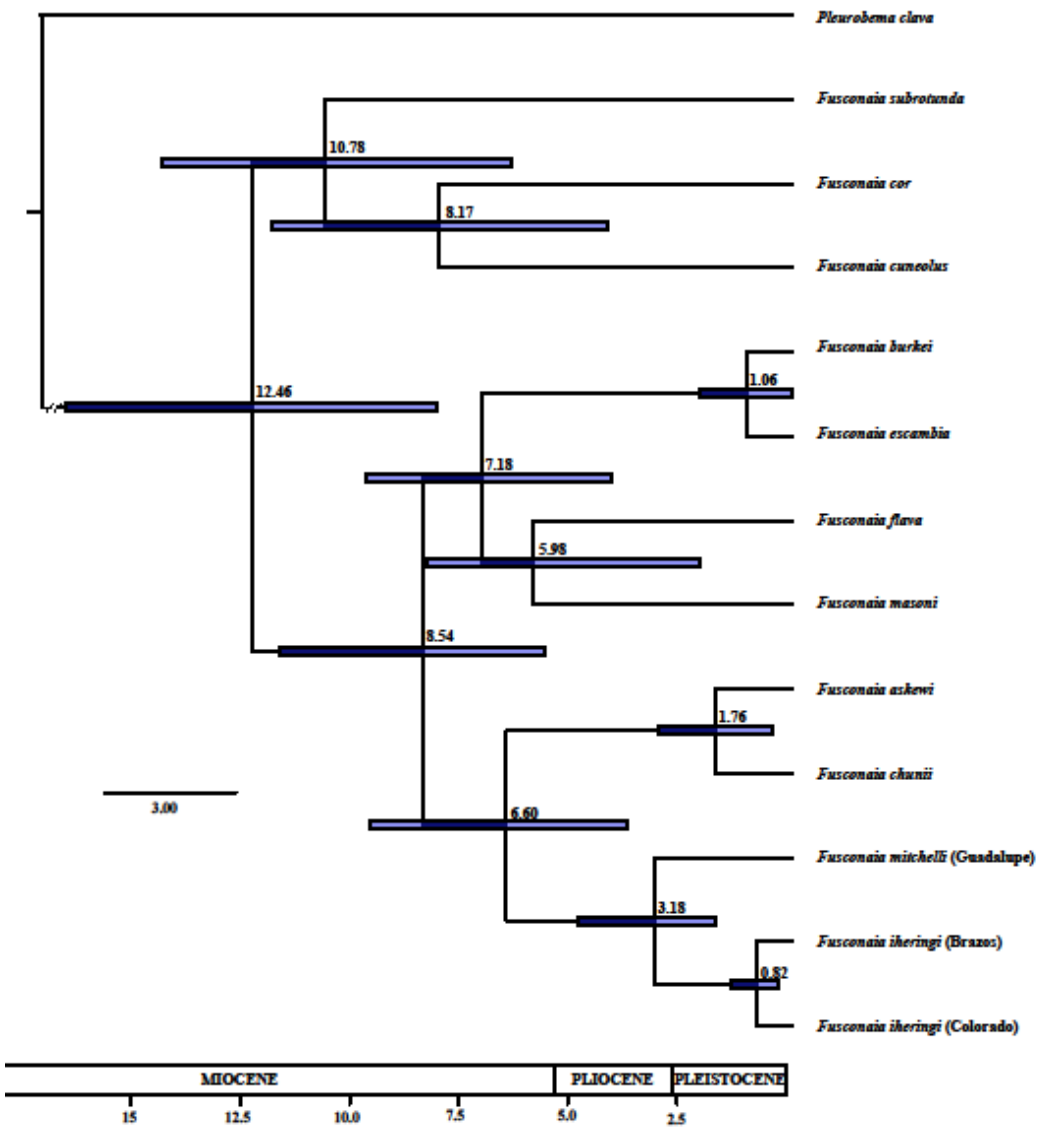

Figure 4.3. Maximum clade credible tree generated from divergence time estimations in *BEAST. Divergence time is scaled to million years before present and node bars represent the 95% CI.

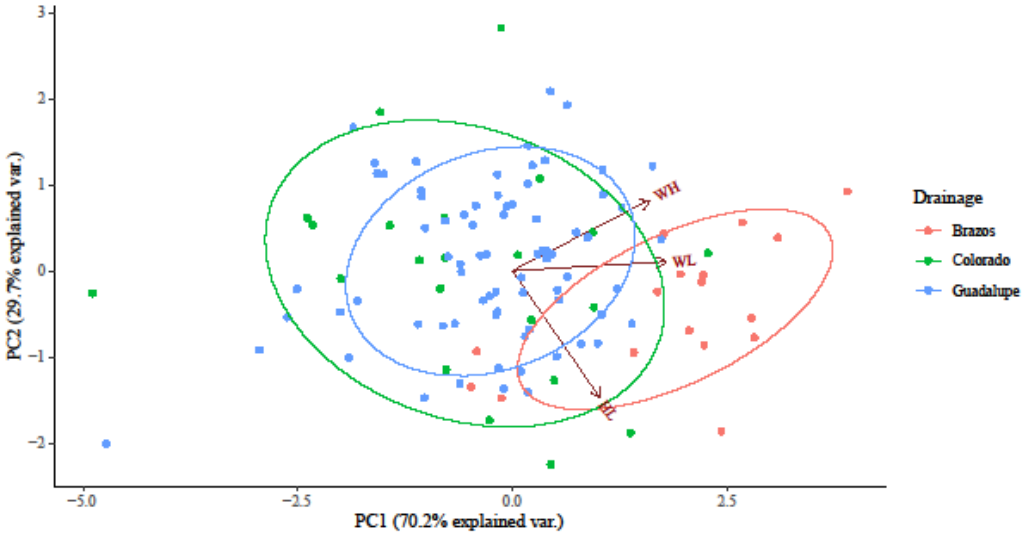

Figure 4.4. PCA biplots from morphometric data with 95% CI ellipses and arrows for biplot variables (HL = height/length, WL = width/length, WH = width/height). Colors indicate the drainage of capture: red (*Fusconaia iheringi* - Brazos), green (*Fusconaia iheringi* - Colorado), and blue (*Fusconaia mitchelli* - Guadalupe).

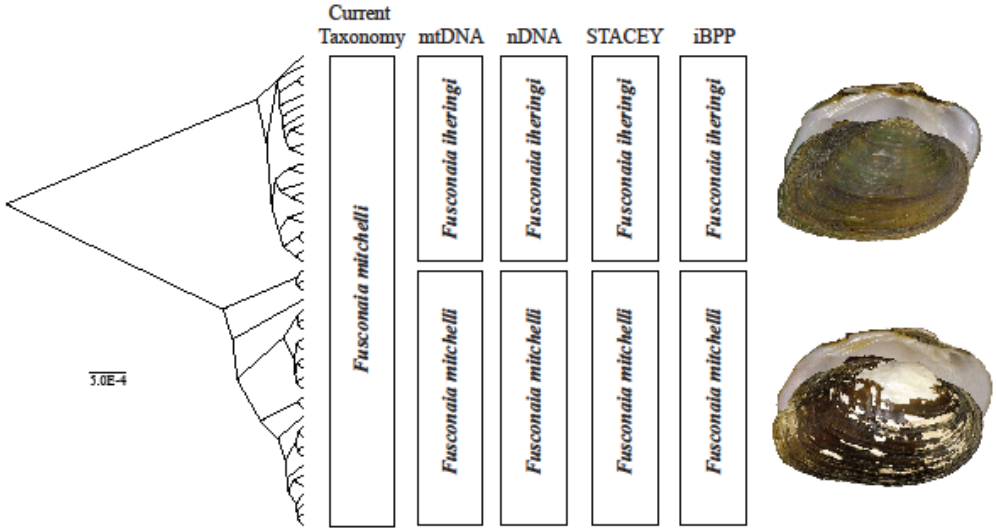

Figure 4.5. Summary of data types collected in this study and the STACEY phylogenetic reconstruction used to guide iBPP analyses. Photographs of shells represent the Holotype of *Fusconaia iheringi* (USNM152171) and Lectotype of *Fusconaia mitchelli* (USNM128364).

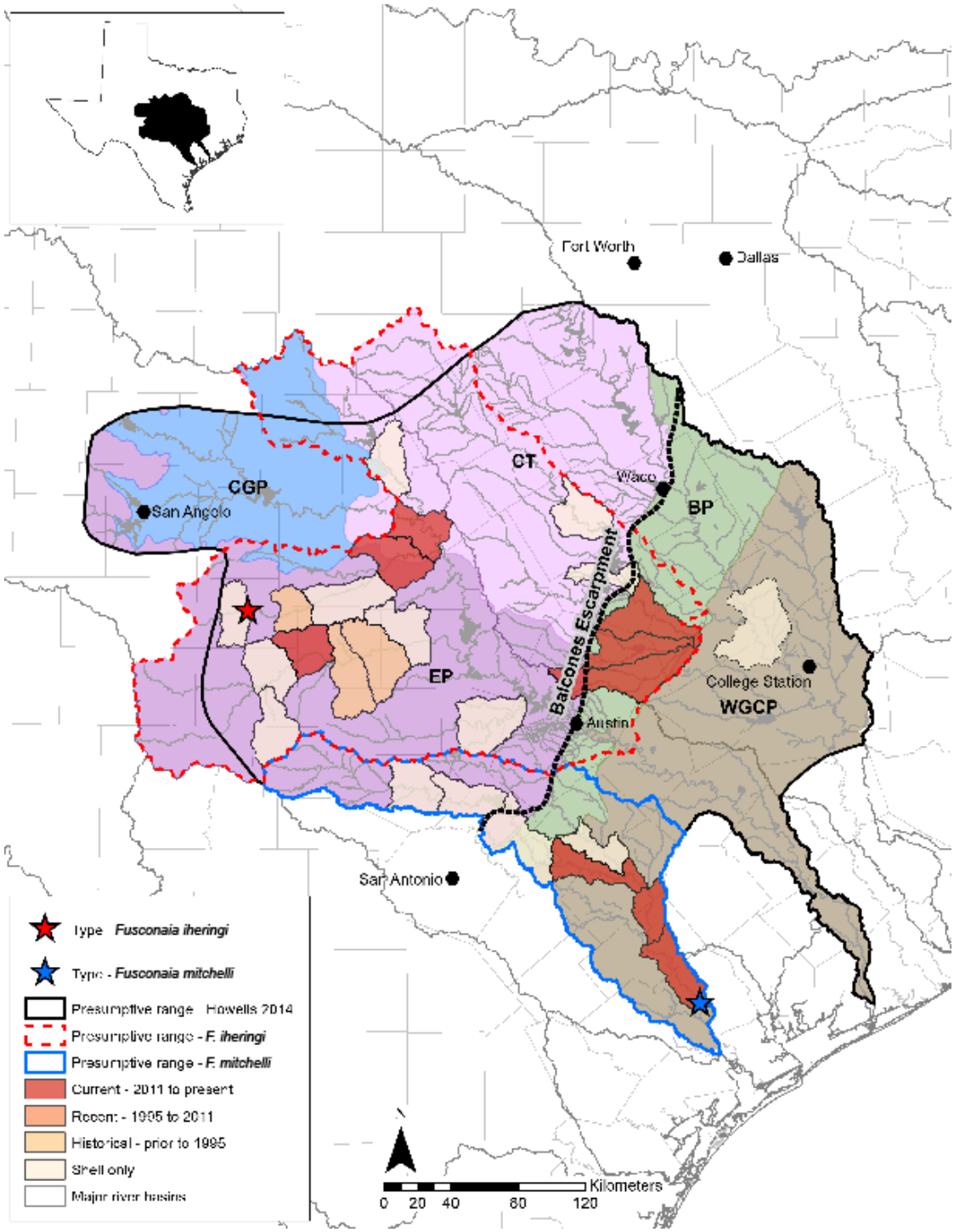

Figure 4.6. Conservation status map for *Fusconaia iheringi* and *Fusconaia mitchelli*. Hydrologic Unit Codes (HUC) 10-level are colored to distinguish between live and shell only records. For the former, HUCs are further shaded by when a live specimen of *F. iheringi* or *F. mitchelli* was collected. The presumptive ranges for *F. iheringi* and *F. mitchelli* are denoted by the dashed red line and solid blue line, respectively. Type localities for *F. iheringi* and *F. mitchelli* are represented by red and blue stars, respectively. Ecoregion designations follow Griffith et al. (2007): Blackland Prairie (BP), Central Great Plains (CGP), Cross Timbers (CT), Edwards Plateau (EP), and Western Gulf Coastal Plain (WGCP).

Tables

Table 4.1. Molecular material examined in this study with indication of river drainage where specimens were collected, catalog numbers, and GenBank accession numbers. Museum abbreviations are as follows: (JBFMC – Joseph Britton Freshwater Mollusk Collection; NCSM – North Carolina Museum of Natural Sciences; UF – Florida Museum of Natural History). NAs represent individuals collected using non-lethal methods or concatenated sequences from GenBank. Novel data generated in this study are represented by GenBank accessions MN649033- MN649180.

Taxon	Drainage	Source	CO1	ND1	ITS1
Elliptio crassidens (Lamarck, 1819)	Ohio	UF441250	MH633634	MH633586	MH362521
Elliptio crassidens	Ohio	UF441250	KT285622	MN649089	KT285666
Elliptoideus sloatianus (Lea, 1840)	Apalachicola	UF441118	KT285623	MN649081	KT285667
Eurynia dilatata (Rafinesque, 1820)	Tennessee	UF441302	MN649035	MN649084	MN649140
Eurynia dilatata	Tennessee	UF441302	MN649036	MN649085	MN649141
Fusconaia askewi (Marsh, 1896)	Sabine	UF441160	MF961824	MH133663	MH133813
Fusconaia askewi	Sabine	UF441253	KT285625	MH133668	KT285669
Fusconaia burkei (Walker, 1922)	Choctawhatchee	UF441129	KT285628	MH133770	KT285672
Fusconaia burkei	Choctawhatchee	UF441129	MN649034	MN649083	MN649139
Fusconaia chunii (Lea, 1862)	Trinity	UF439075	MF961853	MH133715	MH133855
Fusconaia chunii	Trinity	UF439075	MF961854	MH133716	MH133856
Fusconaia cor (Conrad, 1834)	NA	GenBank	HM230369	KT187953	KT188104
Fusconaia cuneolus (Lea, 1840)	NA	GenBank	AY654998	KT187960	KT188107
Fusconaia escambia Clench and Turner, 1956	Escambia	UF428548	KT285631	MH133772	KT285675
Fusconaia escambia	Escambia	UF428548	MN649040	MN649090	MN649145
Fusconaia flava (Rafinesque, 1820)	Red	UF375436	KT285634	MH133764	KT285678
Fusconaia flava	Red	UF375436	KT285636	MH133765	KT285680
Fusconaia iheringi (Wright, 1898)	Brazos	UF438156	KT285638	MN649099	KT285682
Fusconaia iheringi	Brazos	UF438156	KT285639	MN649100	KT285683
Fusconaia iheringi	Brazos	UF438156	MN649045	MN649101	MN649150
Fusconaia iheringi	Brazos	UF438156	KT285637	MN649102	KT285681
Fusconaia iheringi	Brazos	UF439060	MN649053	MN649110	MN649158
Fusconaia iheringi	Brazos	UF439060	MN649054	MN649111	MN649159
Fusconaia iheringi	Brazos	UF439060	MN649055	MN649112	MN649160
Fusconaia iheringi	Brazos	UF439060	MN649056	MN649113	MN649161
Fusconaia iheringi	Brazos	JBFMC8065	MN649078	MN649135	-
Fusconaia iheringi	Brazos	JBFMC8065	MN649079	MN649136	-
Fusconaia iheringi	Brazos	JBFMC8102	MN649057	MN649114	MN649162
Fusconaia iheringi	Brazos	JBFMC8102	MN649058	MN649115	MN649163
Fusconaia iheringi	Colorado	UF441083	MN649076	MN649133	-

Fusconaia iheringi	Colorado	UF438010	KT285650	MN649091	KT285694
Fusconaia iheringi	Colorado	UF438155	KT285640	MN649098	KT285684
Fusconaia iheringi	Colorado	UF438745	MN649052	MN649109	MN649157
Fusconaia iheringi	Colorado	JBFMC8089	MN649080	MN649137	-
Fusconaia iheringi	Colorado	JBFMC8502	MN649066	MN649123	MN649171
Fusconaia iheringi	Colorado	JBFMC8502	MN649067	MN649124	MN649172
Fusconaia iheringi	Colorado	JBFMC8502	MN649068	MN649125	MN649173
Fusconaia iheringi	Colorado	JBFMC8502	MN649069	MN649126	MN649174
Fusconaia iheringi	Colorado	JBFMC8502	MN649070	MN649127	MN649175
Fusconaia iheringi	Colorado	JBFMC8502	MN649071	MN649128	MN649176
Fusconaia iheringi	Colorado	JBFMC8502	MN649072	MN649129	MN649177
Fusconaia iheringi	Colorado	JBFMC8502	MN649073	MN649130	MN649178
Fusconaia iheringi	Colorado	JBFMC8502	MN649074	MN649131	MN649179
Fusconaia iheringi	Colorado	JBFMC8502	MN649075	MN649132	MN649180
Fusconaia masoni (Conrad, 1834)	Neuse	UF438274	MF961941	MH133773	MH133892
Fusconaia masoni	Pamlico	UF438289	MF961942	MH133774	MH133893
Fusconaia mitchelli (Simpson in Dall, 1895)	Guadalupe	UF441081	KT285651	MH133775	KT285695
Fusconaia mitchelli	Guadalupe	UF441082	KT285652	MH133776	KT285696
Fusconaia mitchelli	Guadalupe	Swab	MN649041	MN649092	MN649146
Fusconaia mitchelli	Guadalupe	Swab	MN649042	MN649093	MN649147
Fusconaia mitchelli	Guadalupe	Swab	MN649043	MN649094	MN649148
Fusconaia mitchelli	Guadalupe	Swab	KT285653	MN649095	KT285697
Fusconaia mitchelli	Guadalupe	Swab	KT285654	MN649096	KT285698
Fusconaia mitchelli	Guadalupe	Swab	MN649044	MN649097	MN649149
Fusconaia mitchelli	Guadalupe	UF438139	MN649046	MN649103	MN649151
Fusconaia mitchelli	Guadalupe	UF438139	MN649047	MN649104	MN649152
Fusconaia mitchelli	Guadalupe	UF438139	MN649048	MN649105	MN649153
Fusconaia mitchelli	Guadalupe	UF438139	MN649049	MN649106	MN649154
Fusconaia mitchelli	Guadalupe	UF438139	MN649077	MN649134	-
Fusconaia mitchelli	Guadalupe	UF438549	MN649050	MN649107	MN649155
Fusconaia mitchelli	Guadalupe	UF438549	MN649051	MN649108	MN649156
Fusconaia mitchelli	Guadalupe	JBFMC8188	MN649059	MN649116	MN649164
Fusconaia mitchelli	Guadalupe	JBFMC8188	MN649060	MN649117	MN649165
Fusconaia mitchelli	Guadalupe	JBFMC8188	MN649061	MN649118	MN649166
Fusconaia mitchelli	Guadalupe	JBFMC8188	MN649062	MN649119	MN649167
Fusconaia mitchelli	Guadalupe	JBFMC8188	MN649063	MN649120	MN649168
Fusconaia mitchelli	Guadalupe	JBFMC8233	MN649064	MN649121	MN649169
Fusconaia mitchelli	Guadalupe	JBFMC8233	MN649065	MN649122	MN649170
Fusconaia subrotunda (Lea, 1831)	NA	GenBank	HM230405	KT187998	KT188110
Hemistena lata (Rafinesque, 1820)	Tennessee	UF439083	MN649038	MN649087	MN649143
Parvaspina steinstansana (Johnson and Clarke, 1983)	Pamlico	NCSM43401	MN649033	MN649082	MN649138
Plethobasus cyphyus (Rafinesque, 1820)	Clinch	Swab	MN649039	MN649088	MN649144
Pleurobema clava (Lamarck, 1819)	NA	GenBank	AY655013	AY613802	DQ383449

Pleuronaia barnesiana (Lea, 1838)	Tennessee	UF438232	MN649037	MN649086	MN649142
Quadrula quadrula (Rafinesque, 1820)	Ohio	UF439156	MH633643	MH633595	MH362613

Table 4.2. Intra- and inter-drainage uncorrected p-distance for *Fusconaia iheringi* and *Fusconaia mitchelli*. Pairwise genetic distances are reported as mean (min-max).

Drainage	Locus	Intra-drainage	Comparison	Locus	Inter-drainage
Brazos	CO1	0.003 (0-0.009)	Brazos ~ Colorado	CO1	0.008 (0.005-0.013)
	ND1	0.002 (0-0.004)		ND1	0.002 (0.001-0.005)
	ITS1	0		ITS1	0
Colorado	CO1	0.004 (0 -0.010)	Brazos ~ Guadalupe	CO1	0.016 (0.014-0.017)
	ND1	0.002 (0-0.003)		ND1	0.018 (0.015-0.020)
	ITS1	0		ITS1	0.004 (0.004-0.006)
Guadalupe	CO1	0.001 (0-0.005)	Colorado ~ Guadalupe	CO1	0.019 (0.015-0.021)
	ND1	0.002 (0-0.007)		ND1	0.017 (0.014-0.021)
	ITS1	0.001 (0-0.006)		ITS1	0.004 (0.004-0.006)

CHAPTER FIVE

A Comparative Phylogeographic Approach to Facilitate Recovery of an Imperiled Freshwater Mussel (Bivalvia: Unionida: *Potamilus inflatus*)

This chapter accepted for publication as: Smith, C.H., Johnson, N.A. A Comparative Phylogeographic Approach to Facilitate Recovery of an Imperiled Freshwater Mussel (Bivalvia: Unionida: *Potamilus inflatus*) [Freshwater Mollusk Conservation]. *Diversity*, Accepted.

Abstract

North American freshwaters are among the world's most threatened ecosystems, and freshwater mussels are the most imperiled inhabiting these systems. A critical aspect of conservation biology is delineating patterns of genetic diversity, which can be difficult when a taxon has been extirpated from a significant portion of its historical range. In such cases, evaluating conservation and recovery options may benefit from the use of surrogate species as proxies when assessing overall patterns of genetic diversity. Here, we integrate the premise of surrogate species into a comparative phylogeographic approach to hypothesize genetic relationships between extant and extirpated populations of *Potamilus inflatus* by characterizing genetic structure in co-distributed congeners with similar life histories. Mitochondrial and nuclear sequence data showed variable patterns of genetic divergence between *Potamilus* spp. native to the Mobile and Pascagoula+Pearl+Pontchartrain (PPP) provinces. However, hierarchical Approximate Bayesian Computation indicated diversification between Mobile and PPP clusters was synchronous and represents a genetic signature of a common history of vicariance. Recent fluctuations in sea-level appear to have caused populations of *Potamilus* spp. in

the PPP to be clustered as one grouping, providing justification for using the Amite River population as a source of broodstock to re-establish extirpated populations of *P. inflatus*. Given the imperilment status of freshwater mussel species globally, our study represents a novel and useful methodology for predicting relationships among extant and extirpated populations. Future studies utilizing archaeological, eDNA, and genome-wide molecular data are essential to better understand the distribution of *P. inflatus* and establish robust genetic management plans.

Introduction

Due to anthropogenic alterations to the environment, the world is losing species comparable to mass extinctions during major transitions of geological time periods (Butchart et al., 2010; Rands et al., 2010). North American freshwaters are among the world's most threatened ecosystems (Dudgeon et al., 2006), and freshwater mussels (Bivalvia: Unionida) are the most imperiled group of organisms inhabiting these systems with 65% of all recognized species considered of conservation concern (Haag & Williams, 2014; Strayer et al., 2004; Williams et al., 1993). Several inherent biological characters (e.g., limited locomotive capabilities in many species, extreme sensitivity to pollutants, obligate parasitism, and filter feeding) have disproportionately impacted mussels in anthropogenically dominated landscapes (Bringolf et al., 2007; Wang et al., 2007; Watters, 1993), leading to extensive population decline of both common and rare species (Haag & Williams, 2014; Vaughn & Taylor, 1999; Williams et al., 1993). Given these declines, establishing robust species-specific status assessments is essential toward future implementation of effective conservation and recovery strategies for these highly imperiled organisms (Ferreira-Rodríguez et al., 2019; Haag & Williams, 2014).

One critical aspect of conservation biology is delineating patterns of genetic diversity across geographic ranges of species (Allendorf et al., 2013). In general, freshwater organisms have unique biogeographic constraints as they are restricted by both terrestrial and marine barriers. Thus, dispersal between watersheds is primarily limited to connectivity of freshwaters during rare geologic events and often leaves unique genetic signatures (Oaks, 2014; Unmack, 2001). Comparative phylogeographic approaches offer options for resolving the effects of geological processes on observed genetic diversity in co-distributed taxa with similar life histories (Hickerson et al., 2010; Moritz & Faith, 1998). Multiple studies have used comparative phylogeography to resolve the evolutionary history of aquatic taxa in the southeastern United States and showed concordance in phylogeographic clustering across co-distributed taxa (Avise, 1992; Bermingham & Avise, 1986; Walker & Avise, 1998). However, these examples have concentrated on relatively common species, and determining relationships among populations of imperiled species can be problematic when taxa have been extirpated from a significant portion of their historical range. The use of surrogate species is increasingly being used in conservation practices of rare species (Grantham et al., 2010), but this practice has not been explored in many freshwater taxa (Stewart et al., 2018), or to our knowledge, within a comparative phylogeographic framework. Here, we explore the use of comparative phylogeography for hypothesizing relationships among extant and extirpated populations of imperiled aquatic species by characterizing genetic structure in co-distributed taxa with congruent life histories.

The freshwater mussel genus *Potamilus* is a highly specialized group of freshwater mussels consisting of ten currently recognized species (Smith et al., 2020;

Williams et al., 2017). All species in this genus have similar life history characteristics, including long-term brooding of larvae, miniaturized larvae, larval growth during encystment, and specialized infection of *Aplodinotus grunniens* (Haag, 2012; Smith et al., 2020). One species, *Potamilus inflatus*, is listed as threatened under the Endangered Species Act (ESA; USFWS, 1990) and was historically distributed throughout the Mobile, Pearl, and Lake Pontchartrain drainages (R. L. Jones et al., 2019; Williams et al., 2008). Systematic habitat destruction has extirpated the species from much of its historical range and extant populations are restricted to the Tombigbee and Black Warrior rivers in the Mobile Basin, and a 40 km-long stretch of the Amite River in the Lake Pontchartrain drainage (Brown & Daniel, 2014; Hartfield, 1988). Concomitant to extirpation throughout large portions of the Lake Pontchartrain drainage, *P. inflatus* is believed to be extirpated from the entire Pearl River system (Hartfield, 1988; USFWS, 2014). Only two live individual have ever been collected (Frierson 1911; MMNS13211) and only three dead shells have subsequently been collected (George & Reine, 1996) despite extensive surveys throughout the basin (Brown et al., 2010; Brown & Banks, 2001). Further, a mill spill in 2011 led to extensive fish and mussel kills (estimated total - 591,561 fish and mussels) throughout the presumptive range of *P. inflatus* in the Pearl River, however, no specimens of *P. inflatus* were salvaged (Brown & Daniel, 2012; LDWF, 2011).

Understanding genetic diversity across populations of *P. inflatus* is critical to determine threats to extant populations and establishing effective recovery plans to re-establish the species throughout its historical range. This problem is of the upmost importance given the threatened status of *P. inflatus* under the ESA and the possibility of

recovery if viable populations are re-established where presumed extirpated (USFWS, 2014). To facilitate conservation and recovery, we use phylogeographic techniques to evaluate range-wide genetic diversity within *P. inflatus* as well as sympatric congeners *Potamilus fragilis* and *Potamilus purpuratus* using multi-locus sequence data. Next, we utilized *P. fragilis* and *P. purpuratus* as surrogate species to hypothesize the genetic relationships between extirpated and extant populations of *P. inflatus* to better inform conservation and recovery planning.

Materials and Methods

Taxon Sampling

We examined genetic diversity from co-distributed members of *Potamilus* native to the Mobile, Pascagoula, Pearl, and Pontchartrain drainages (Table 5.1; Fig. 5.1). Genomic DNA was extracted from mantle tissue clips stored in cell lysis buffer using the PureGene DNA extraction kit with the standard extraction protocol (Gentra Systems, Inc., Minneapolis, MN, USA). We amplified and sequenced two mitochondrial (mtDNA) loci commonly used in freshwater mussel phylogenetic studies: a partial portion of *cytochrome c oxidase subunit 1* (CO1), *NADH dehydrogenase subunit 1* (ND1). For a subset of individuals, we sequenced three nuclear (nDNA) loci: the commonly used nuclear-encoded *ribosomal internal transcribed spacer 1* (ITS1), and two additional protein-coding loci *Fem-1 like protein C* (FEM1) and *UbiA prenyltransferase domain-containing protein 1* (UBiA). We developed two novel primer sets to amplify FEM1 and UBiA based on data generated in phylogenetic studies using the recently developed AHE probe set Unioverse (Pfeiffer et al., 2019; Smith et al., 2020). Primers for all loci and

thermal cycling conditions for CO1, ND1, and ITS1 are reported in Table 5.2. Thermal cycling conditions for FEM1 and UBiA were as follows: an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 51/60°C (FEM1/UBiA) for 30 s, and 72°C for 1 min and 30 s.

PCRs were conducted using a 25 µl mixture of the following: molecular grade water (9.5 µl), MyTaq^{TM} Red Mix (12.5 µl; Bioline), primers (1.0 µl each) and DNA template (100 ng). Products were sent to Molecular Cloning Laboratories (McLAB, South San Francisco, CA, USA) for bi-directional sequencing on an ABI 3730. Geneious v 10.2.3 was used to assemble contigs and edit chromatograms (Kearse et al., 2012), and sequences were aligned in Mesquite v 3.31 (Maddison & Maddison, 2017) using MAFFT v 7.311 (Katoh & Standley, 2013). Loci were aligned independently using the L-INS-i method in MAFFT and translated into amino acids to ensure absence of stop codons and gaps. Incomplete codons at each terminal end were removed.

Phylogenetic Analyses

Phylogenetic reconstruction was performed on a concatenated alignment of individuals represented by all five loci using IQ-TREE v 2.0-rc1 (Chernomor et al., 2016; Minh et al., 2020). Both mtDNA and nDNA protein coding genes were partitioned by codon position. Partitions and substitution models for the analysis were determined by ModelFinder (Kalyaanamoorthy et al., 2017) using Bayesian inference criteria. We used 10 independent runs of an initial tree search and 1,000 ultrafast bootstrap replicates (BS) for nodal support (Hoang et al., 2018).

Coalescent-based approaches have been repeatably criticized to delimit populations and not species (Sukumaran & Knowles, 2017), including in freshwater

mussels (Pfeiffer et al., 2016; Smith et al., 2018, 2019). However, this methodology is promising toward the designation of population clusters from genetic data (Sukumaran & Knowles, 2017); and here, we use the Bayesian coalescent-based model STACEY (G. Jones, 2017) in BEAST v 2.6.2 (Bouckaert et al., 2014) to define clusters in our molecular dataset for downstream analysis. STACEY allows for the inclusion of individuals with missing data, so we included all available data for the 5 loci in the analysis. *Potamilus* spp. were binned by drainage of capture, and we allowed the model to freely assign drainages to appropriate clusters. A substitution model for each locus alignment was determined using ModelFinder, a strict molecular clock was set at 1.0 for CO1, and clock rates for the four additional loci were estimated by STACEY. The Epi Tree prior was used as the species tree prior with a collapse height of 0.0001. Our analyses executed 10^9 generations and logged every 5000 trees with an initial 10% burn-in. Effective sample size (ESS) was ensured using Tracer v 1.7 (Bouckaert et al., 2014), and the most likely number of clusters was calculated by SpeciesDelimitationAnalyser (SpeciesDA) v 1.8.0 (G. Jones, 2017) with a collapse height of 0.0001, a 1.0 simcutoff, and an initial 10% burn-in (2000 trees).

To estimate divergence times among well supported clusters, we used the Bayesian coalescent-based model *BEAST (Heled & Drummond, 2010) in BEAST. We chose a coalescent approach to account for concatenation methods, which typically overestimate the divergence times across species trees (Arbogast et al., 2002; Ogilvie et al., 2016). Similar to STACEY, *BEAST allows for the inclusion of individuals with missing data and all available data for the five loci in the analysis. For each species, individuals were grouped based on the most likely clusters resolved by STACEY: 1)

Mobile; and 2) Pascagoula+Pearl+Pontchartrain (herein referred to as PPP). A strict molecular clock and an HKY model of nucleotide evolution was fit to each locus to better match priors for comparative phylogeographic analyses (see below). The substitution rate for CO1 was set to $2.56 \times 10^{-9} \pm 0.6 \times 10^{-9}$ substitutions per site per year (Froufe et al., 2016), and substitution rates were estimated for the four additional loci. Yule process was used as the species tree prior paired with a piecewise linear and constant root population size model. The analysis was run for $1.5*10^{9}$ MCMC generations sampling every 5000 generations and a 10% burn-in. Effective sample size (ESS) was ensured using Tracer v 1.7 (Bouckaert et al., 2014), and a maximum clade credibility tree was created using TreeAnnotator v 2.5 (Bouckaert et al., 2014).

Phylogeographic Analyses

To visualize genetic divergence with respect to geographic distribution, we created a median joining haplotype network (Bandelt et al., 1999) for each of the three *Potamilus* spp. independently in PopART 1.7 (Leigh & Bryant, 2015) with the default epsilon value set at 0. Additionally, an analysis of molecular variance (AMOVA) was conducted for each species independently in PopART to further evaluate genetic diversity with regard to geography. Each analysis was performed on a concatenated alignment of CO1 and ND1, and missing data in both PopART analyses was handled using complete deletion. To further assess genetic variation within *Potamilus* spp. with regard to geography, we calculated DNA sequence divergence between groups of *Potamilus* spp. using uncorrected pairwise genetic distances in MEGAX (Kumar et al., 2018). Partial deletion was used to handle missing data in MEGAX calculations. For haplotype networks, species were grouped by drainage and groups for all other analyses were as

follows: *P. fragilis* from the Mobile and Pearl+Pontchartrain, *P. inflatus* from the Mobile and Pontchartrain, and *P. purpuratus* from the Mobile and PPP.

Comparative Phylogeography

We tested for simultaneous divergence between clusters of *Potamilus* spp. defined by STACEY under a hierarchical Approximate Bayesian Computation (hABC) approach as implemented in the PyMsBayes package (Oaks et al., 2014). Specifically, we tested if divergence between Mobile and PPP clusters of *P. fragilis*, *P. inflatus*, and *P. purpuratus* was synchronous. PyMsBayes implements a modified version of *msBayes* (Huang et al., 2011) that specifies a Dirichlet-process prior (*dpp*) to compare fit of empirical data to simulated data under user-informed priors (Oaks, 2014). We used *dpp-msbayes* to test for synchronous divergence between Mobile and PPP clusters of *Potamilus* spp. using alignments from all available loci. We used results from our *BEAST divergence time analysis to guide prior selection for *dpp-msbayes* as follows: the concentration parameter [1000, 0.00141] in which there was prior probability for one or two, or three divergence events, population size (θ) [1, 0.0005], and divergence times (τ) [1, 0.01]. To allow *dpp-msbayes* to freely explore different divergence scenarios, we allowed the model to estimate independent parameters for each species (θ parameter = 012) and the number of divergence events (τ classes = 0). Transition-transversion rate of the HKY substitution model was estimated for each alignment independently using IQ-TREE. Our *dpp-msbayes* run performed a total of 10^7 simulations with 10,000 standardizing samples and reported every 20,000 simulations. We retained the 1000 simulations with the best fit to empirical data to estimate posterior probability (PP) values for each divergence scenario.

To measure support for the number of divergence events, Bayes factors were measured using twice the difference of $-ln$ likelihood (Kass & Raftery, 1995).

Results

Molecular Analyses

Our five-locus concatenated molecular matrix included 28 individuals aligned to 3368 bp (CO1 = 657 bp; ND1 = 900 bp; FEM1 = 501 bp; UBiA = 765 bp; ITS1 = 545 bp). The total number of individuals sequenced for each locus are as follows: CO1 – 102, ND1 – 103, FEM1 – 29, UBiA – 29, and ITS1 – 31. Additional details regarding the individuals used in molecular analyses are available in Table 5.1, GenBank (*Will be added upon publication), and ScienceBase (*Will be added upon publication).

Five partitions and substitution models were determined by ModelFinder for ML analyses in IQ-TREE: TN+F+I for mtDNA codon 1 and nDNA codon 3, TN+F+I for mtDNA codon 2 and nDNA codon 2, K3Pu+F+G4 for mtDNA codon 3, F81+F for nDNA codon 1, and K2P+I for ITS1. All species-level relationships had full support (BS=100) and the only two major nodes that were not strongly support (i.e., BS ≥ 95) were the PPP clade of *P. fragilis* (BS = 94) and the Mobile clade of *P. purpuratus* (BS = 92). All three taxa were resolved as monophyletic with *P. inflatus* sister to *P. fragilis* and *P. purpuratus*, aligning with findings in a previous study (Smith et al., 2020).

Substitution models determined by ModelFinder for locus alignments in the STACEY analysis were: HKY+I for CO1, HKY+I for ND1, JC for FEM1, F81+I for UBiA, and K2P+I (=K80+I) for ITS1. Convergence of the analysis was supported by all parameters having ESS values > 200, and all nodes were strongly supported (PP ≥ 95). SpeciesDA

supported six clusters (54%): 1) *P. inflatus* from the Mobile, 2) *P. inflatus* from the Pontchartrain, 3) *P. fragilis* from the Mobile, 4) *P. fragilis* from the Pearl+Pontchartrain, 5) *P. purpuratus* from the Mobile, and 6) *P. purpuratus* from the PPP. The second most likely clustering scenario supported 7 clusters (18.5%), with the Pearl population of *P. purpuratus* recognized as a distinct cluster.

The topological reconstruction from *BEAST was congruent with IQ-TREE and STACEY topologies, and all nodes were strongly supported (Fig. 5.2). Mobile and PPP clusters of *Potamilus* spp. were resolved as monophyletic with full support (PP = 100; Fig. 5.2). Convergence of the analysis was supported by all parameters having ESS values > 200. Divergence estimates differed slightly among Mobile and PPP clusters of *Potamilus* spp. The split between *P. inflatus* was estimated to have occurred ~2.13 Mya (95% CI 0.28-3.92 Mya; Fig. 5.2), and the splits between *P. fragilis* and *P. purpuratus* were estimated to have occurred more recently: ~1.35 Mya (95% CI 0.54-2.27 Mya) and ~0.72 Mya (95% CI 0.27-1.39 Mya), respectively (Fig. 5.2).

Mean uncorrected p-distances between Mobile and PPP groups for all species were larger than 1% and are reported in Table 5.3. Distance values were larger in *P. inflatus* (2.33%) when compared to *P. fragilis* (1.11%) and *P. purpuratus* (1.31%). Haplotype networks were concordant with phylogenetic analyses and showed clear separation between the Mobile and PPP groupings of all three *Potamilus* spp. (Fig. 5.3). However, within the PPP province there was haplotype sharing between drainages in *P. fragilis* and *P. purpuratus* (Fig. 5.3). AMOVAs indicated the majority of molecular variation occurred between Mobile and PPP groups of all *Potamilus* spp. (Table 5.3).

Molecular variance was higher within *P. fragilis* (19.1%) than *P. inflatus* (1.1%) and *P. purpuratus* (3.7%).

The *dpp-msbayes* analysis supported synchronous divergence between clusters of *Potamilus* spp. (Fig. 5.2). Support for a single divergence event was 55.7 PP with the next best supported scenario of two divergence events (*P. inflatus* and *P. purpuratus* equal, and *P. fragilis* subsequently diverged independently) at 15.7 PP (Fig. 5.2). Similarly, Bayes factors indicated positive support for one divergence event ($2ln$BF = 1.7), and negative support for two ($2ln$BF = -0.74) and three ($2ln$BF = -2.19) divergence events (Fig. 5.2). The overlap of confidence intervals for divergence estimates in the *BEAST analysis and *dpp-msbayes* further supports evidence of synchronous divergence between *Potamilus* spp. (Fig. 5.2).

Discussion

Accurate evaluations of genetic diversity is a critical component in developing effective conservation and recovery strategies. The specific goal of our study was to characterize range-wide genetic variation of *P. inflatus*. Given the overall rarity of the species and plausible extirpation from multiple river systems, estimating genetic relationships across the historical range of *P. inflatus* is completely dependent on understanding the genetic composition of closely related and co-distributed species with similar life histories. Our comparative phylogeographic approach that integrates the premise of surrogate species is promising for predicting relationships among extant and extirpated populations of imperiled species. Although the use of surrogate species to prioritize areas for conservation has become commonplace (Grantham et al., 2010); to our knowledge, the use of surrogate species within a comparative phylogeographic

framework is novel, not only to freshwater mussels, but across all taxa. Below, we discuss the evolutionary forces driving congruent patterns of genetic divergence within *Potamilus* spp., and how our novel methodology impacts future conservation and recovery efforts for *P. inflatus*.

Patterns of Genetic Variation in Potamilus Species

Large-scale environmental change has substantial effects on communities of species and associated microbiota (Hoberg, 1997; Oaks, 2014; Thompson, 2005). This is certainly the case in mussels and their hosts, as biogeographical processes are a driver of faunal structure and genetic diversity (Beaver et al., 2019; Haag, 2012; Inoue et al., 2015; Scott et al., 2020; Smith et al., 2018, 2020). Given biogeography is a critical driver of genetic variation, identifying faunal provinces is the first step toward understanding specific patterns of phylogeography (Whittaker et al., 2005). Multiple attempts have been made to classify North American mussel fauna into biogeographic provinces (Burlakova et al., 2011; Haag, 2010; R. I. Johnson, 1970; Neck, 1982; Sepkoski Jr. & Rex, 1974), and understanding the processes that have driven faunal shifts across these regions has been integral toward understanding the evolution of the group (Inoue et al., 2015; Lopes-Lima et al., 2019; Smith et al., 2020). In the case of the Mobile and PPP provinces, the drainages have been linked in hierarchical classifications of mussel diversity based on species composition (Haag, 2010). Prior to our study, however, these relationships have not been tested in a molecular context. Our molecular analyses align with the hypothetical historical connection between the Mobile and PPP, as our coalescent-based species delimitation analysis strongly supported *Potamilus* spp. in these biogeographic provinces as distinct clusters. These results align with other mussel species showing

genetic distinctiveness across these drainages (Gangloff et al., 2013; N. A. Johnson et al., 2018; Lopes-Lima et al., 2019; Smith et al., 2018), as well as other aquatic species (Egge & Hagbo, 2015; Ennen et al., 2010; Halas & Simons, 2014; Ross, 2001; Warren et al., 2000).

The geological connection between the Mobile and PPP drainages has been hypothesized by numerous authors (reviewed by (Otvos, 2018)) and a vicariance event between the two systems has likely driven the observed genetic differentiation in *Potamilus* spp. If a vicariance event was the source for all the species, we would expect to see similar patterns of divergence across *Potamilus* spp. Molecular analyses, however, differed from these expected patterns of genetic drift and showed varying levels of sequence divergence (Table 5.3). Specifically, genetic distance values between populations of *P. inflatus* were larger than those in *P. fragilis* and *P. purpuratus* (Table 5.3). However, it is an unrealistic expectation to assume that rates of evolution are identical between species, especially across geographically isolated populations (Avise, 1992; Charlesworth, 2009; Laporte & Charlesworth, 2002). Variable rates of molecular diversification within *Potamilus* spp. could be indicative of a variety of confounding variables, such as differing population demographics (e.g., population size, age structure), evolutionary processes (e.g., mutation rate, genetic drift, selection), or species-specific traits (e.g., habitat preferences, dispersal capabilities) rather than multiple hypothetical vicariance events. To address this issue, we used a hABC approach to explicitly test whether divergence between Mobile and PPP populations of *Potamilus* spp. occurred synchronously. Our results suggest that the divergence between Mobile and PPP clusters of *Potamilus* spp. occurred simultaneously and further support previously described

biogeographic provinces (Haag, 2010). The causative event driving genetic differentiation between these groupings is uncertain, but further molecular investigations in other freshwater mussels, as well as host fishes, may further elucidate the timing and patterns of faunal exchange between these two provinces.

Despite extensive geographic range within the PPP, our molecular data showed no diagnostic divergence between drainages within the province (Fig. 5.3; Table 5.3). Limited genetic diversity was suspected within *P. inflatus* given there is only one extant population; however, the more common and wide-ranging species, *P. fragilis* and *P. purpuratus,* both showed haplotype sharing between drainages and no evidence of drainage specific structuring within the PPP (Fig. 5.3; Table 5.3). A signal for incomplete lineage sorting at nDNA loci is expected due to the effective population size being nearly four times that of mtDNA loci (Moore, 1995; Toews & Brelsford, 2012); however, incomplete lineage sorting of mtDNA loci likely indicates relatively recent gene flow between populations. Approximately 18 Kya during the last glacial low stand, geological evidence suggests the PPP drainages were connected (Flocks et al., 2009; Otvos, 2018), which would allow for gene flow to occur between currently isolated populations. Subsequent sea level rise from deglaciation began to form modern fluvial systems in the PPP (Flocks et al., 2009), causing genetic isolation of populations of *Potamilus* spp. Given the hypothetical mtDNA mutation rates of freshwater mussels (Bolotov et al., 2016; Froufe et al., 2016), it is an unrealistic expectation that mtDNA markers would become fixed across these drainages and using more rapidly evolving markers (GBS, WGR) would be necessary to molecularly diagnose these drainages or test for ongoing gene flow. However, only one extant population of *P. inflatus* occurs within the PPP

(Amite River – Pontchartrain drainage) and it is a realistic expectation that the presumed extirpated populations of *P. inflatus* in the Pontchartrain and Pearl drainages would have a similar genetic makeup as the Amite River population given the patterns of genetic diversity seen in *P. fragilis* and *P. purpuratus*.

Implications on Conservation

Captive propagation of freshwater mussels is a critical component of recovery planning for many species (McMurray & Roe, 2017; Neves, 2004) and likely the only viable recovery option for *P. inflatus* (USFWS, 2014). Within the PPP province, we found that all sampled populations were consistently clustered as one grouping across *Potamilus* spp. (Fig. 5.2; Fig. 5.3), which provides justification for using the Amite River population rather than the Mobile population of *P. inflatus* as a source of brood stock for recovery efforts that include translocation or captive propagation in the Pearl and Pontchartrain drainages. Based on the likely scenario that extant populations of *P. inflatus* are restricted to the Amite and Mobile rivers, possible reintroduction sites to historically occupied river systems would include the Bogue Chitto, Comite, Pearl, and Tangipahoa rivers.

Although a useful tool, without proper guidance and planning efforts, introduction of captive raised individuals has the potential to harm existing populations or nontarget species (Olden et al., 2011; Snyder et al., 1996). Findings from our study provide direction for future recovery efforts; however, we encourage further evaluations of population genetic structure and characterization of population genetic diversity using fine scale genomic markers (e.g., GBS, WGR) to develop robust genetic management plans before captive propagation efforts. Ideally, characterizing genetic diversity in

captively bred individuals and identifying and screening for potentially adaptive loci that may increase survivability would be performed before re-establishing extirpated populations.

Future Directions

Although most species found in the PPP also occur in the Mobile drainage, distributional patterns of *Potamilus* spp. within the PPP are inconsistent. *Potamilus inflatus* has never been recorded within the Pascagoula watershed, while *P. purpuratus* is widely distributed across the basin (R. L. Jones et al., 2019). Furthermore, *P. fragilis* was previously hypothesized to not occur in Pascagoula drainage (R. L. Jones et al., 2005), however, it appears to be extremely rare within the system based on newly found records (R. L. Jones et al., 2019). The Pascagoula drainage mussel fauna consists of 33 species, and multiple other species extant in both the Mobile and Pearl drainage have not been found in the system, such as *Arcidens confragosus*, *Ligumia recta*, *Obliquaria reflexa*, and *Truncilla donaciformis* (R. L. Jones et al., 2005, 2019). The causation for these inconsistencies is unknown and also unexpected given the Pascagoula drainage has been classified as the least impacted major river system in the continental United States (Dynesius & Nilsson, 1994). Archaeological data is a useful tool to establish baseline community composition information for conservation efforts (Peacock, 2012; Rick & Lockwood, 2013). Specifically, archeological records hold useful information about the geographic range of species and community composition of systems prior to human-mediated environmental impacts. Recent zooarchaeological studies on the freshwater mussel fauna has identified that most mussel species were more common and/or widely distributed prior to widespread human impacts, including some species that are extirpated

from the respective system (Randklev et al., 2010; Wolverton & Randklev, 2016). Given the absence of many common and rare mussel taxa in the Pascagoula, using archaeological data to assess the community composition of mussel fauna could be a useful conservation technique, and the identification of historical populations of *P. inflatus* in the Pascagoula River could be possible.

In recent years, a resurgence of sampling effort by state and federal agencies has resulted in hundreds of surveys each year, and several mussel species that were presumed extinct have been recently rediscovered (Holcomb et al., 2015; N. Johnson et al., 2016; Randklev et al., 2012). This may be a possibility for *P. inflatus* in the PPP drainages, where extant populations may be discovered by thorough survey efforts. Further, environmental DNA (eDNA) sampling techniques represent a promising approach to detect freshwater mussels (Cho et al., 2016; Currier et al., 2018; Sansom & Sassoubre, 2017) and are likely an integral tool toward guiding effective traditional surveys of imperiled species. Within the PPP, *P. inflatus* is only known from the Amite River (Brown & Daniel, 2014; Hartfield, 1988); however, historical records have also been reported from the Tangipahoa River in the Pontchartrain drainage (USFWS, 1990), and more recently the Pearl River drainage (Frierson, 1911; George & Reine, 1996). It is also possible that the Pascagoula River and adjacent coastal drainages such as the Biloxi, Jourdan, Tchoutacabouffa, and Wolf rivers have not been sampled thoroughly enough to detect the species (R. L. Jones et al., 2019). Recovery planning would greatly benefit from accurate distributional information for *P. inflatus,* and future efforts utilizing both eDNA sampling and traditional surveys would help resolve whether the species is absent from select drainages.

Conclusion

Given the imperilment status of freshwater mussel species globally (Lopes-Lima et al., 2018), our study represents a novel and useful methodology for hypothesizing the genetic relationships of extant and extirpated populations of imperiled species to facilitate recovery planning. The use of mtDNA may be limited on a regional scale in most species; however, comparative phylogeographic approaches incorporating more rapidly evolving genome-wide markers such as GBS and WGR introduces a more robust methodology for evaluating population dynamics within drainages and even at a local scale using surrogate species. As the understanding of phylogeny and life history characteristics continues to improve, utilizing comparative phylogeographic methodologies is a promising tool toward effective species recovery and long-term viability of freshwater mussels.

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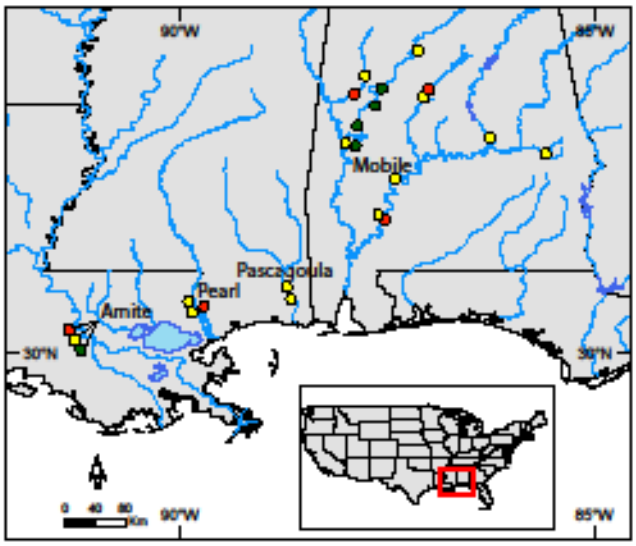

Figure 5.1. Collection locations for *Potamilus fragilis* (red), *P. inflatus* (green), and *P. purpuratus* (yellow) in the Mobile, Pascagoula, Pearl, and Pontchartrain drainages.

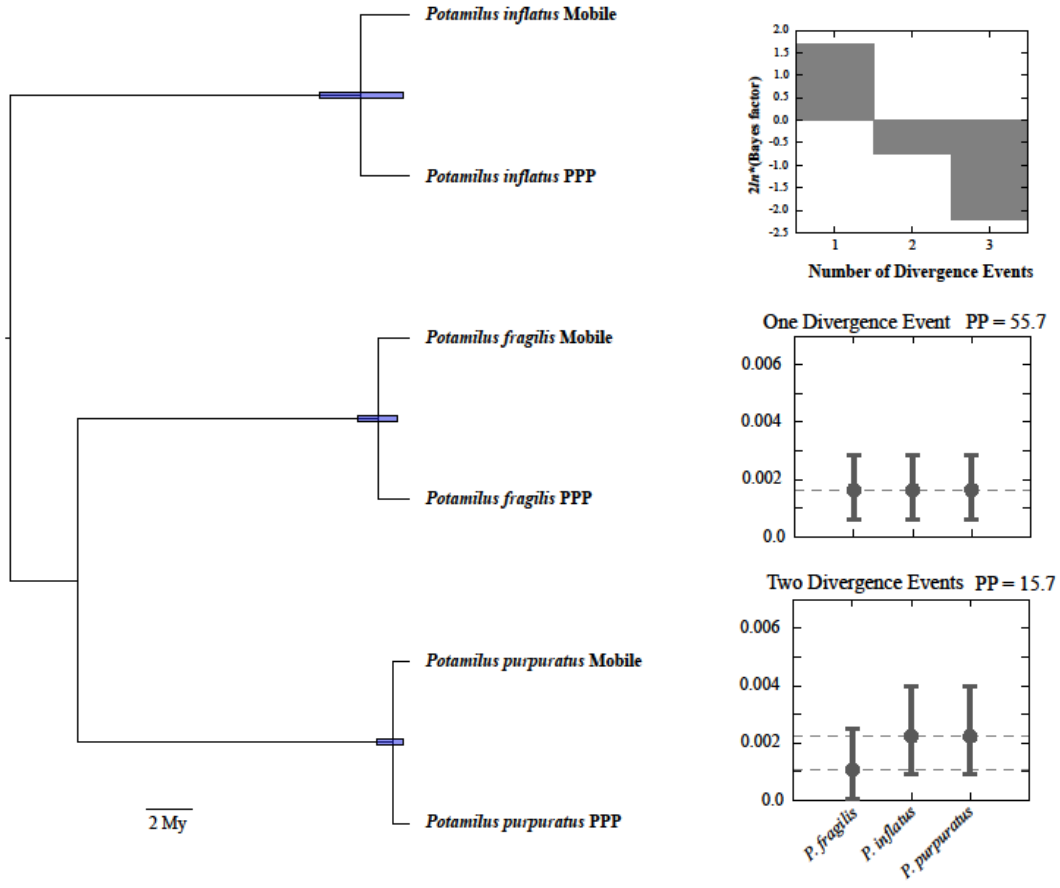

Figure 5.2. *BEAST phylogenetic reconstruction with divergence time scaled in million years before present and node bars represent the 95% CI. All nodes were strongly supported with posterior probability greater than 97. *Dpp-msbayes* output regarding Bayes Factor support for the number of divergence events, and the two most likely divergence scenarios.

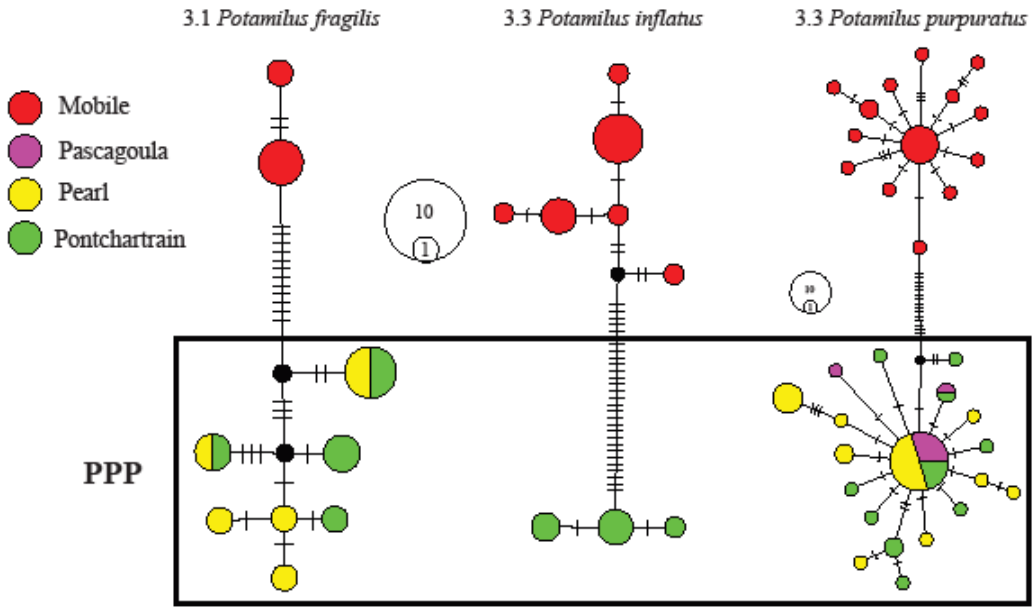

Figure 5.3. Haplotype networks based on a concatenated alignment of CO1 and ND1 for *Potamilus fragilis* (3.1), *P. inflatus* (3.2), and *P. purpuratus* (3.3). Each circle represents a unique haplotype and size is relative to the number of individuals. Black circles represent unsampled haplotypes and individual tick marks indicate nucleotide substitutions. Specimens are grouped by drainage of capture: Mobile, Pascagoula, Pearl, and Pontchartrain.

Tables

Table 5.1. Molecular material examined in this study. Museum abbreviations are as follows: UA – Alabama Museum of Natural History; UF – Florida Museum. GenBank or SRA accession numbers are provided for each locus, and missing values were not used in phylogenetic analyses. * indicates will be added upon publication.

Taxon	Source	CO1	ND1	ITS1	FEM1	UBiA
Potamilus fragilis	UF438237	*	*	*		
Potamilus fragilis	UF439330	*	*	*	*	*
Potamilus fragilis	UF439352	*	*			
Potamilus fragilis	UF439352	*	*			
Potamilus fragilis	UF439332	*	*			
Potamilus fragilis	UF439332	*	*	*	*	*
Potamilus fragilis	UF439365	*	*			
Potamilus fragilis	UF439343	*	*			
Potamilus fragilis	UF439343	*	*			
Potamilus fragilis	UF439343	*	*			
Potamilus fragilis	*	*	*			
Potamilus fragilis	*	*	*			
Potamilus fragilis	*	*	*			
Potamilus fragilis	*	*	*			
Potamilus fragilis	*	*	*			
Potamilus fragilis	*	*	*	*	*	*
Potamilus fragilis	UF439131	*	*	*	*	*
Potamilus fragilis	UF439131	MK044952	MK045103	MK036203	*	*
Potamilus fragilis	UF439131	*	*	*	*	*
Potamilus fragilis	UF439131	MK044953	MK045104	MK036204	*	*
Potamilus fragilis	UF439131	*	*	*	*	*
Potamilus fragilis	UF439131	*	*	*	*	*
Potamilus inflatus	*	*	*	*	*	*

Potamilus inflatus	*	*	*	*	*	*
Potamilus inflatus	*	*	*	*	*	*
Potamilus inflatus	*	*	*	*	*	*
Potamilus inflatus	*	*	*	*	*	*
Potamilus inflatus	*	*	*	*	*	*
Potamilus inflatus	UF439514	*	*	*	*	*
Potamilus inflatus	UF439514	*	*	*	*	*
Potamilus inflatus	UF439514	*	*	*	*	*
Potamilus inflatus	UF439514	*	*	*	*	*
Potamilus inflatus	UF439514	*	*	*	*	*
Potamilus inflatus	UF439513	*	*	*	*	*
Potamilus inflatus	UF439513	*	*	*	*	*
Potamilus inflatus	UA2696		*	*	*	*
Potamilus purpuratus	UF438434	*	*			
Potamilus purpuratus	UF439145	*	*			
Potamilus purpuratus	UF439145	MK044960	MK045111	MK036211		
Potamilus purpuratus	UF439145	MK044961	MK045112	MK036212		
Potamilus purpuratus	UF439145	*	*			
Potamilus purpuratus	UF439145	*	*			
Potamilus purpuratus	UF439452	*	*			
Potamilus purpuratus	UF439452	*	*			
Potamilus purpuratus	UF439452	*	*			
Potamilus purpuratus	UF439452	*	*			
Potamilus purpuratus	UF439452	*	*			
Potamilus purpuratus	UF439453	*	*			
Potamilus purpuratus	UF439453	*	*			
Potamilus purpuratus	UF439453	*	*	*	*	*
Potamilus purpuratus	UF439453	*	*			

Potamilus purpuratus	UF439453	*	*			
Potamilus purpuratus	UF439454	*	*			
Potamilus purpuratus	UF439454	*	*			
Potamilus purpuratus	UF439454	*	*			
Potamilus purpuratus	UF439454	*	*			
Potamilus purpuratus	UF439456	*	*			
Potamilus purpuratus	UF439456	*	*			
Potamilus purpuratus	UF439457	*	*			
Potamilus purpuratus	UF439457	*	*			
Potamilus purpuratus	UF439457	*	*			
Potamilus purpuratus	UF439457	*	*			
Potamilus purpuratus	UF439457	*	*			
Potamilus purpuratus	UF439456	*	*			
Potamilus purpuratus	UF439456	*	*			
Potamilus purpuratus	UF439456	*	*			
Potamilus purpuratus	UF439456	*	*			
Potamilus purpuratus	UF439456	*	*			
Potamilus purpuratus	UF439458	*	*			
Potamilus purpuratus	UF439459	*	*			
Potamilus purpuratus	UF439459	*	*			
Potamilus purpuratus	UF439459	*	*			
Potamilus purpuratus	UF439459	*	*			
Potamilus purpuratus	UF439459	*	*			
Potamilus purpuratus	UA62	*	*			
Potamilus purpuratus	UA2469	*	*			
Potamilus purpuratus	UA2510	*	*			
Potamilus purpuratus	UA2562	*	*			
Potamilus purpuratus	UA2740	*	*	*	*	*

Potamilus purpuratus	UA3100	*	*				
Potamilus purpuratus	UA3123	*	*				
Potamilus purpuratus	UA3205	*	*				
Potamilus purpuratus	UA3417	*	*				
Potamilus purpuratus	UA3482	*	*				
Potamilus purpuratus	UF439510	*	*				
Potamilus purpuratus	UF439510	*	*				
Potamilus purpuratus	UF439510	*	*				
Potamilus purpuratus	UF439510	*	*				
Potamilus purpuratus	UF439510	*	*	*	*	*	
Potamilus purpuratus	UF439510	*	*				
Potamilus purpuratus	UF439510	*	*				
Potamilus purpuratus	*	*	*	*	*	*	
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				
Potamilus purpuratus	*	*	*				

Table 5.2. Primers and PCR conditions used in this study to generate molecular data.

Locus	Primers	Source	Conditions
CO1	F: 5'-GTTCCACAAATCATAAGGATATTGG-3'	Campbell et	Johnson et
	R: 5'-TACACCTCAGGGTGACCAAAAAACCA-3'	al. 2005	al. 2018
ND1	F: 5'-TGGCAGAAAAGTGCATCAGATTAAAGC-3'	Serb et al.	Serb et al.
	R: 5'-CCTGCTTGGAAGGCAAGTGTACT-3'	2003	2003
ITS1	F: 5'-AAAAAGCTTCCGTAGGTGAACCTGCG-3'	King et al.	King et al.
	R: 5'-AGCTTGCTGCGTTCTTCATCG-3'	1999	1999
FEM1	F: 5'- GTRATGGAGTATCGCAGTGT-3'	This Study	This Study
	R: 5'-ACRCTYTTCCTGTTAACATC-3'		
UBiA	F: 5'- TTTACTCCTGTTGCACTTGGGA-3'	This Study	This Study
	R: 5'-AGCATCTGTCATGAAGACTCCAAC-3'		

Table 5.3. Summary of AMOVA analyses in PopArt. Sample sizes for each taxon from the Mobile drainage and Pascagoula+Pearl+Pontchartrain (PPP) are reported. All values for each comparison were found to be significant ($\alpha < 0.0001$).

Taxa	N Mobile	N PPP	Amova between	Amova within	Distance between (uncorrected p)
Potamilus fragilis	4	12	80.9%	19.1%	1.11%
Potamilus inflatus	13	6	98.9%	1.1%	2.33%
Potamilus purpuratus	22	45	96.3%	3.7%	1.31%

CHAPTER SIX

Conclusion

The incorporation of molecular data with multiple independent lines of evidence represents a powerful approach to resolve evolutionary relationships in freshwater mussels. For my doctoral research, I used an integrative approach to resolve enigmatic questions pertaining to numerous groups of mussels, including accurately defining systematic placement, resolving species-level diversity, as well as advancing knowledge of functional traits that have driven lineage diversification in freshwater mussels. The multi-locus sequence data I present support the hypothesis that diversification in Lampsilini was broadly shaped by life history characters, as we resolved a strong correlation between host fish use, host infection strategies, and phylogenetic placement, similar to previous evaluations (Hewitt et al., 2019). In particular, molecular data resolved a novel monophyletic grouping consisting of *Ellipsaria*, *Leptodea*, *Potamilus*, and *Truncilla*, which are linked by being host specialists, with glochidia only transforming on freshwater drum, *Aplodinotus grunniens* (Barnhart et al., 2008; Sietman et al., 2018). Though investigations using multi-locus sequence data could not resolve strong support for phylogenetic relationships between *A. grunniens* specialists, the use of hybrid enrichment strategies resolved phylogenetic relationships between these taxa and represent a significant improvement in the ability to reconstruct accurate phylogeny when compared to traditional multi-locus datasets. Specifically, integrating anchored hybrid enrichment and life history data revealed a complex pattern of evolution within *A.*

grunniens specialists, including host switching and multiple instances of convergence, including reduction in size of larvae, increased fecundity, and growth during encapsulation to increase survival post-metamorphosis. Additionally, integrative assessments recovered the non-monophyly of multiple genera (i.e., *Leptodea* and *Potamilus*) and the associated traits used for previous taxonomic hypotheses. Multiple systematic changes were made to more accurately reflect the evolutionary history of this group, including the description of the new genus *Atlanticoncha*, and the synonymy of the genus *Leptodea* under *Potamilus*. Resolving the evolutionary history of this group and illustrating the functional significance of lineage specific life history traits will be critical towards determining conservation priorities and predicting species-specific responses in the development of effective recovery strategies.

Species conservation is largely dependent on the ability to distinguish one species from another, which has been an ongoing issue hindering recovery practices in freshwater mussels (e.g., Inoue et al., 2013, 2020; Johnson et al., 2018; Keogh & Simons, 2019; Smith et al., 2018, 2019). Integrative assessments resolved species-level diversity within two species complexes: 1) the *P. ohiensis* species complex, and 2) the *F. mitchelli* species complex. Within the *P. ohiensis* species complex, inference from an integrative framework unveiled overwhelming support for a new species of freshwater mussel, *P. streckersoni* or Brazos Heelsplitter. Data showed clear separation of three taxonomic entities: *P. amphichaenus*, *P. ohiensis*, and *P. streckersoni*; all molecularly, geographically, and morphologically diagnosable. Within *F. mitchelli*, phylogenetic analyses depicted deep molecular divergence between two clades of *F. mitchelli* (Guadalupe and Brazos+Colorado), which were strongly supported as distinct species by

models integrating both molecular and morphological data coupled with biogeographic information. Based on these results, the junior synonym *F. iheringi*, or Balcones Spike, was elevated to represent the Brazos and Colorado populations and restrict the distribution of *F. mitchelli* to the Guadalupe River drainage. These findings have profound implications on both the understanding of evolution and taxonomy in freshwater mussels, highlights the importance of an integrative approach in species delineation, and will aid in resolving species-specific status assessments, management practices, and recovery planning.

Given the imperilment of freshwater mussels globally, there remains a critical need for a standardized methodology to determine relationships among populations when taxa have been extirpated from a significant portion of their historical range. A comparative phylogeographic approach integrating the premise of surrogate species represents a promising framework for hypothesizing relationships among extant and extirpated populations of imperiled mussel species. In the case of *P. inflatus*, by characterizing genetic structure in the sympatric congeners *P. fragilis* and *P. purpuratus*, I provide justification for using the Amite River population as a source of broodstock to re-establish extirpated populations of *P. inflatus* in the Pearl and Pontchartrain drainages. The use of this approach serves as a model study for future molecular studies in imperiled mussel species and results supply natural resource managers with modes of action that may ultimately lead to species recovery and subsequent delisting of the federally threatened species, *P. inflatus* (USFWS, 2014).

Despite findings from all research objectives, the basic biology and ecology of many mussel species remains poorly understood. Fortunately, the utilization of

phylogenomic data will help infer the basic biology of freshwater mussels globally, and as genome-scale studies integrating multiple data types continue to be performed, the bases of many enigmatic ecologically and evolutionary questions will be unraveled in these highly imperiled organisms.

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APPENDIX

APPENDIX

Supplemental Figures and Tables

Figures

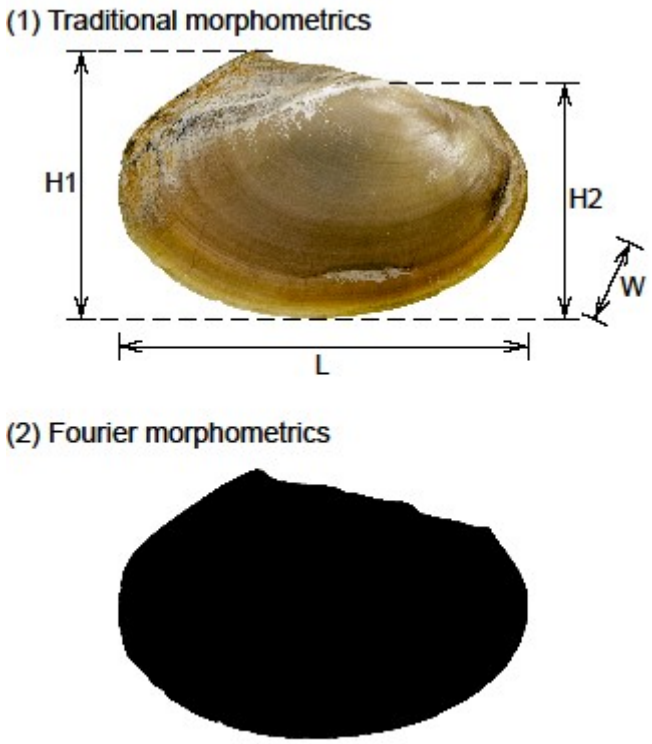

Figure S2.1. Examples of measurements of shell characteristics used for traditional morphometric analysis (S2.1.1) and shell outline used for Fourier shape morphometrics (S2.1.2).

Figure S2.2. Maximum Likelihood (ML) reconstruction generated by IQ-TREE on a concatenated molecular matrix (CO1, ND1, ITS1, 28S). Values above branches represent ultrafast bootstrap support (BS). Strongly supported nodes (i.e., BS ≥ 95) are indicated by asterisks.

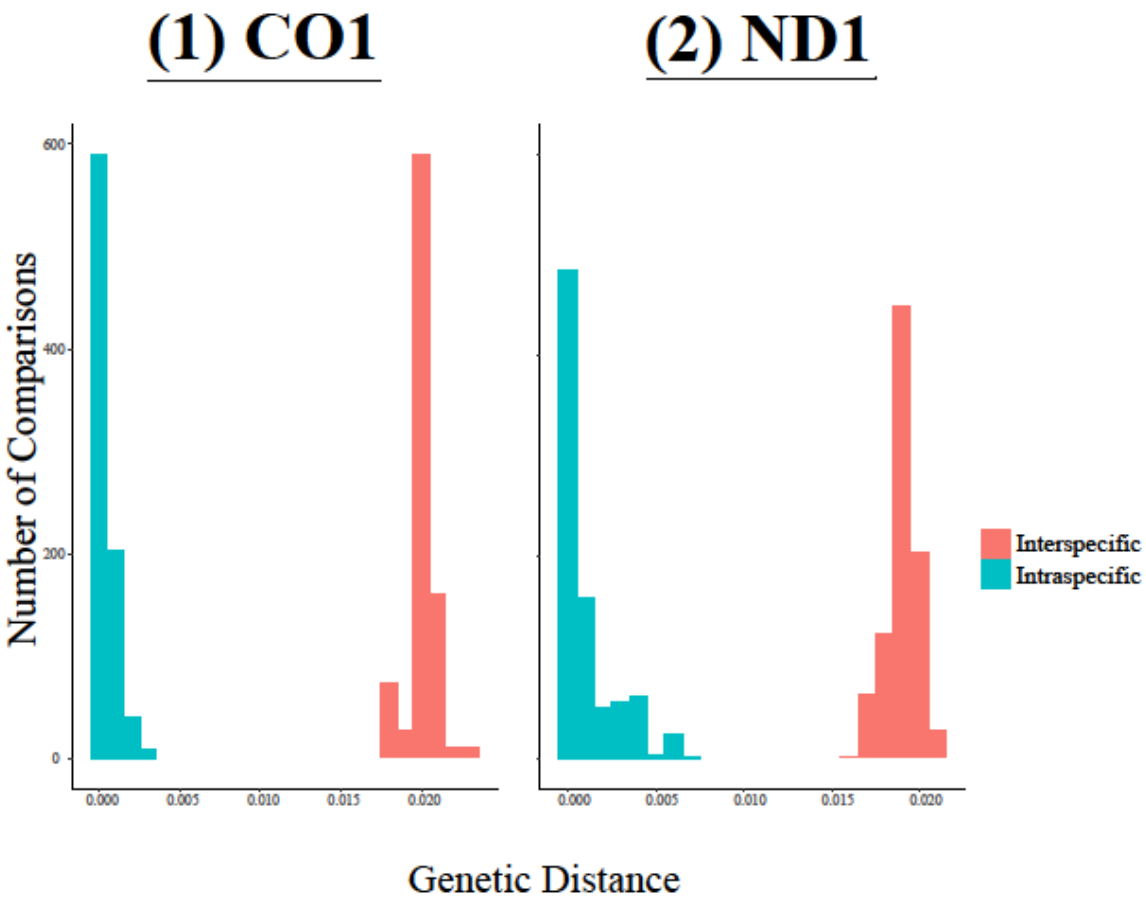

Figure S2.3. Histograms for CO1 (S2.3.1) and ND1 (S2.3.2) illustrating intraspecific and interspecific pairwise uncorrected-p distances for *Potamilus amphichaenus* and *Potamilus streckersoni* sp. nov.

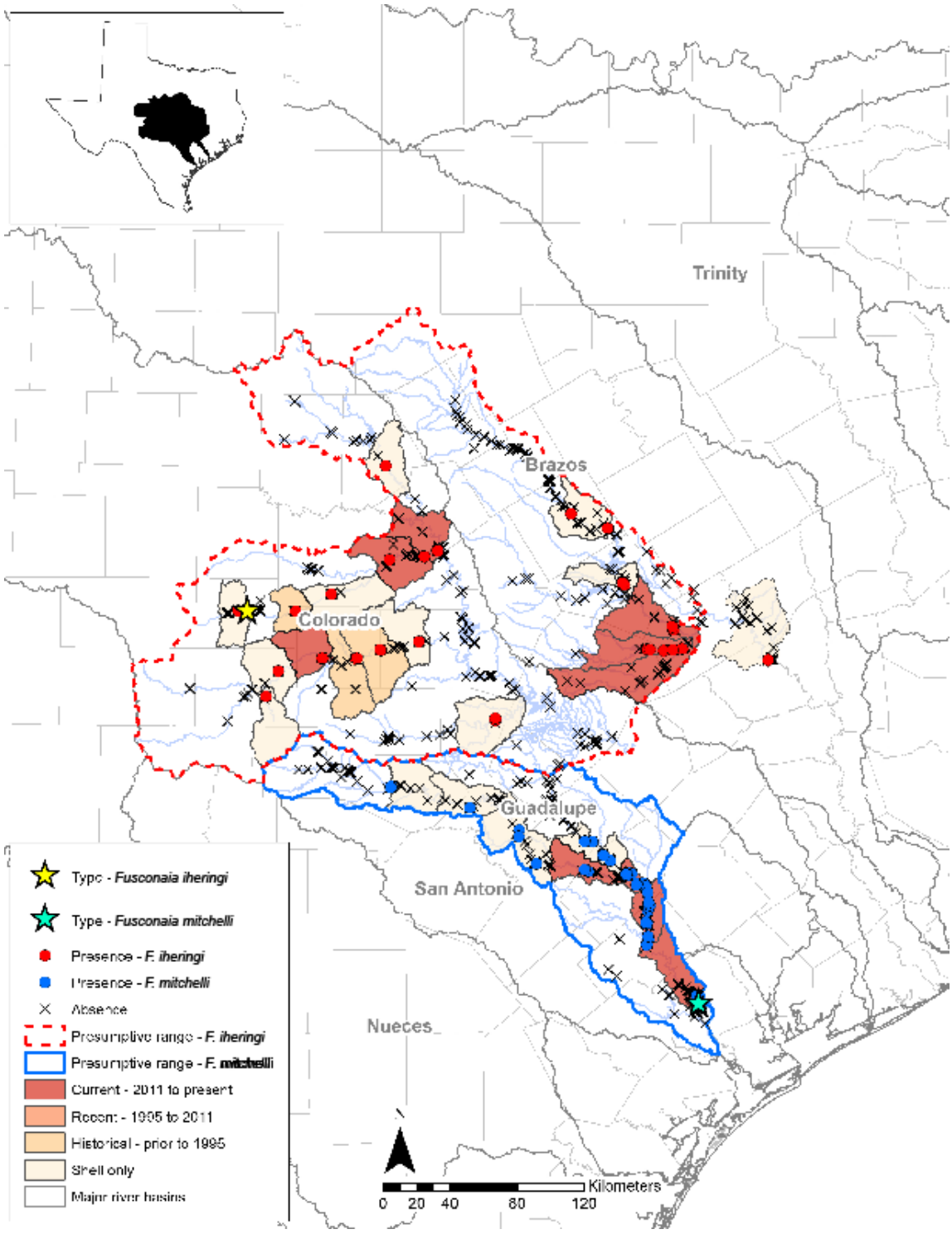

Figure S4.1. Conservation status map for *Fusconaia iheringi* and *Fusconaia mitchelli*. Shaded circles denote presence and "X" indicates absence. Hydrologic Unit Codes (HUC) 10-level are colored to distinguish between live and shell only records. For the former, HUCs are further shaded by when a live specimen of *F. iheringi* (red) or *F. mitchelli* (blue) was collected. The presumptive ranges for *F. iheringi* and *F. mitchelli* are denoted by the dashed red line and solid blue line, respectively. Type localities for *F. iheringi* and *F. mitchelli* are represented by yellow and turquoise stars, respectively.

Tables

Table S2.1. Details for all molecular material examined for phylogenetic analyses and additional material examined in the *P. ohiensis* species complex, including taxon labels, museum catalog numbers, and GenBank accession numbers. Museum abbreviations are as follows: (ASU – Arkansas State University Museum; INHS – Illinois Natural History Survey; JBFMC – Joseph Britton Freshwater Mollusk Collection; UA – Alabama Museum of Natural History; UF – Florida Museum of Natural History).

Taxon label	Catalog number	CO1	ND1	ITS1	28S
Actinonaias ligamentina	UA1941	MK044901	MK045051	MK036152	MK036068
Actinonaias pectorosa	UF439496.6286	MK044902	MK045052	MK036153	MK036069
Amblema plicata 1	UF438247.6267	MK044903	MK045053	MK036154	MK036070
Amblema plicata 2	UF438247.6268	MK044904	MK045054	MK036155	MK036071
Cyprogenia stegaria	UA3899	MK044905	MK045055	MK036156	MK036072
Cyrtonaias tampicoensis 1	UF441144.007	KT285620	MK045056	KT285664	MK036073
Cyrtonaias tampicoensis 2	UF441145.008	MK044906	MK045057	MK036157	MK036074
Ellipsaria lineolata	UF439368.11430	MK044907	MK045058	MK036158	MK036075
Epioblasma rangiana 1	INHS85842-4	MK044908	MK045059	MK036159	MK036076
Epioblasma rangiana 2	INHS85842-5	MK044909	MK045060	MK036160	MK036077
Glebula rotundata 1	UF440911.055	MK044910	MK045061	MK036161	MK036078
Glebula rotundata 2	UF440908.083	MK044911	MK045062	MK036162	MK036079
Hamiota subangulata 1	N/A	MK044912	MK045063	MK036163	MK036080
Hamiota subangulata 2	UF441105.004	MK044913	MK045064	MK036164	MK036081
Lampsilis cardium	INHS49380	MK044914	MK045065	MK036165	MK036082
Lampsilis dolabraeformis 1	UF439211.001	MK044915	MK045066	MK036166	MK036083
Lampsilis dolabraeformis 2	UF439211.002	MK044916	MK045067	MK036167	MK036084
Lampsilis ovata 1	UF438255.6285	MK044917	MK045068	MK036168	MK036085
Lampsilis ovata 2	UF438257.6291	MK044918	MK045069	MK036169	MK036086
Lampsilis satura 1	UF441168.002	MK044919	MK045070	MK036170	MK036087
Lampsilis satura 2	UF441170.004	MK044920	MK045071	MK036171	MK036088
Lemiox rimosus 1	N/A	MK044921	MK045072	MK036172	MK036089
Lemiox rimosus 2	N/A	MK044922	MK045073	MK036173	MK036090
Leptodea fragilis 1	INHS39037	MK044923	MK045074	MK036174	MK036091
Leptodea fragilis 2	INHS39037	MK044924	MK045075	MK036175	MK036092
Leptodea fragilis 3	INHS39037	MK044925	MK045076	MK036176	MK036093
Leptodea leptodon	INHS44305	MK044926	MK045077	MK036177	MK036094
Leptodea ochracea 1	UF438217.6173	MK044927	MK045078	MK036178	MK036095
Leptodea ochracea 2	UF438217.6176	MK044928	MK045079	MK036179	MK036096
Ligumia recta 1	UF438249.6274	MK044929	MK045080	MK036180	MK036097
Ligumia recta 2	INHS49383	MK044930	MK045081	MK036181	MK036098
Ligumia recta 3	INHS79831	MK044931	MK045082	MK036182	MK036099
Medionidus conradicus 1	UF439086.9848	MK044932	MK045083	MK036183	MK036100
Medionidus conradicus 2	UF439086.9849	MK044933	MK045084	MK036184	MK036101

Obliquaria reflexa 1	UF438246.6265	MK044934	MK045085	MK036185	MK036102
Obliquaria reflexa 2	UF438940.6910	MK044935	MK045086	MK036186	MK036103
Obovaria subrotunda 1	UF438391.005	MK044936	MK045087	MK036187	MK036104
Obovaria subrotunda 2	UF438391.006	MK044937	MK045088	MK036188	MK036105
Plectomerus dombeyanus 1	UF438973.7009	MK044939	MK045090	MK036190	MK036107
Plectomerus dombeyanus 2	UF438823.018	MK044938	MK045089	MK036189	MK036106
Popenaias popeii 1	UF438742.6643	MK044940	MK045091	MK036191	MK036108
Popenaias popeii 2	UF438742.6641	MK044941	MK045092	MK036192	MK036109
Popenaias popeii 3	UF438742.6642	MK044942	MK045093	MK036193	MK036110
Potamilus alatus 1	UF438248.6269	MK044946	MK045097	MK036197	MK036114
Potamilus alatus 2	INHS79834	MK044947	MK045098	MK036198	MK036115
Potamilus amphichaenus 1	UF439482.237	MK044950	MK045101	MK036201	MK036118
Potamilus amphichaenus 10	UF439483.238	MK045013	MK045165	N/A	N/A
Potamilus amphichaenus 11	JBFMC8043.2	MK045010	MK045162	N/A	N/A
Potamilus amphichaenus 12	JBFMC8043.3	MK045011	MK045163	N/A	N/A
Potamilus amphichaenus 13	JBFMC8043.4	MK045012	MK045164	N/A	N/A
Potamilus amphichaenus 14	JBFMC8442.1	MK045019	MK045171	N/A	N/A
Potamilus amphichaenus 15	JBFMC8442.2	MK045020	MK045172	N/A	N/A
Potamilus amphichaenus 16	JBFMC8442.3	MK045021	MK045173	N/A	N/A
Potamilus amphichaenus 17	JBFMC8442.4	MK045022	MK045174	N/A	N/A
Potamilus amphichaenus 18	JBFMC8442.5	MK045023	MK045175	N/A	N/A
Potamilus amphichaenus 19	JBFMC8442.6	MK045024	MK045176	N/A	N/A
Potamilus amphichaenus 2	N/A	MK044948	MK045099	MK036199	MK036116
Potamilus amphichaenus 20	JBFMC8444.1	MK045025	MK045177	N/A	N/A
Potamilus amphichaenus 21	JBFMC8444.2	MK045026	MK045178	N/A	N/A
Potamilus amphichaenus 22	JBFMC8444.3	MK045027	MK045179	N/A	N/A
Potamilus amphichaenus 23	JBFMC8444.4	MK045028	MK045180	N/A	N/A
Potamilus amphichaenus 24	JBFMC8444.5	MK045029	MK045181	N/A	N/A
Potamilus amphichaenus 25	JBFMC8444.6	MK045030	MK045182	N/A	N/A
Potamilus amphichaenus 26	JBFMC8450.1	MK045031	MK045183	N/A	N/A
Potamilus amphichaenus 27	JBFMC8450.3	MK045032	MK045184	N/A	N/A
Potamilus amphichaenus 28	JBFMC8450.4	MK045033	MK045185	N/A	N/A

Potamilus amphichaenus 29	JBFMC8468.1	MK045034	MK045186	N/A	N/A
Potamilus amphichaenus 3	UF439095.9875	MK044949	MK045100	MK036200	MK036117
Potamilus amphichaenus 4	UF438920.6849	MK045009	MK045161	N/A	N/A
Potamilus amphichaenus 5	UF438957.6959	MK045014	MK045166	N/A	N/A
Potamilus amphichaenus 6	N/A	MK045015	MK045167	N/A	N/A
Potamilus amphichaenus 7	N/A	MK045016	MK045168	N/A	N/A
Potamilus amphichaenus 8	UF439095.9876	MK045017	MK045169	N/A	N/A
Potamilus amphichaenus 9	UA2997	MK045018	MK045170	N/A	N/A
Potamilus capax	N/A	MK044951	MK045102	MK036202	MK036119
Potamilus inflatus 1	UF439131.10456	MK044952	MK045103	MK036203	MK036120
Potamilus inflatus 2	UF439131.10458	MK044953	MK045104	MK036204	MK036121
Potamilus metnecktayi 1	UF438911.6814	MK044954	MK045105	MK036205	MK036122
Potamilus metnecktayi 2	UF438911.6813	MK044955	MK045106	MK036206	MK036123
Potamilus metnecktayi 3	UF438911.6816	MK044956	MK045107	MK036207	MK036124
Potamilus ohiensis 1	INHS49472	MK044958	MK045109	MK036209	MK036126
Potamilus ohiensis 10	UF439129.10796	MK045041	MK045193	N/A	N/A
Potamilus ohiensis 11	UF439204.11071	MK045042	MK045194	N/A	N/A
Potamilus ohiensis 12	UF439204.11072	MK045043	MK045195	N/A	N/A
Potamilus ohiensis 13	UF439204.11073	MK045044	MK045196	N/A	N/A
Potamilus ohiensis 14	UF439204.11074	MK045045	MK045197	N/A	N/A
Potamilus ohiensis 15	UF439204.11075	MK045046	MK045198	N/A	N/A
Potamilus ohiensis 16	INHS35320	MK045047	MK045199	N/A	N/A
Potamilus ohiensis 17	INHS39560	MK045048	MK045200	N/A	N/A
Potamilus ohiensis 18	INHS39054	MK045049	MK045201	N/A	N/A
Potamilus ohiensis 19	INHS39054	MK045050	MK045202	N/A	N/A
Potamilus ohiensis 2	UF438806.6741	MK044957	MK045108	MK036208	MK036125
Potamilus ohiensis 3	INHS41658	MK044959	MK045110	MK036210	MK036127
Potamilus ohiensis 4	UF439451.03	MK045035	MK045187	N/A	N/A
Potamilus ohiensis 5	UF439451.04	MK045036	MK045188	N/A	N/A
Potamilus ohiensis 6	UF439451.05	MK045037	MK045189	N/A	N/A
Potamilus ohiensis 7	UF438806.6740	MK045038	MK045190	N/A	N/A
Potamilus ohiensis 8	UF438806.6742	MK045039	MK045191	N/A	N/A
Potamilus ohiensis 9	UF439129.10795	MK045040	MK045192	N/A	N/A
Potamilus purpuratus 1	UF441141.002	MK044980	MK045132	MK036231	MK036150
Potamilus purpuratus 2	UF438562.6544	MK044981	MK045133	MK036232	MK036151
Potamilus purpuratus 3	UF439145.9905	MK044960	MK045111	MK036211	MK036128
Potamilus purpuratus 4	UF439145.9906	MK044961	MK045112	MK036212	MK036129
Potamilus streckersoni 1	UF441294.001	MK044944	MK045095	MK036195	MK036112
Potamilus streckersoni 10	UF439478.170	MK044988	MK045140	N/A	N/A

Potamilus streckersoni 11	UF439478.171	MK044989	MK045141	N/A	N/A
Potamilus streckersoni 12	UF439478.172	MK044990	MK045142	N/A	N/A
Potamilus streckersoni 13	UF439478.173	MK044991	MK045143	N/A	N/A
Potamilus streckersoni 14	UF439479.216	MK044992	MK045144	N/A	N/A
Potamilus streckersoni 15	UF439480.220	MK044993	MK045145	N/A	N/A
Potamilus streckersoni 16	UF439481.231	MK044994	MK045146	N/A	N/A
Potamilus streckersoni 17	UF439481.232	MK044995	MK045147	N/A	N/A
Potamilus streckersoni 18	JBFMC8176.1	MK044996	MK045148	N/A	N/A
Potamilus streckersoni 19	JBFMC8402.2	MK044997	MK045149	N/A	N/A
Potamilus streckersoni 2	UF439477.021	MK044943	MK045094	MK036194	MK036111
Potamilus streckersoni 20	JBFMC8402.4	MK044998	MK045150	N/A	N/A
Potamilus streckersoni 21	JBFMC8402.5	MK044999	MK045151	N/A	N/A
Potamilus streckersoni 22	JBFMC8402.6	MK045000	MK045152	N/A	N/A
Potamilus streckersoni 23	JBFMC8406.1	MK045001	MK045153	N/A	N/A
Potamilus streckersoni 24	JBFMC8406.2	MK045002	MK045154	N/A	N/A
Potamilus streckersoni 25	JBFMC8411.1	MK045003	MK045155	N/A	N/A
Potamilus streckersoni 26	JBFMC8411.2	MK045004	MK045156	N/A	N/A
Potamilus streckersoni 27	JBFMC8433.1	MK045005	MK045157	N/A	N/A
Potamilus streckersoni 28	JBFMC8492.2	MK045006	MK045158	N/A	N/A
Potamilus streckersoni 29	JBFMC8492.3	MK045007	MK045159	N/A	N/A
Potamilus streckersoni 3	UF441294.004	MK044945	MK045096	MK036196	MK036113
Potamilus streckersoni 30	JBFMC8492.4	MK045008	MK045160	N/A	N/A
Potamilus streckersoni 4	UF439475.019	MK044982	MK045134	N/A	N/A
Potamilus streckersoni 5	UF439476.020	MK044983	MK045135	N/A	N/A
Potamilus streckersoni 6	UF441294.002	MK044984	MK045136	N/A	N/A
Potamilus streckersoni 7	UF441294.003	MK044985	MK045137	N/A	N/A
Potamilus streckersoni 8	UF438262.6305	MK044986	MK045138	N/A	N/A
Potamilus streckersoni 9	UF439497.169	MK044987	MK045139	N/A	N/A

Ptychobranchus fasciolaris 1	UF438231.6230	MK044962	MK045113	MK036213	MK036130
Ptychobranchus fasciolaris 2	UF438231.6226	MK044963	MK045114	MK036214	MK036131
Ptychobranchus fasciolaris 3	UF438231.6227	MK044964	MK045115	MK036215	MK036132
Quadrula quadrula	UA3563	MH633643	MH633595	MH362613	MK036133
Reginaia ebenus 1	UF438233.003	MK044965	MK045116	MK036216	MK036134
Reginaia ebenus 2	UF438233.004	MK044966	MK045117	MK036217	MK036135
Toxolasma lividum 1	UF438185.6055	MK044967	MK045118	MK036218	MK036136
Toxolasma lividum 2	UF438185.6057	MK044968	MK045119	MK036219	MK036137
Truncilla cognata 1	UF438552.6496	MK044969	MK045120	MK036220	MK036138
Truncilla cognata 2	UF438552.6502	MK044970	MK045121	MK036221	MK036139
Truncilla donaciformis 1	UF439324.35	MK044972	MK045123	MK036223	MK036141
Truncilla donaciformis 2	UF438243.001	MK044971	MK045122	MK036222	MK036140
Truncilla macrodon 1	UF441137.005	MK044973	MK045124	MK036224	MK036142
Truncilla macrodon 2	UF440984.001	KT285658	MK045125	KT285702	MK036143
Truncilla macrodon 3	UF439090.9867	MK044974	MK045126	MK036225	MK036144
Truncilla truncata 1	ASU1253.1	MK044975	MK045127	MK036226	MK036145
Truncilla truncata 2	UF438976.7025	MK044976	MK045128	MK036227	MK036146
Venustaconcha ellipsiformis	INHS36120-2	MK044977	MK045129	MK036228	MK036147
Villosa villosa 1	UF441040.067	MK044978	MK045130	MK036229	MK036148
Villosa villosa 2	UF438638.070	MK044979	MK045131	MK036230	MK036149

Table S2.2. Details for all morphological material examined for morphometric analyses including museum catalog numbers, drainage, and waterbody of collection. Museum abbreviations are as follows: (JBFMC – Joseph Britton Freshwater Mollusk Collection; UF – Florida Museum of Natural History).

Taxon	Catalog_Number	Drainage	Waterbody
Potamilus amphichaenus	JBFMC184A	Trinity	Lake Worth
Potamilus amphichaenus	JBFMC226B	Trinity	Lake Bridgeport
Potamilus amphichaenus	JBFMC416	Trinity	Grapevine Lake
Potamilus amphichaenus	JBFMC416.4	Trinity	Grapevine Lake
Potamilus amphichaenus	JBFMC8043.1	Neches	Neches River
Potamilus amphichaenus	JBFMC8043.2	Neches	Neches River
Potamilus amphichaenus	JBFMC8043.3	Neches	Neches River
Potamilus amphichaenus	JBFMC8043.4	Neches	Neches River
Potamilus amphichaenus	JBFMC8259.1	Sabine	Sabine River
Potamilus amphichaenus	JBFMC8259.2	Sabine	Sabine River
Potamilus amphichaenus	JBFMC8259.3	Sabine	Sabine River
Potamilus amphichaenus	JBFMC8376.1	Neches	Neches River
Potamilus amphichaenus	JBFMC8376.2	Neches	Neches River
Potamilus amphichaenus	JBFMC8376.3	Neches	Neches River
Potamilus amphichaenus	JBFMC8442.1	Trinity	Trinity River

Potamilus amphichaenus	JBFMC8442.2	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8442.3	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8442.4	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8442.5	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8442.6	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8444.1	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8444.2	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8444.3	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8444.4	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8444.5	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8444.6	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8450.1	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8450.2	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8450.3	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8450.4	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8468.1	Trinity	Trinity River
Potamilus amphichaenus	JBFMC8634.1	Sabine	Sabine River
Potamilus amphichaenus	UF439482.237	Sabine	Sabine River
Potamilus amphichaenus	UF439483.238	Sabine	Sabine River
Potamilus ohiensis	JBFMC8632.1	Red	Red River
Potamilus ohiensis	JBFMC8632.2	Red	Red River
Potamilus ohiensis	JBFMC8632.3	Red	Red River
Potamilus ohiensis	JBFMC8643.1	Red	Red River
Potamilus ohiensis	JBFMC8643.2	Red	Red River
Potamilus ohiensis	JBFMC8646.1	Red	Red River
Potamilus ohiensis	JBFMC8655.1	Red	Red River
Potamilus ohiensis	JBFMC8655.2	Red	Red River
Potamilus ohiensis	JBFMC8659.1	Red	Red River
Potamilus ohiensis	JBFMC8659.2	Red	Red River
Potamilus ohiensis	JBFMC8663.1	Red	Red River
Potamilus ohiensis	JBFMC8663.2	Red	Red River
Potamilus streckersoni	JBFMC26.1	Brazos	Possum Kingdom Reservoir
Potamilus streckersoni	JBFMC41D	Brazos	Brazos River
Potamilus streckersoni	JBFMC41E	Brazos	Brazos River
Potamilus streckersoni	JBFMC41G	Brazos	Brazos River
Potamilus streckersoni	JBFMC147	Brazos	Brazos River
Potamilus streckersoni	JBFMC178	Brazos	Clear Fork Brazos River
Potamilus streckersoni	JBFMC292	Brazos	Brazos River
Potamilus streckersoni	JBFMC433.1	Brazos	Yegua Creek
Potamilus streckersoni	JBFMC433.2	Brazos	Yegua Creek
Potamilus streckersoni	JBFMC433.3	Brazos	Yegua Creek
Potamilus streckersoni	JBFMC8176.1	Brazos	Brazos River
Potamilus streckersoni	JBFMC8210.1	Brazos	Brazos River

Potamilus streckersoni	JBFMC8402.1	Brazos	Brazos River
Potamilus streckersoni	JBFMC8402.2	Brazos	Brazos River
Potamilus streckersoni	JBFMC8402.3	Brazos	Brazos River
Potamilus streckersoni	JBFMC8402.4	Brazos	Brazos River
Potamilus streckersoni	JBFMC8402.5	Brazos	Brazos River
Potamilus streckersoni	JBFMC8402.6	Brazos	Brazos River
Potamilus streckersoni	JBFMC8406.1	Brazos	Brazos River
Potamilus streckersoni	JBFMC8406.2	Brazos	Brazos River
Potamilus streckersoni	JBFMC8406.3	Brazos	Brazos River
Potamilus streckersoni	JBFMC8411.1	Brazos	Brazos River
Potamilus streckersoni	JBFMC8411.2	Brazos	Brazos River
Potamilus streckersoni	JBFMC8433.1	Brazos	Brazos River
Potamilus streckersoni	JBFMC8492.2	Brazos	Brazos River
Potamilus streckersoni	JBFMC8492.3	Brazos	Brazos River
Potamilus streckersoni	JBFMC8492.4	Brazos	Brazos River
Potamilus streckersoni	JBFMC8492.5	Brazos	Brazos River
Potamilus streckersoni	JBFMC8492.6	Brazos	Brazos River
Potamilus streckersoni	UF439479.216	Brazos	Brazos River
Potamilus streckersoni	UF439480.220	Brazos	Brazos River
Potamilus streckersoni	UF439481.231	Brazos	Brazos River
Potamilus streckersoni	UF439475.019	Brazos	Brazos River
Potamilus streckersoni	UF439476.020	Brazos	Brazos River
Potamilus streckersoni	UF439477.021	Brazos	Brazos River
Potamilus streckersoni	UF439478.169	Brazos	Brazos River
Potamilus streckersoni	UF439478.170	Brazos	Brazos River
Potamilus streckersoni	UF439478.171	Brazos	Brazos River
Potamilus streckersoni	UF439478.172	Brazos	Brazos River
Potamilus streckersoni	UF439478.173	Brazos	Brazos River
Potamilus streckersoni	UF439481.232	Brazos	Brazos River

Table S2.3. Distribution data used to create the conservation map of *P. streckersoni* sp. nov. Sources of the distribution data were as follows: Baylor University Mayborn Museum (BU-MMC_MO); Fort Worth Museum of Science and History (FWMSH); Texas Parks and Wildlife Department (TPWD), Texas A&M Institute of Renewable Natural Resources (Texas A&M NRI); Joseph Britton Freshwater Mollusk Collection (JBFMC); Ohio State University Museum (OSUM); Texas A&M Natural Resource Institute (Texas A&M NRI); University of Florida Museum of Natural History (FLMNH); University of Michigan Museum of Zoology (UMMZ); University of Science and Arts of Oklahoma (USAO); and U.S. Fish and Wildlife Service (USFWS). Null values indicate missing information.

Species	Temporal period	Year	Waterbody	Source	HUC_10
Potamilus streckersoni	Shell	1994	Clear Fork Brazos River	Howells 1996 MDS 120	1206010401
Potamilus streckersoni	Shell	2010	Collins Creek	Texas A&M NRI	1206010401

Potamilus streckersoni	Shell	1994	Clear Fork Brazos River	Howells 1996 MDS 120	1206010402
Potamilus streckersoni	Recent	1999	Hubbard Creek Reservoir	Howells 2000 MDS 170	1206010506
Potamilus streckersoni	Recent	1999	Hubbard Creek Reservoir	Howells 2000 MDS 170	1206010506
Potamilus streckersoni	Shell	2012	Hubbard Creek	Texas A&M NRI	1206010506
Potamilus streckersoni	Shell	1998	Possum Kingdom Reservoir	USAO 9038	1206020105
Potamilus streckersoni	Shell	2007	Brazos River	Texas A&M NRI	1206020105
Potamilus streckersoni	Shell	1969	Brazos River	FWMSH_94V 1923	1206020107
Potamilus streckersoni	Shell	1969	Brazos River	FWMSH_94V 1922	1206020107
Potamilus streckersoni	Shell	1962	Brazos River	FWMSH_1001	1206020107
Potamilus streckersoni	Shell	1996	Brazos River	Howells 1997 MDS 144	1206020110
Potamilus streckersoni	Shell	1996	Brazos River	Howells 1997 MDS 144	1206020110
Potamilus streckersoni	Shell	1996	Brazos River	Howells 1997 MDS 144	1206020111
Potamilus streckersoni	Shell	1996	Brazos River	Howells 1997 MDS 144	1206020111
Potamilus streckersoni	Shell	1971	Brazos River	Texas A&M NRI	1206020113
Potamilus streckersoni	Shell	1976	Brazos River	Texas A&M NRI	1206020113
Potamilus streckersoni	Shell	1975	Brazos River	Texas A&M NRI	1206020113
Potamilus streckersoni	Shell	1996	Brazos River	Howells 1997 MDS 144	1206020113
Potamilus streckersoni	Recent	1998	Brazos River	Howells 1999 MDS 161	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31281 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31282 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31283 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31289 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31290 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31291 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31284 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31285 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31286 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31287 -A-B	1206020203

Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31288 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31292 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31293 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31294 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31295 -A-B	1206020203
Potamilus streckersoni	Shell	N/A	Paluxy Creek	BU-MMC_MO 31296 -A-B	1206020203
Potamilus streckersoni	Shell	2007	Brazos River	Texas A&M NRI	1206020203
Potamilus streckersoni	Shell	2007	Brazos River	Texas A&M NRI	1206020203
Potamilus streckersoni	Shell	N/A	Aquilla Creek	BU-MMC_MO 32281 -A-B	1206020205
Potamilus streckersoni	Shell	N/A	Aquilla Creek	BU-MMC_MO 32282 -A-B	1206020205
Potamilus streckersoni	Shell	N/A	Aquilla Creek	BU-MMC_MO 32283 -A-B	1206020205
Potamilus streckersoni	Shell	N/A	Aquilla Creek	BU-MMC_MO 32284 -A-B	1206020205
Potamilus streckersoni	Historical	N/A	Brazos River	UMMZ_83009	1206020208
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31508 -A-B	1206020208
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31509 -A-B	1206020208
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31510 -A-B	1206020208
Potamilus streckersoni	Shell	1994	Brazos River	Howells 1996 MDS 120	1206020208
Potamilus streckersoni	Shell	1994	Brazos River	Howells 1996 MDS 120	1206020208
Potamilus streckersoni	Shell	2016	Brazos River	Texas A&M NRI	1206020208
Potamilus streckersoni	Shell	N/A	Brazos River	UMMZ 83009	1206020208
Potamilus streckersoni	Shell	1934	Middle Bosque River	UMMZ 58929	1206020301
Potamilus streckersoni	Shell	1980	Lake Waco	USAO 3744	1206020303
Potamilus streckersoni	Shell	1938	South Bosque River	UMMZ 132520	1206020303
Potamilus streckersoni	Historical	N/A	North Bosque River	UMMZ_83007	1206020404
Potamilus streckersoni	Shell	1934	North Bosque River	UMMZ 58926	1206020404
Potamilus streckersoni	Shell	N/A	North Bosque River	UMMZ 83007	1206020404
Potamilus streckersoni	Shell	N/A	North Bosque River	UMMZ 88992	1206020404
Potamilus streckersoni	Current	2017	Brazos River	UF439477	1207010101

Potamilus streckersoni	Shell	1994	Brazos River	Howells 1996 MDS 120	1207010101
Potamilus streckersoni	Current	2017	Brazos River	JBFMC_8433	1207010103
Potamilus streckersoni	Current	2017	Brazos River	JBFMC_8492	1207010103
Potamilus streckersoni	Current	2017	Brazos River	UF439476	1207010103
Potamilus streckersoni	Current	2018	Brazos River	UF439479	1207010103
Potamilus streckersoni	Current	2018	Brazos River	UF439480	1207010103
Potamilus streckersoni	Shell	1996	Brazos River	Howells 1997 MDS 144	1207010103
Potamilus streckersoni	Current	2018	Brazos River	UF439481	1207010106
Potamilus streckersoni	Current	2017	Brazos River	UF439475	1207010106
Potamilus streckersoni	Current	2017	Brazos River	UF439478	1207010106
Potamilus streckersoni	Historical	1994	Brazos River	Howells 1996 MDS 125	1207010106
Potamilus streckersoni	Shell	1999	Brazos River	Howells 2000 MDS 170	1207010106
Potamilus streckersoni	Shell	1994	Brazos River	Howells 1996 MDS 120	1207010106
Potamilus streckersoni	Shell	2006	Little Brazos River	Texas A&M NRI	1207010106
Potamilus streckersoni	Shell	1977	Brazos River	Texas A&M NRI	1207010106
Potamilus streckersoni	Current	2013	Brazos River	JBFMC_292	1207010107
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010107
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010107
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010107
Potamilus streckersoni	Shell	1994	Brazos River	Howells 1996 MDS 120	1207010107
Potamilus streckersoni	Current	2014	Lower Brazos River	Texas A&M NRI	1207010108
Potamilus streckersoni	Current	2012	Brazos River	JBFMC_8210	1207010108
Potamilus streckersoni	Current	2012	Brazos River	JBFMC_8210	1207010108
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010108
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010108
Potamilus streckersoni	Shell	2006	Brazos River	Texas A&M NRI	1207010108
Potamilus streckersoni	Shell	1994	Brazos River	Howells 1996 MDS 120	1207010108
Potamilus streckersoni	Current	2011	Yegua Creek	Texas A&M NRI	1207010203

Potamilus streckersoni	Shell	2011	Yegua Creek	Texas A&M NRI	1207010203
Potamilus streckersoni	Current	2012	Yegua Creek	Texas A&M NRI	1207010205
Potamilus streckersoni	Current	2012	Yegua Creek	Texas A&M NRI	1207010205
Potamilus streckersoni	Current	2012	Yegua Creek	Texas A&M NRI	1207010205
Potamilus streckersoni	Current	2012	Yegua Creek	Texas A&M NRI	1207010205
Potamilus streckersoni	Shell	2006	Yegua Creek	Texas A&M NRI	1207010205
Potamilus streckersoni	Shell	1974	Navasota River	Littleton 1979/Calnan 1976	1207010301
Potamilus streckersoni	Current	2016	Navasota River	Texas A&M NRI	1207010308
Potamilus streckersoni	Current	2016	Navasota River	Texas A&M NRI	1207010308
Potamilus streckersoni	Current	2016	Navasota River	Texas A&M NRI	1207010308
Potamilus streckersoni	Current	2016	Navasota River	Texas A&M NRI	1207010308
Potamilus streckersoni	Current	2016	Navasota River	Texas A&M NRI	1207010308
Potamilus streckersoni	Shell	1984	Navasota River	USAO 2744	1207010308
Potamilus streckersoni	Shell	1973	Navasota River	Littleton 1979/Calnan 1976	1207010308
Potamilus streckersoni	Current	2012	Brazos River	JBFMC_8411	1207010401
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010401
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010401
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010401
Potamilus streckersoni	Recent	2006	Brazos River	Texas A&M NRI	1207010401
Potamilus streckersoni	Historical	1982	Brazos River	USAO 1539	1207010401
Potamilus streckersoni	Shell	1981	Brazos River	USAO 662	1207010401
Potamilus streckersoni	Current	2012	Brazos River	JBFMC_8176	1207010403
Potamilus streckersoni	Current	2012	Brazos River	JBFMC_8176	1207010403
Potamilus streckersoni	Current	2012	Brazos River	JBFMC_8402	1207010403
Potamilus streckersoni	Current	2012	Brazos River	JBFMC_8406	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403

Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Historical	N/A	Brazos River	UMMZ_83011	1207010403
Potamilus streckersoni	Historical	1945	Brazos River	UMMZ_165435	1207010403
Potamilus streckersoni	Shell	N/A	Brazos River	Strecker 1931	1207010403
Potamilus streckersoni	Shell	1945	Brazos River	UMMZ 165435	1207010403
Potamilus streckersoni	Shell	N/A	Unnamed Pond	UMMZ 83011	1207010403
Potamilus streckersoni	Shell	2006	Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Shell	2012	Lower Brazos River	Texas A&M NRI	1207010403
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Current	2013	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010404

Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Current	2012	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Shell	2006	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Shell	2011	Brazos River	Texas A&M NRI	1207010404
Potamilus streckersoni	Current	2015	Little River	Texas A&M NRI	1207020401
Potamilus streckersoni	Current	2015	Little River	Texas A&M NRI	1207020401
Potamilus streckersoni	Current	2015	Brushy Creek	Texas A&M NRI	1207020504
Potamilus streckersoni	Current	2015	Brushy Creek	Texas A&M NRI	1207020504
Potamilus streckersoni	Historical	1977	Brazos River	OSUM	N/A
Potamilus streckersoni	Historical	1970	Brazos River Canal	OSUM	N/A
Potamilus streckersoni	Historical	N/A	North Bosque River	UMMZ_58926	N/A
Potamilus streckersoni	Historical	N/A	North Bosque River	UMMZ_88992	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31252 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31254 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31255 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31256 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31257 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31258 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31259 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31272 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31273 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31274 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31275 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31260 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31261 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31262 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31263 -A-B	N/A

Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31264 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31265 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31266 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31267 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31268 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31269 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31270 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31271 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31298 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31299 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31300 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31301 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31302 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31303 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31304 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31305 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31306 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31307 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31308 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31309 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31310 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31311 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31312 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31313 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31314 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31315 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 31316 -A-B	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	BU-MMC_MO 34334 -A-B	N/A

Potamilus streckersoni	Shell	N/A	Navasota River	BU-MMC_MO 31253 -A-B	N/A
Potamilus streckersoni	Shell	N/A	North bosque River	BU-MMC_MO 31429 -A-B	N/A
Potamilus streckersoni	Shell	N/A	North bosque River	BU-MMC_MO 31496 -A-B	N/A
Potamilus streckersoni	Shell	N/A	North Bosque River	BU-MMC_MO 31497 -A-B	N/A
Potamilus streckersoni	Shell	1974	Diversion Reservoir	Texas A&M NRI	N/A
Potamilus streckersoni	Shell	1975	Unrecorded	Texas A&M NRI	N/A
Potamilus streckersoni	Shell	1974	Brazos River	Texas A&M NRI	N/A
Potamilus streckersoni	Shell	1970	Possom Kingdom Lake	Texas A&M NRI	N/A
Potamilus streckersoni	Shell	1974	Possom Kingdom Lake	Texas A&M NRI	N/A
Potamilus streckersoni	Shell	1971	Brazos River	Texas A&M NRI	N/A
Potamilus streckersoni	Shell	1981	Brazos River	OSUM	N/A
Potamilus streckersoni	Shell	N/A	Aquilla Creek	Strecker 1931	N/A
Potamilus streckersoni	Shell	N/A	Aquilla Creek	Strecker 1931	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	Strecker 1931	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	Strecker 1931	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	Strecker 1931	N/A
Potamilus streckersoni	Shell	N/A	Brazos River	Strecker 1931	N/A
Potamilus streckersoni	Shell	1974	Navasota River	Littleton 1979/Calnan 1976	N/A
Potamilus streckersoni	Shell	N/A	North Bosque River	Strecker 1931	N/A

Table S3.1. Fecundity data for *Leptodea ochracea*, *Potamilus inflatus*, and *P. streckersoni.*

Taxa (Number of individuals)	Catalog Number	Mean Length (mm)	Mean Fecundity	Fecundity Range
Leptodea ochracea (2)	UF438217	47	30,500	28,400-32,600
Potamilus inflatus (3)	UF439514	46.67	45,666.67	25,200-61,400
Potamilus streckersoni (3)	UF441294	93.67	1,876,000	1,332,000-2,272,000

Table S4.1. Morphological material examined in this study with catalog numbers and locality information of where specimens were collected, including river drainage, waterbody, and county. Museum abbreviations are as follows: (HMNS– Houston Museum of Natural Science; JBFMC – Joseph Britton Freshwater Mollusk Collection; UF – Florida Museum of Natural History).

Taxon	Catalog_Number	Drainage	Waterbody	County
Fusconaia iheringi	BV127	Colorado	San Saba River	Menard
Fusconaia iheringi	BV128	Colorado	San Saba River	Menard
Fusconaia iheringi	BV129	Colorado	San Saba River	Menard
Fusconaia iheringi	BV130	Colorado	San Saba River	Menard
Fusconaia iheringi	BV131	Brazos	Leon River	Coryell
Fusconaia iheringi	BV132	Brazos	Leon River	Coryell
Fusconaia mitchelli	BV133	Guadalupe	Guadalupe River	Comal
Fusconaia mitchelli	BV134	Guadalupe	Guadalupe River	Victoria
Fusconaia mitchelli	BV135	Guadalupe	Guadalupe River	Comal
Fusconaia mitchelli	BV144	Guadalupe	Guadalupe River	Kendall
Fusconaia iheringi	BV1544	Brazos	Leon/Little River	Bell
Fusconaia iheringi	BV1545	Brazos	Leon/Little River	Bell
Fusconaia iheringi	BV187	Colorado	Llano River	Mason
Fusconaia iheringi	BV188	Colorado	Llano River	Mason
Fusconaia iheringi	BV189	Colorado	Llano River	Mason
Fusconaia iheringi	BV190	Colorado	Llano River	Mason
Fusconaia iheringi	BV2501	Colorado	Colorado River	Travis
Fusconaia iheringi	BV3552	Colorado	Llano River	Mason
Fusconaia iheringi	BV3553	Colorado	Llano River	Mason
Fusconaia iheringi	BV3554	Colorado	Llano River	Mason
Fusconaia iheringi	BV3555	Colorado	Llano River	Mason
Fusconaia iheringi	BV3556	Colorado	Llano River	Mason
Fusconaia iheringi	BV3557	Colorado	Llano River	Mason
Fusconaia iheringi	BV5286	Brazos	Leon River	Coryell
Fusconaia mitchelli	BV5287	Guadalupe	Guadalupe River	Kendall
Fusconaia iheringi	BV6064	Brazos	Leon River	Coryell
Fusconaia iheringi	BV6065	Brazos	Leon River	Coryell
Fusconaia mitchelli	HMNS32346	Guadalupe	Geronimo Creek	Guadalupe
Fusconaia iheringi	JBFMC8065.1	Brazos	San Gabriel River	Williamson
Fusconaia iheringi	JBFMC8065.2	Brazos	San Gabriel River	Williamson
Fusconaia iheringi	JBFMC8102.1	Colorado	Little River	Milam
Fusconaia iheringi	JBFMC8102.2	Colorado	Little River	Milam
Fusconaia iheringi	JBFMC8102.3	Colorado	Little River	Milam
Fusconaia mitchelli	JBFMC8188.2	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC8188.3	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC8188.4	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC8188.7	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC8188.8	Guadalupe	Guadalupe River	DeWitt

Fusconaia mitchelli	JBFMC8188.9	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt

Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	JBFMC9594	Guadalupe	Guadalupe River	DeWitt
Fusconaia iheringi	UF438010	Colorado	San Saba River	San Saba
Fusconaia iheringi	UF438155	Colorado	Llano River	Mason
Fusconaia mitchelli	UF438139	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	UF438139	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	UF438139	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	UF438139	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	UF438139	Guadalupe	Guadalupe River	DeWitt
Fusconaia iheringi	UF438156	Brazos	San Gabriel River	Williamson
Fusconaia iheringi	UF438156	Brazos	San Gabriel River	Williamson
Fusconaia iheringi	UF438156	Brazos	San Gabriel River	Williamson
Fusconaia iheringi	UF438156	Brazos	San Gabriel River	Williamson
Fusconaia mitchelli	UF438549	Guadalupe	Guadalupe River	DeWitt
Fusconaia mitchelli	UF438549	Guadalupe	Guadalupe River	DeWitt
Fusconaia iheringi	UF438745	Colorado	Llano River	Mason
Fusconaia iheringi	UF439060	Brazos	Little River	Milam
Fusconaia iheringi	UF439060	Brazos	Little River	Milam
Fusconaia iheringi	UF439060	Brazos	Little River	Milam
Fusconaia iheringi	UF439060	Brazos	Little River	Milam
Fusconaia mitchelli	UF441081	Guadalupe	Guadalupe River	Gonzalez
Fusconaia mitchelli	UF441082	Guadalupe	Guadalupe River	Gonzalez
Fusconaia iheringi	UF441083	Colorado	San Saba River	San Saba

Table S4.2. Distribution data used to create the conservation maps of *Fusconaia iheringi* and *Fusconaia mitchelli*. Sources of the distribution data were as follows: Auburn University Museum of Natural History (AUMNH), Baylor University Mayborn Museum (BU), Florida Museum of Natural History (FLMNH), Fort Worth Museum of Science and History (FWMNH), Houston Museum of Natural Science (HMNS), Joseph Britton Freshwater Mollusk Collection (JBFMC), Smithsonian National Museum of Natural History (USNM), Texas A&M Natural Resources Institute (NRI), Texas Department of Transportation (TXDOT), Texas Parks and Wildlife Department (TPWD), University of Michigan Museum of Zoology (UMMZ), and U.S. Fish and Wildlife Service (USFWS).

Taxon	Temporal period	Year	Waterbody	Drainage	County	Source	HUC-10
Fusconaia iheringi	Historical	1980	Llano River	Colorado	Mason	TPWD	1209020405
Fusconaia iheringi	Historical	1980	Llano River	Colorado	Llano	TPWD	1209020406
Fusconaia iheringi	Historical	1980	San Saba River	Colorado	Menard	TPWD	1209010904
Fusconaia iheringi	Historical	1992	Pedernales River	Colorado	Blanco	TPWD	1209020603
Fusconaia iheringi	Recent	2000	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Recent	2000	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Historical	1992	Pedernales River	Colorado	Blanco	TPWD	1209020603
Fusconaia iheringi	Recent	2001	Salado Creek	Guadalupe	Bexar	TPWD	1210030101
Fusconaia iheringi	Historical	1993	San Saba River	Colorado	McCulloch	TPWD	1209010907
Fusconaia iheringi	Recent	1995	Pecan Bayou	Colorado	Brown	TPWD	1209010704
Fusconaia iheringi	Current	2011	San Saba River	Colorado	San Saba	TPWD	1209010908
Fusconaia iheringi	Current	2011	San Saba River	Colorado	San Saba	TPWD	1209010606
Fusconaia iheringi	Historical	1949	Guadalupe River	Guadalupe	Guadalupe	TPWD	1210020201
Fusconaia iheringi	Historical	1974	Guadalupe River	Guadalupe	Kendall	TPWD	1210020103
Fusconaia iheringi	Historical	1974	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Historical	1979	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Historical	1982	San Marcos River	Guadalupe	Caldwell	TPWD	1210020305
Fusconaia iheringi	Historical	1982	San Marcos River	Guadalupe	Caldwell	TPWD	1210020305
Fusconaia iheringi	Historical	1985	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Historical	1993	Leon River	Brazos	Bell	TPWD	1207020111
Fusconaia iheringi	Historical	1993	Guadalupe River	Guadalupe	Gonzales	TPWD	1210020202
Fusconaia iheringi	Historical	1993	Guadalupe River	Guadalupe	Gonzales	TPWD	1210020202

Fusconaia iheringi	Recent	1997	Guadalupe River	Guadalupe	Gonzales	TPWD	1210020204
Fusconaia iheringi	Recent	2005	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Recent	2006	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Recent	2009	Guadalupe River	Guadalupe	Kendall	TPWD	1210020103
Fusconaia iheringi	Current	2012	San Gabriel River	Brazos	Milam	TXDOT	1207020505
Fusconaia iheringi	Historical	1905	Llano River	Colorado	Llano	AUMNH	1209020406
Fusconaia iheringi	Historical	1905	Llano River	Colorado	Llano	AUMNH	1209020406
Fusconaia iheringi	Historical	1905	Llano River	Colorado	Llano	AUMNH	1209020406
Fusconaia iheringi	Historical	1905	Llano River	Colorado	Llano	AUMNH	1209020406
Fusconaia iheringi	Historical	1974	Llano River	Colorado	Mason	TPWD	1209020405
Fusconaia iheringi	Historical	1974	Llano River	Colorado	Llano	TPWD	1209020406
Fusconaia iheringi	Historical	1974	Llano River	Colorado	Mason	TPWD	1209020406
Fusconaia iheringi	Historical	1974	Llano River	Colorado	Llano	TPWD	1209020407
Fusconaia iheringi	Historical	1980	Johnson Fork Creek	Colorado	Kimble	TPWD	1209020401
Fusconaia iheringi	Historical	1980	Llano River	Colorado	Kimble	TPWD	1209020402
Fusconaia iheringi	Recent	2009	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Recent	2009	San Marcos River	Guadalupe	Gonzales	TPWD	1210020305
Fusconaia iheringi	Unknown	N/A	Brazos River	Brazos	Burleson	FLMNH	1207010106
Fusconaia iheringi	Unknown	N/A	Leon River	Brazos	Bell	BU	1207020111
Fusconaia iheringi	Unknown	N/A	Leon River	Brazos	Bell	BU	1207020111
Fusconaia iheringi	Unknown	N/A	Leon River	Brazos	Coryell	BU	1207020109
Fusconaia iheringi	Unknown	N/A	Leon River	Brazos	Coryell	BU	1207020109
Fusconaia iheringi	Unknown	N/A	Leon River	Brazos	Coryell	BU	1207020109
Fusconaia iheringi	Historical	1978	Not recorded	Brazos	Coryell	BU	1207020109
Fusconaia iheringi	Historical	1978	Not recorded	Brazos	Coryell	BU	1207020109
Fusconaia iheringi	Unknown	N/A	Colorado River	Colorado	Travis	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown

Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	BU	Unknown
Fusconaia iheringi	Unknown	N/A	San Saba River	Colorado	Menard	BU	Unknown
Fusconaia iheringi	Unknown	N/A	San Saba River	Colorado	Menard	BU	Unknown
Fusconaia iheringi	Unknown	N/A	San Saba River	Colorado	Menard	BU	Unknown
Fusconaia iheringi	Unknown	N/A	San Saba River	Colorado	Menard	BU	Unknown
Fusconaia iheringi	Unknown	N/A	San Saba River	Colorado	Menard	FLMNH	Unknown
Fusconaia iheringi	Historical	1995	Llano River	Colorado	Kimble	TPWD	Unknown
Fusconaia iheringi	Historical	1982	Llano River	Colorado	Llano	TPWD	Unknown
Fusconaia iheringi	Historical	1981	Brazos River	Brazos	Somervell	TPWD	Unknown
Fusconaia iheringi	Unknown	N/A	Leon River	Brazos	Bell	TPWD	Unknown
Fusconaia iheringi	Unknown	N/A	Leon River	Brazos	Coryell	TPWD	Unknown
Fusconaia iheringi	Unknown	N/A	Llano River	Colorado	Mason	TPWD	Unknown
Fusconaia iheringi	Historical	1974	Llano River	Colorado	Llano/Mason	TPWD	Unknown
Fusconaia iheringi	Historical	1972	Llano River	Colorado	Kimble	TPWD	Unknown
Fusconaia iheringi	Historical	1972	Llano River	Colorado	Llano	TPWD	Unknown
Fusconaia iheringi	Historical	1973	Llano River	Colorado	Llano	TPWD	Unknown
Fusconaia iheringi	Historical	1972	Llano River	Colorado	Llano	TPWD	Unknown
Fusconaia iheringi	Historical	1972	Llano River	Colorado	Mason	TPWD	Unknown
Fusconaia iheringi	Historical	1973	Llano River	Colorado	Mason	TPWD	Unknown
Fusconaia iheringi	Unknown	N/A	San Saba River	Colorado	Menard	TPWD	Unknown
Fusconaia iheringi	Historical	1905	San Saba River	Colorado	Not Recorded	TPWD	Unknown

Fusconaia iheringi	Unknown	N/A	Leon River	Brazos	Bell	TPWD	Unknown
Fusconaia iheringi	Unknown	N/A	San Saba River	Colorado	Menard	TPWD	Unknown
Fusconaia iheringi	Historical	1898	San Saba River	Colorado	Menard	USNM	Unknown
Fusconaia mitchelli	Current	2011	Guadalupe River	Guadalupe	Comal	TXDOT	1210020104
Fusconaia mitchelli	Current	2012	San Marcos River	Guadalupe	Caldwell	USFWS	1210020305
Fusconaia mitchelli	Current	2012	San Marcos River	Guadalupe	Gonzales	USFWS	1210020305
Fusconaia mitchelli	Current	2012	San Marcos River	Guadalupe	Gonzales	USFWS	1210020305
Fusconaia mitchelli	Current	2016	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2016	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Comal	NRI	1210020201
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Comal	BU	1210020201
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Kendall	BU	1210020103
Fusconaia mitchelli	Current	2012	San Gabriel River	Brazos	Milam	TXDOT	1207020505
Fusconaia mitchelli	Current	2013	San Gabriel River	Brazos	Milam	TXDOT	1207020505
Fusconaia mitchelli	Current	2013	San Gabriel River	Brazos	Williamson	NRI	1207020505
Fusconaia mitchelli	Current	2015	Little River	Brazos	Milam	NRI	1207020401
Fusconaia mitchelli	Current	2015	Little River	Brazos	Milam	NRI	1207020401
Fusconaia mitchelli	Current	2015	Little River	Brazos	Milam	NRI	1207020401
Fusconaia mitchelli	Current	2015	Little River	Brazos	Milam	NRI	1207020401
Fusconaia mitchelli	Current	2015	Brushy Creek	Brazos	Milam	NRI	1207020504
Fusconaia mitchelli	Current	2015	San Gabriel River	Brazos	Williamson	NRI	1207020505
Fusconaia mitchelli	Current	2015	San Gabriel River	Brazos	Williamson	NRI	1207020505
Fusconaia mitchelli	Historical	1971	San Saba River	Colorado	Menard	FWMNH	1209010906
Fusconaia mitchelli	Historical	1974	Llano River	Colorado	Mason	TPWD	1209020405
Fusconaia mitchelli	Historical	1974	Llano River	Colorado	Llano	TPWD	1209020406
Fusconaia mitchelli	Current	2013	Guadalupe River	Guadalupe	Gonzales	TPWD	1210020202
Fusconaia mitchelli	Current	2013	Guadalupe River	Guadalupe	Gonzales	TPWD	1210020202
Fusconaia mitchelli	Current	2013	Guadalupe River	Guadalupe	Gonzales	TPWD	1210020202

Fusconaia mitchelli	Current	2013	Guadalupe River	Guadalupe	Victoria	TPWD	1210020401
Fusconaia mitchelli	Current	2013	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2013	Guadalupe River	Guadalupe	Gonzales	TPWD	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2014	Guadalupe River	Guadalupe	Gonzales	NRI	1210020202
Fusconaia mitchelli	Current	2015	Guadalupe River	Guadalupe	De Witt	NRI	1210020204

Fusconaia mitchelli	Current	2012	San Saba River	Colorado	San Saba	IRNR	1209010908
Fusconaia mitchelli	Current	2016	Guadalupe River	Guadalupe	De Witt	NRI	1210020204
Fusconaia mitchelli	Current	2012	San Saba River	Colorado	San Saba	IRNR	1209010606
Fusconaia mitchelli	Current	2012	Llano River	Colorado	Mason	NRI	1209020403
Fusconaia mitchelli	Current	2015	Llano River	Colorado	Mason	NRI	1209020403
Fusconaia mitchelli	Current	2015	Llano River	Colorado	Mason	NRI	1209020403
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Kendall	BU	Unknown
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Victoria	BU	Unknown
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Victoria	BU	Unknown
Fusconaia mitchelli	Historical	1982	San Marcos River	Guadalupe	Caldwell	TPWD	Unknown
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Kendall	TPWD	Unknown
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Kerr	TPWD	Unknown
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Victoria	TPWD	Unknown
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Victoria	TPWD	Unknown
Fusconaia mitchelli	Historical	1904	Guadalupe River	Guadalupe	Comal	TPWD	Unknown
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Kendall	TPWD	Unknown
Fusconaia mitchelli	Historical	1974	Guadalupe River	Guadalupe	Kendall	TPWD	Unknown
Fusconaia mitchelli	Historical	1974	Guadalupe River	Guadalupe	Kendall	TPWD	Unknown
Fusconaia mitchelli	Historical	1974	Guadalupe River	Guadalupe	Kendall	TPWD	Unknown
Fusconaia mitchelli	Historical	1974	Guadalupe River	Guadalupe	Kendall	TPWD	Unknown
Fusconaia mitchelli	Recent	2005	San Marcos River	Guadalupe	Gonzales	TPWD	Unknown
Fusconaia mitchelli	Unknown	N/A	Guadalupe River	Guadalupe	Victoria	USNM	Unknown
Fusconaia mitchelli	Historical	1902	Guadalupe River	Guadalupe	Kerr	USNM	Unknown
Fusconaia mitchelli	Unknown	N/A	Geronimo Creek	Guadalupe	Guadalupe	HMNS	Unknown

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