PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE CAROLINA HEELSPLITTER (*LASMIGONA DECORATA*) A CRITICALLY-ENDANGERED FRESHWATER MUSSEL

A Thesis
by
VICTORIA CLARK FOWLER

Submitted to the School of Graduate Studies
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2023
Department of Biology

PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE CAROLINA HEELSPLITTER (*LASMIGONA DECORATA*) A CRITICALLY-ENDANGERED FRESHWATER MUSSEL

A Thesis
by
VICTORIA CLARK FOWLER
December 2023

APPROVED BY:

Michael M. Gangloff, Ph.D.
Chairperson, Thesis Committee

Matt C. Estep, Ph.D.
Member, Thesis Committee

Lynn M. Siefferman, Ph.D.
Member, Thesis Committee

Ava Udvadia, Ph.D.
Chairperson, Department of Biology

Ashley Colquitt, Ph.D.
Associate Vice Provost and Dean, Cratis D. Williams School of Graduate Studies

Abstract

PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE CAROLINA HEELSPLITTER (*LASMIGONA DECORATA*) A CRITICALLY-ENDANGERED FRESHWATER MUSSEL

Victoria Clark Fowler
B.S., Appalachian State University
M.S., Appalachian State University

Chairperson: Michael M. Gangloff, Ph.D.

Freshwater mussel biodiversity has declined substantially in many southern Atlantic Slope streams during the last four decades and several species endemic to the region are now critically-endangered. The Carolina heelsplitter (*Lasmigona decorata*) is a federally-endangered freshwater mussel that historically occurred in the Pee Dee, Santee and Savannah basins in Georgia, North Carolina, and South Carolina. Currently, Carolina heelsplitters persist as small, isolated populations, primarily within the Charlotte and Carolina Slate Belt physiographic provinces. Captive propagation and population augmentation efforts are underway, however the degree of genetic variation existing within these populations is unknown. Additionally, earlier work provided evidence that the genus *Lasmigona* is not monophyletic. I obtained non-lethal DNA samples from wild animals and individuals in hatchery facilities to assess genetic diversity and historical gene flow patterns within and among Carolina heelsplitter populations. I generated and examined 93 *COI* sequences from 6 Carolina heelsplitters populations from the Santee, Pee Dee, and Savannah basins. I found

surprisingly low levels of genetic divergence (≤0.14%) both within and among populations. Three *COI* haplotypes were identified and two of them were shared among populations in all three river basins. A third haplotype was restricted to Goose and Duck creeks, tributaries to the Pee Dee River. To better understand the evolutionary history of *Lasmigona,* I re-constructed the phylogeny of this genus to analyze genetic variation within and among all currently-recognized *Lasmigona* taxa using *COI, NDI* and 28s markers. Haplotype networks revealed that Carolina heelsplitters are most closely related to the Green floater, a species that is morphologically similar and shares low levels of range-wide genetic diversity. Both single and multi-gene phylogenies revealed *Lasmigona* as highly polyphyletic. Moreover the clade containing both *L. decorata* and *L. subviridis* did not contain the type species for *Lasmigona*, *L. costata*. Although genetic coverage is limited to one nuclear and two mitochondrial markers, my data provide evidence for historical connections among Carolina heelsplitter populations in the Pee Dee, Santee, and Savannah basins. Future surveys for Carolina heelsplitters should increase the geographical coverage of study sites and use genetic screening to verify field identifications of ambiguous individuals. More variable genetic marker systems such as microsatellites or SNPs should be utilized to help assess whether finer-scale genetic differentiation is occurring and to help guide propagation and augmentation efforts. Finally, taxonomic revision is needed to describe evolutionary patterns more accurately within *Lasmigona* and other closely-related groups.

Acknowledgments

This research was funded by the U.S. Fish and Wildlife Service, and I would like to thank service biologists Morgan Wolf and Jason Mays for their assistance. I would also like to thank Dr. Gangloff and Dr. Siefferman for inviting me to their lab and for the countless meals, gatherings, and overall family atmosphere they created. To Mike specifically, thank you for being an awesome mentor through your patience and guidance along this journey. Our adventures have included traveling across many states, staying in haunted houses, and chatting for hours about crazy ideas and life; I'll forever be grateful for your friendship. To Lynn specifically, thank you for your guidance during this journey and for always being there for support and reassurance that everything is going to be okay. I will forever do the superwoman pose in times of need! I would also like to thank Dr. Estep for challenging me to think about molecular techniques and for your encouraging words early on. I also owe many thanks to Rachael Hoch, Michael Perkins, and T.R. Russ for their help with data collection and for always showing up to my numerous talks, it truly means a lot.

To all the members of the ACRL lab, thank you for the countless laughs and most importantly for being a big family. Jon Wells, thank you for being my secret weapon, sounding board, and for being someone I admire and hope to be as cool as some day. Ashley Yaun and Chantelle Rondel, you both impacted my life in such positive ways. I can never thank you enough for making me laugh when I wanted to cry, for helping me see the good when all I saw was a bumpy road, and most importantly for always being there. Vincent

Santini, we have been best mates, desk mates and lab mates, I honestly cannot wait to see what crazy adventure we embark on next in the streams.

To my family, thank you for your endless support in every way possible. I could not have completed this without you guys. Finally, credit must be given to Peggy who became a lab mascot and the beloved Rankin cat.

Dedication

I would like to dedicate this work to my "real mother," Tammy Moore, who taught me to appreciate the small things and live life to the fullest. Though she passed away during my graduate career, her inspiration and guidance continue to shape who I am today.

Table of Contents

List of Tables

List of Figures

Foreword

The research detailed in this thesis will be submitted to the peer-reviewed journal *Conservation Genetics*. The body of this thesis has been formatted according to the style requirements for publication in this journal.

Introduction

Global species loss rates are currently 1,000 to 10,000x greater than of pre-Anthropocene (i.e., natural) extinction rates (Pimm et al. 2014). Freshwater ecosystems cover <1% of the Earth's surface and are one of its most threatened biomes. Humans and >100,000 species rely on these habitats for our continued existence (Dudgeon et. al. 2006). Freshwater mussels (Unioniformes) and operculate freshwater snails (Pleuroceridae) are among the most imperiled mollusk groups globally and the total number of extinct, endangered, threatened, or imperiled species exceeds that of any other freshwater faunal group (Haag and Williams 2014; Regnier et al. 2009). These declines are largely attributable to degradation of aquatic systems via urban and ex-urban development, introduction of exotic species, increased pollutants, and modification of streams by channelization and dams (Fuller 1974; Smith et al. 2003; Strayer and Dudgeon 2010).

Freshwater mussels are indicators of stream water quality and assemblages that are characterized by high abundance or richness are largely restricted to systems with high water quality and stable habitat conditions. Toxicology data reveals that juvenile freshwater mussels are acutely sensitive to sediment, nutrient, and inorganic chemicals (Goudreau et al. 1993). Freshwater mussels also play a critical role in nutrient cycling through filter feeding, bioturbation, and biodeposition, linking both benthic and pelagic food webs (Hoellein et al. 2017; Spooner et al. 2012; Vaughn and Hakenkamp 2001). Mussel aggregations appear to stabilize streambed substrates and increase habitat heterogeneity (Haag 2012; Haag and Williams 2014; Zimmerman and de Szalay 2007). Freshwater mussels utilize a unique reproductive strategy in which fertilized eggs are released from the mother into the water column where they act as obligate ectoparasites on fish to continue morphological

development (Watters and O'Dee 1998). Due to their dependence on stable substrate, clean water and suitable host fishes for survival, mussels are more susceptible to environmental perturbations than other benthic invertebrates and are intimately linked to the dynamics of freshwater ecosystems (Hoftyzer et al. 2008).

Southeastern Atlantic Slope Geography

North America has the highest diversity of freshwater mussels in the world and ~300 species are currently recognized (Williams et al. 1993). Rivers in the southeastern region of North America support diverse and often highly endemic faunas including >62% of the continent's fishes and ~200 freshwater mussel species (Bogan and Roe 2008; Neves et al. 1997; Warren et al. 2000). The Eastern Continental Divide separates the watersheds flowing into the Atlantic Ocean from those flowing into the Gulf of Mexico. Atlantic Slope drainages extend from the St. Mary's River on the Georgia-Florida border to the St. Lawrence River system in Canada (Johnson 1970; Maurakis and Lipscomb 1999). Previous biogeographic studies have identified distinct northern and southern species assemblages among Atlantic Slope watersheds with the subdivision believed to occur near the James River Basin in Virginia (Kozak et al. 2006; McAlpine and Smith 2010). Numerous fish and mollusk species in the southern Atlantic Slope drainages are believed to have entered these drainages via headwater capture of Gulf of Mexico Drainage streams during the pre-Pleistocene era (Johnson 1970). Overall, 35 mussel species in the Southern Atlantic Slope region are believed to have origins in the Interior Basin. Nine of these mussels are endemic to the region and 7 are endemic to individual river basins (Johnson 1970).

High rates of mussel and fish endemism in Atlantic Slope rivers suggest that many of these systems were less glaciated and subject to frequent isolation during portions of the Pleistocene, thereby facilitating radiation of endemic species via allopatric speciation (Haag 2009; Hocutt et al. 1986; Sepkoski and Rex 1974; Starnes and Etnier 1986). More recently, the eastern and Piedmont regions of the Carolinas were historically impacted by in-stream gold mining, forest clearing and dam construction beginning in the late 18th Century and these landuse legacies likely shaped the current level of fragmentation (Agpaoa 2012). Numerous freshwater species in the region are restricted to a few small populations and presumably occupy only a fraction of their historical ranges (Alderman 2006; Oswald et al. 2009). For example, the Pee Dee River is one of the largest river drainages in the Carolinas but a series of large impoundments along its mainstem have resulted in widespread habitat fragmentation that has imperiled many species including the Robust redhorse (*Moxostoma robustum*), a large potadramous sucker that was presumed extinct for nearly 100 y (Benke and Cushing 2011; Grabowski and Isely 2007; Jenkins and Burkhead 1994). Mussel species declines in this region are frequently attributed to recent human population growth as well as an increased frequency of drought events and these stressors are exacerbated by historical habitat fragmentation leading to the existence of small or geographically-isolated populations (Archambault et al. 2018). Increasing stream connectivity with the goal of increasing genetic connectivity is a focal goal of recovery plans for many at-risk species in Southern Atlantic Slope drainages.

Genetic Variation in Atlantic Slope Taxa

Mussel life history traits are unusual among freshwater biota as they exhibit both gamete- (i.e., sperm dispersal) and zygote- (i.e., fish-aided movement of glochidia larvae) mediated gene flow dispersal (Watters 1992; Ferguson et al. 2013). Some mussels release sperm aggregates called spermatozeugmata, where thousands of sperm are embedded in a thin spherical membrane, allowing them to travel up to 16 km downstream thus increasing the potential to facilitate gamete dispersal among adjoining populations (Barnhart and Robert 1997; Waller and Lasee 1997). Glochidia dispersal is dependent on both the number and behavior of host fishes, leading to high variability in colonization rates and the subsequent impact on gene flow both within and among mussel species. Host fish mobility also affects glochidia dispersal, with less mobile fish contributing less to zygote-mediated gene flow (Chong 2016; Schwalb et al. 2011). The key difference between gamete and zygote-mediated gene flow is that glochidia are not limited to only downstream dispersal. This unique evolutionary adaptation allows mussels to colonize habitats both up- and downstream, as well as into adjoining tributaries and drainages. This has led to both high rates of vicariance as well as to the evolution of species with very broad geographic ranges (Haag 2012).

Isolated mussel populations have likely become increasingly geographically and thus genetically isolated, predisposing them to the effects of genetic drift, inbreeding depression and localized extinction (Lesica and Allendorf 1995). However, some isolated rivers today were components of larger, ancient river systems (i.e., Pee Dee and Catawba-Santee basins) were historically connected leading to gene flow across drainages during low sea stands and complex patterns of genetic divergence (Corcoran 1981; Hocutt et al. 1986). Oswald et al.

(2009) found that two rare *Etheostoma* species endemic to the Pee Dee and Catawba drainages shared alleles and exhibited low divergence rates suggestive of historical gene flow between these taxa/drainages. Similarly, investigation of two taxa in the mussel genus *Parvaspina* revealed low intra- (0.0003-0.0016%) and inter-species divergence rates (0.013%) and a single mtDNA haplotype present in the majority of the sampled populations among drainages and taxa (Perkins et al. 2017). King et al. (1999) observed similarly low levels of divergence (i.e., only two variable nucleotide substitution sites) and three mtDNA haplotypes among 9 *Lasmigona subviridis* populations in three states (Virginia, West Virginia, and North Carolina). High levels of haplotype sharing were hypothesized to be attributable to the fact that most Atlantic Slope drainages are geologically and were more recently linked with other adjoining drainages on the continental shelf during low sea-stand intervals.

Study Species

The Carolina heelsplitter (*Lasmigona decorata* Lea*,* 1852) was first described as *Unio decoratus* from specimens collected in the Saluda-Wateree River System in what was formerly known as the Abbeville District in west-central South Carolina. *Unio insolidus* Lea, 1872 and *Unio charlottensis* Lea, 1863 were described from the Yadkin-Pee Dee Basin along the North Carolina border in Mecklenburg County and are considered synonyms of *Lasmigona decorata*. Both *Unio insolidus* and *Unio charlottensis* have a slightly less distinct transition from the umbo and a wider double ridge along the posterior-dorsal shell margin than observed in *Unio decoratus*. These taxa were synonymized under *L. decorata* by Clarke (1980) in his revision of the genus *Lasmigona*.

The Carolina heelsplitter is endemic to the southeastern Atlantic Slope and is currently one of the most imperiled mussels in North America. Over the past two decades both Carolina heelsplitter abundance and the extent of occupied habitat appear to have declined rangewide (USFWS 2012). Historically, Carolina heelsplitters occurred in Piedmont streams and small rivers in the Carolina and Charlotte Slate belts. Populations are known from the Saluda, Santee, Savannah, and Yadkin-Pee-Dee basins in North and South Carolina (Bogan and Alderman 2008). Athearn (1992) reported a specimen from the Oconee River in Georgia, however this specimen cannot be located and is believed to be a misidentification (Athearn 1992; Bogan and Raley 2012). Currently, 11 populations are believed to be extant including 5 in the Santee, 2 in the Pee Dee, 2 in the Savannah and 2 in the Saluda basins (Three Oaks Engineering 2017, Table 1). These populations are believed to be small and are isolated from one other by impoundments or other extensive reaches of unsuitable habitat. Surveys conducted during 2004-2011 found only 152 individuals from among the 11 populations (USFWS 2012). The Lynches River and Flat Creek in South Carolina are believed to support the largest populations whereas populations in streams around Charlotte including Goose, Duck and Waxhaw creeks are extremely small and may not be reproducing.

Carolina heelsplitters occur primarily in small to mid-sized streams with stable, vegetated banks and substrates ranging from muddy sand to muddy gravel (Bogan and Raley 2012; Clarke 1985; Keferel 1991). Declines of Carolina heelsplitter populations in the Catawba-Santee Basin are likely due to habitat alterations associated with rapidly-increasing human populations across much of the North and South Carolina Piedmont. North Carolina was once an important producer of gold and numerous abandoned mining sites present in the Carolina Slate Belt region continue to impact water quality through acid and metal

contamination (Roghair et al. 2017). Human population growth around Charlotte, NC has subsequently altered stream physical habitat, water quality, and quantity across the core of this species' range (USFWS 2012). Carolina heelsplitters in Waxhaw, Goose, and Duck creeks were also impacted by historical agriculture, logging, and development. Additionally, portions of 8 streams with extant Carolina heelsplitter populations have recently been added to the EPA's 303d list of impaired waters because they exceed sediment and nutrient thresholds (Three Oaks Engineering 2017). The combination of low population densities and declining habitat quality makes Carolina heelsplitters highly vulnerable to extirpation from stochastic and chronic events.

Prior published research on Carolina heelsplitters is limited and has primarily focused on aspects of reproductive ecology, habitat ecology and distribution (Bogan 2002; Bogan et al. 2008; Bogan and Raley 2012; Eads et al. 2010; Ward et al. 2007). The overall goal of this research was to determine the number of management units present among extant Carolina heelsplitter populations. To do this I used two approaches. First, I examined variation in the mitochondrial *COI* gene to assess genetic diversity within and among Carolina heelsplitter populations in the Santee, Pee Dee and Savannah basins and examine historical gene flow patterns among populations. Second, I analyzed variation in the mitochondrial *COI, NDI* and nuclear 28s markers to assess diversity within and among all currently-recognized *Lasmigona* taxa. This allowed me to investigate current divergence levels within the genus and to re-construct the phylogeny of *Lasmigona sensu strictu* to better understand its evolutionary history. These data will help inform propagation and habitat management strategies for this endangered freshwater mussel.

Methods

Mussel collections

During 2017-2019 I collected 75 Carolina heelsplitter DNA samples from wild animals and individuals held at the Marion Conservation Aquaculture Center (MCAC) and Orangeburg National Fish Hatchery (ONFH) facilities (Table 2). Additionally, 18 Carolina heelsplitter sequences were used from previous unpublished work in the Gangloff lab (N = 93 animals, Table 2). Non-lethal DNA samples were collected using buccal swabs (Isohelix SK-1S swabs, Boca Scientific Inc., Boca Raton, FL) by gently swabbing each side of the foot 5-10x, using the alternate side of the swab for each side of the foot; mussels were returned to the substrate immediately afterwards. Surveys were conducted using visual tactile searches aided by mask and snorkel or bathyscopes (Aquascope Jointed, Nuova Rade, Genova, Italy). Larger, more stable populations (e.g., Lynches River, Stevens Creek Sub-basin) were sampled at multiple locations to ensure proper genetic representation (Table 3).

Three putative species were described by Lea in the 1850s (and subsequently synonymized by Clarke in 1980) from across the current range of Carolina heelsplitters suggesting that phenotypic plasticity is evident among Carolina heelsplitter populations. Variable shell morphology that may be similar to other taxa can make it difficult to conclusively identify Carolina heelsplitters in the field. I used buccal swabs to obtain DNA samples and verify field identifications of problematic specimens. In addition to specimens that were clearly diagnosable as Carolina heelsplitters, individuals displaying evidence of a slightly upturned dorsal shell margin, a double ridge along the posterior-dorsal shell margin, a relatively thin shell compared to similar-appearing mussels and the presence of a salmon-to-orange colored foot were also swabbed to verify their identities. Carolina heelsplitters in

the Savannah Basin can be very similar in appearance to *Elliptio complanata* and *Uniomerus carolinianus* and all three taxa co-occur across the range of Carolina heelsplitters (Bogan 2002; Bogan et al. 2008).

Genetic analyses

A DNA bar-coding approach was used to verify field identifications of all Carolina heelsplitters sampled from wild populations. Sequences from all putative Carolina heelsplitters were compared with sequences obtained from known Carolina heelsplitters housed in propagation facilities and the single *COI* sequence available on Genbank. Sample tubes containing only buffer and water were used as negative controls to ensure that no sample contamination had occurred. Buccal swabs were preserved in 95% non-denatured ethanol in the field and stored on ice during transportation to Appalachian State University for analyses. Samples collected prior to 2018 were extracted using the MoBio Ultra Clean Tissue and Cells DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following manufacturer protocols, including the optional Proteinase K step. DNA was extracted from buccal swabs collected in 2018 using the Buccal-Prep Plus DNA Isolation Kit (Isohelix, Harrietsham, UK) with modifications to the buffer and digestion components in manufacturers protocol. DNA concentration and purity were evaluated using a NanoDrop 2000 nano-spectrophotometer (Thermo Scientific, Walkham, MA). DNA concentration values were standardized to either 5 ng/µL or 20 ng/µL (depending on original concentration readings) using nuclease-free water to optimize DNA amplification.

To determine the identity of putative Carolina heelsplitters and to assess range-wide (i.e., among and within populations) genetic diversity, I amplified a fragment of the

mitochondrial cytochrome oxidase subunit I (*COI*) gene. Polymerase chain reaction (PCR) amplifications were carried out under the following conditions: 12.5 µL of GoTaq® Green Master Mix 2X (Promega Corporation, Madison, WI), 40 ng of template DNA, 1.25 µL of both forward and reverse primers (0.5µ M), and nuclease-free water to a final volume of 25 µL per sample.

Reactions were completed using both the primers developed by Campbell et al. (2012) as well as a more targeted primer set developed by Bogan and Raley (2012) that was modified from the Folmer 1994 universal *COI* primer, yielding higher amplification success rates when using buccal swabs during DNA collection (forward 5'-TAAACTTCAGGGTGACCAAAAAATCA- 3'; reverse 5'-GGTCAACAAATCATAAAGATATTGG- 3').

Reactions were conducted on an Eppendorf Mastercycler Nexus thermal cycler (Hauppauge, NY, USA) with conditions as follows; 95°C for 2:00, then 40 cycles of 95°C for 0:45, 52°C for 0:45, 72°C for 1:00, before final extension at 72°C for 7:00. PCR products were visually examined via gel electrophoresis on a 1% agarose gel stained with ethidium bromide in 1X TAE buffer, successful reactions were sent to Retrogen Inc. (San Diego, CA) for sequencing.

To further verify the results of bar-code analyses at the *COI* locus, I also sequenced a fragment of the mitochondrial NADH subunit I (*NDI*) gene and the nuclear *28s* ribosomal RNA gene to obtain coverage at three loci. This was done to more confidently identify individuals swabbed during field surveys that displayed problematic morphological characteristics. Primers were adapted from Serb et al. 2003 and Therriault et al. 2004 for the *NDI* and *28s* genes, respectively.

COI sequence reads were compiled and edited using Geneious R7 (Biomatters Ltd., Auckland, New Zealand). Sequences with HQ scores below 50% were discarded, as were sequence reads shorter than 512 nucleotides. All reads were examined for the presence of stop codons, mitochondrially-derived nuclear fragments and male mitotypes (Buhay 2009). A final alignment of 512 base pairs was constructed using the ClustalW algorithm and manually trimmed in MEGA 7 (Kumar et al. 2016). Genetic distances were generated using maximum composite likelihood in MEGA 7. Haplotype data and genetic differentiation values were determined using DNAsp (Librado and Rozas 2009). ARLEQUIN software was utilized for AMOVA values (Excoffier and Lischer 2010). Haplotype networks were constructed in PopArt using a median-spanning algorithm with the epsilon value set to zero (Clement et al. 2002).

To investigate deeper phylogenetic relationships within *Lasmigona* I compiled a three gene dataset (*COI, NDI* and 28s) representing all recognized taxa: *L. costata, L. compressa, L. subviridis, L. alabamensis, L. holstonia, L. etowaensis, L. complanata*. Sequences from taxa within *Alasmidonta*, *Amblema, Anodonta, Anodontoides, Margaritifera, Pyganodon, Quadrula, Simpsonaias, Strophitus* and *Utterbackia* were utilized as outgroups. Outgroup specimens were obtained from museum collections at Appalachian State University and Auburn University. Sequences were also obtained from Genbank when museum animals were not available (Table 4). Sequence reads were compiled, edited, and aligned using Geneious R7. Intra- and inter-specific genetic distances were generated using maximum composite likelihood in MEGA 7. The best-fit model of nucleotide substitution was inferred for each dataset using IQTree (Trifinopoulos et al. 2016). Bayesian inference by Markov Chain Monte Carlo (MCMC) analyses were conducted on each single-gene dataset, the

mitochondrial dataset and a concatenated alignment consisting of all 3 genes using the MrBayes 3.2.2 plug-in (Huelsenback and Ronquist 2001) within Geneious. Phylogenies were visualized and edited using FigTree v1.4.2 (Rambaut 2014).

Results

Phylogeography

Initially, I tested the ability of three primer sets to generate sequenceable PCR product (Bogan and Raley 2012; Campbell and Lydeard 2012; Folmer et al. 1994). Although all three primers yielded sequences, the Bogan and Raley (2012) primer set consistently yielded higher quality reads from *Lasmigona* and closely-related taxa. Modification of manufacturer protocols for DNA extraction from buccal swabs produced higher nucleic acid concentrations, increasing material for downstream analysis.

Analysis of the *COI* gene revealed low levels of genetic divergence and a high rate of haplotype sharing among Carolina heelsplitter populations in the Pee Dee, Santee, and Savannah basins. I observed a mean pairwise substitution rate of 0.0014 (0.14%) among all sampled Carolina heelsplitter populations. Haplotype diversity (Hd) for all 93 sequences was 0.5653 $\pm$ SD 0.036 and the average number of pairwise nucleotide differences (k) and nucleotide diversity (π) were found to be 0.640 and 0.00135, respectively. Two non-synonymous polymorphisms were identified in the data set. Individuals from Goose and Duck creeks (Pee Dee Basin) had a C instead of a T at nucleotide 424, a substitution unique to this population. Additionally, individuals in haplotype 3 possessed a C at nucleotide 278, whereas all others had a T.

Within-drainage divergence rates were highest in the Catawba River Drainage and the Pee Dee Basin (Table 5). In the Catawba Drainage, a maximum genetic distance of 0.0021 (0.21%) was observed when comparing individuals from Sixmile Creek to specimens collected in the Rocky and Fishing creek sub-basins. The maximum genetic distance observed among Savannah Basin individuals (0.0020) was found when comparing specimens from Mountain Creek and Turkey Creek to individuals sampled from tributaries to Stevens Creek. Within the Pee Dee Basin, the highest divergence rates were observed among Carolina heelsplitters from the Lynches River and Goose and Duck creeks (0.0039). Interestingly, the greatest pairwise genetic distances were observed between individuals from the adjoining Pee Dee and Santee (Catawba) basins (Table 5).

Within-population genetic distances were highest in the Lynches River (0.08%) and Stevens Creek sub-basins (0.04%, Table 6). Among-population genetic distances were highest when comparing individuals from the Goose and Duck creek populations to individuals from Rocky Creek (0.43%) as well as when comparing the Lynches River to the Goose and Duck creek populations (0.26%, Table 6).

Examination of range-wide genetic diversity revealed that three distinct haplotypes were broadly distributed among basins supporting Carolina heelsplitter populations (Figure 1). All three haplotypes were found in Pee Dee populations whereas Santee and Savannah populations only had two haplotypes present (Figure 1). Additionally, haplotype 2 was geographically restricted to populations in Goose and Duck creeks, tributaries of the Pee Dee River (Figure 2). I did not observe any other examples of haplotype isolation. Haplotype 1 occurred in 5 populations, and haplotype 3 occurred in 3 populations (Figure 2). Both

haplotypes 1 and 3 were present in several streams including the Lynches River and Flat Creek in the Pee Dee Basin as well as Mountain and Turkey creeks in the Savannah Basin.

Genetic divergence rates were generally low both within and among *COI* haplotypes detected in each population and drainage (Table 7). Individuals in Fishing Creek, Sixmile Creek (haplotype 1) and Rocky Creek (haplotype 3) were all genetically identical (i.e., only one haplotype was detected). Santee Basin specimens in haplotype 1 are 0.21% (0.0021) different from haplotype 3 detected in Rocky Creek, the dominant Santee Basin haplotype. Haplotype 2 was only found in individuals sampled from the Goose and Duck Creek population and is 0.43% (0.0043) different from Pee Dee haplotypes observed in the Lynches River population. Specimens sampled from Mountain and Turkey creeks were the most genetically distinct among Savannah Basin individuals, and I only detected haplotype 3 in these samples. Haplotype 3 is 0.21% (0.0021) different from Haplotype 1, the dominant Savannah Basin haplotype.

Population Genetics

Analysis of molecular variance (AMOVAs) conducted within basins revealed that 88% of genetic variation is encompassed among populations whereas 12% is harbored within populations. Furthermore, the polymorphism at locus 424 drives 56% of variation among basins, whereas 81% of within-basin variation is driven by the polymorphism at site 238.

Gene flow indices showed relatively low amounts of nucleotide and haplotype diversity both on a basin and population scale (Table 8). Within-basins, nucleotide and gene diversities were low; most populations were invariant at the *COI* gene except for the Lynches River and Stevens Creek populations which both supported two haplotypes. Positive

Tajima's D and Fu's Fs values indicate that these populations are decreasing in size and approaching balancing selection (Table 8). Interestingly, when the Savannah Basin sites were considered to be a single population, these statistics appear to suggest that the population may be expanding. However, neutrality tests were non-significant and deviation from neutral selection could not be inferred. Analysis of genetic differentiation revealed moderate amounts of gene flow and population structuring among basins (Table 9). Moderate to high amounts of genetic variation are due to structuring among populations, as individuals in the Goose, Duck and Rocky creek populations were significantly different from all other populations, potentially showing an adaptive mutation leading to genetically distinct populations. (Table 10).

Phylogeny of Lasmigona

Phylogenetic analyses revealed that *Lasmigona*, as currently recognized, is polyphyletic. All analyses consistently revealed that *L. decorata* is most closely-related to *L. subviridis* and *L. compressa* (Figures 3, 4, 5, and 6). I found similarly low levels of divergence among *L. subviridis*, another Atlantic Slope taxon, but much higher levels of intraspecific divergence among Interior Basin taxa (e.g., *L. costata* and *L. complanata*). However sample sizes for most putative *Lasmigona* taxa were much smaller than my *L. decorata* dataset. Within-species divergence rates were higher in *L. costata* in all analyses (Table 11). Among species variation was highest when comparing *L. costata* specimens to *L. holstonia* and *L. etowaensis* (Table 12). Haplotype maps showed both high divergence and geographic structuring across *Lasmigona* (Figures 7 and 8).

Discussion

My research found that Carolina heelsplitters exhibit a low degree of genetic diversity suggestive of a high degree of historical connectivity both within and among populations. This is surprising because extant populations are presently geographically disjunct and scattered across small and isolated patches of habitat in three river drainages. Small geographic range, habitat specialization, limited mobility and resultant genetic bottlenecking are among the intrinsic characteristics that predispose species to extinction, and these life history traits characterize many unionid taxa (Harcourt et al. 2002; Purvis et al. 2000). To fulfill objectives outlined in the species recovery plan, critical habitat for the Carolina heelsplitter has been designated for 148.4 km within 10 North and South Carolina streams (USFWS 2002). Additionally, the use of captive breeding programs to propagate individuals for augmentation of wild populations and for the re-introduction of Carolina heelsplitter to habitats within its historical distribution are currently underway in hatchery facilities.

Phylogeography

A thorough understanding of phylogeographic patterns among *L. decorata* populations is critical to meeting range-wide species recovery goals. These data reveal that the *COI* gene is highly conserved within *Lasmigona decorata* and this is consistent with results obtained by previous studies of congeners (e.g., *L. subviridis*- King et al. 1999, *L. costata*- Galbraith et al. 2011) as well as from other studies of highly-endemic, Atlantic Slope mussels (e.g., *Parvaspina*, Perkins et al. 2017).

Despite a significant degree of contemporary isolation, it appears that historical processes may explain phylogeographic patterns observed among *L. decorata* populations

(Bernatchez and Wilson 1998). Limited levels of genetic divergence observed within and among widespread and highly-isolated populations suggest Carolina heelsplitters historically occurred throughout the Santee (Catawba), Pee-Dee and Savannah basins and that the geographic isolation of populations is likely a relatively recent phenomenon. Much of the Southeastern U.S. was subject to intensive surface and placer mining decades before the first mussel surveys were conducted in the mid-19th century (Foley and Ayuso 2012). It is likely that widespread mining, forest clearing and other forms of habitat degradation that persisted throughout much of the 19th and 20th centuries may have done as much or more than current anthropogenic stressors to isolate populations of Carolina heelsplitters. The mitochondrial *COI* gene typically mutates at a rate of 0.67-1.21% per million years and the high degree of genetic similarity observed suggests that these populations were connected as recently as the Pleistocene (Inoue et al. 2014; Marko 2002). Alternatively, some *L. compressa* and *L. subviridis* have been found to be hermaphrodites (Breton et al. 2010; King et al. 1999; Van der Schalie 1966). Thus, it is possible that some degree of facultative or obligate hermaphroditic reproduction is possible in Carolina heelsplitter populations and that this may explain the low levels of observed diversity as well as how some populations are able to persist at vanishingly low numbers (Cyr et al. 2007; Hinzmann et al. 2013).

In order to get a more complete picture of the genetic variability within *L. decorata,* I did not limit surveys for wild individuals to known localities within stream systems and instead identified sites based on the presence of suitable habitats. These surveys extended *L. decorata's* known range within the Stevens Creek sub-basin by ~4.5 km and yielded new locations for *L. decorata* in Mountain, Sleepy and Little Stevens creeks. Detection of gravid individuals coupled with relatively high detection rates (e.g., 4-7 *L. decorata* per site) suggest

that populations in these streams may be larger than previously thought, or that Carolina heelsplitter detectability may vary substantially across seasons. Although this has not been the subject of a focused study, detectability has been found to vary considerably among seasons for other freshwater mussel taxa (Lurman et al. 2014; Rondel 2019; Wacker et al. 2019). Nevertheless, it seems that the Stevens Creek sub-basin is a stronghold for Carolina heelsplitters, and it is likely that other streams in the sub-basin may support populations. Surveys targeting stable stream channels in forested sub-catchments may expand the known range of Carolina heelsplitters in the Savannah Basin.

I found that 2 of 3 *COI* haplotypes identified were broadly distributed and occurred in Carolina heelsplitter populations across the Pee Dee, Santee, and Savannah basins. The population in Goose and Duck creeks contains a unique haplotype suggesting that stocking with propagated individuals may have led to some genetic swamping. However, this is not the only well-sampled (n >20 samples) population where only a single haplotype was present. Other populations including Rocky (n=8 samples) and Sixmile (n=13 samples) creeks also supported a single haplotype. Moreover, because overall genetic diversity is so low (haplotypes differed by only 1 or 2 base pair substitutions and single haplotype populations were common), managers may want to consider attempting to increase intra-population heterozygosity by translocation or cross-breeding individuals from populations exhibiting different haplotypes (Lane et al. 2019; Scott et al. 2014).

A non-significant neutrality test revealed that each population is evolving via random genetic drift, thereby selective pressures (i.e., directional, balancing, or demographic selection) are likely not significantly impacting populations. Genetic structuring among basins is moderate when comparing the Pee-Dee to both the Santee and Savannah basins.

Lower levels of genetic differentiation suggest recent gene flow between the Santee and Savannah basins. Further, when comparing Goose and Duck creeks to all other localities and when comparing individuals from the Rocky Creek sub-basin to other populations within the Catawba Drainage, a significant differentiation in population structure was detected, potentially providing evidence for local adaptations.

Phylogeny

Results show that *Lasmigona*, as currently recognized, is not a monophyletic genus and that deep-rooted radiation has occurred multiple times in the tribe Anodontini (Table 13). *Lasmigona decorata* was found to be sister to *L. subviridis* and *L. compressa* in both single- and multi-gene analyses (Figures 3, 4, 5, and 6). These data are consistent with Ortmann's (1913) hypothesis that *L. subviridis* originated when Interior Basin headwater streams supporting *L. compressa* were captured by Atlantic Slope headwaters. Multi-gene analyses revealed that *L. decorata*, *L. subviridis* and *L. costata* are more closely related to *Alasmidonta* and *Pyganodon* than they are to *L. holstonia* and *L. etowaensis*.

Intraspecific genetic variation was also low within both *L. subviridis* and *L. compressa* (putative sister taxa to *L. decorata*) and is similar to values observed among other Southern Atlantic Slope species. Interestingly, *L. costata* had the highest intra-specific divergence among all taxa examined. High levels of genetic divergence in this taxon may be attributable to the fact that large, stable *L. costata* populations are found across the Interior Basin as well as in rivers draining to both the Atlantic and Arctic oceans (Watters et al. 2009). It would be interesting to examine whether *L. costata* populations in Atlantic or Arctic slope drainages are less diverse than Interior Basin conspecifics.

Management considerations and recommendations

Goose and Duck creeks support what are believed to be the smallest remaining Carolina heelsplitter populations, and these streams should be candidates for translocation or stocking of propagated mussels. However, these streams are also located in the Charlotte, North Carolina metro-area and are increasingly impacted by development, nutrient-loading and increased concentrations of associated pollutants (Goose Creek Watershed Management Plan, 2009). All individuals detected in these streams during the last decade have been moved to ark (captive rearing and propagation) facilities and it seems unlikely that adding genetic diversity from other localities to these populations would increase the likelihood that this species will persist in the Goose and Duck creek sub-basins.

Results of this study suggest that current survey methods for detecting Carolina heelsplitters could be improved in several ways. First, the geographical extent of surveys should be expanded to include streams with hydrological connections to streams supporting Carolina heelsplitter populations. Second, field identifications should be verified using DNA bar-coding whenever possible. Reproductive strategies utilized by unionids allow for the movement of individuals across broad geographic scales; this dispersal mechanism also promotes the spread of individuals through relatively impacted streams and the colonization of other suitable reaches (Archambault et al. 2018; Bauer 1987; Dudding et al. 2019). More geographically-widespread surveys coupled with DNA bar-coding studies conducted across the Carolina Slate Belt Physiographic Provence may detect additional populations that could serve as sources of brood stock or refugia for this highly range-restricted mussel.

Freshwater mussel taxonomists commonly rely on morphological characteristics to identify species in the field and this reliance on phenotypically plastic traits has contributed to conflicting views of species relationships in many groups. Shell growth and shape are strongly influenced by environmental factors (Mitton and Koehn 1985). Differences in chemical, physical, and biological factors among environments drive phenotypic variation within freshwater mollusk species (Dillon 2000). Convergent shell morphology exhibited by Carolina heelsplitters and sympatric unionid taxa (including the widespread and numerically dominant *Elliptio complanata*) may complicate identifications and lead to very different conclusions about the condition of a stream's mussel populations. Inexperienced unionid taxonomists and individuals with little experience working with Carolina heelsplitters may wish to err on the side of caution and use genetic screening to verify the identity of morphologically problematic individuals. Primers developed by Bogan and Raley (2012) successfully produced sequences only for *Lasmigona* and closely-related taxa (i.e., the tribe Anodontini) in this study, demonstrating a potential method to rapidly differentiate Carolina heelsplitters from similar appearing taxa including *E. complanata*.

The Carolina heelsplitter has been listed by the US Fish and Wildlife Service as an endangered species for over two decades. Recovery strategies, including controlled propagation and release have likely increased the likelihood that this species will persist through the Anthropocene. However, the population in Goose and Duck creeks appears to be on the brink of disappearing and efforts to increase genetic diversity are unlikely to recover this population. Conversely, populations in the Lynches River in the Pee Dee Basin along with the Stevens and Rocky Creek Sub-basins in the Savannah Basin appear to be stable and possibly adapting to local pressures, therefore maintaining the genetic structure of these

populations may prove to be advantageous. Future studies should examine microsatellites or other less-conserved markers to examine whether the genetic patterns observed for the *COI* gene are a product of historical connections or recent isolation. Additionally, very little is known about the habitat ecology and life history of Carolina heelsplitters. Adjustment of survey methods to take advantage of seasonal changes in surface activity associated with reproduction may improve detection rates and lead to the discovery of additional conservation or management units.

References

Alderman JM (2006) Mussel surveys in the vicinity of the lee nuclear station, Cherokee County, SC. Prepared for Duke Power Company, Charlotte, NC

Agpaoa E (2012) Uwharrie national forest land and resource management plan. U.S. Department of Agriculture Forest Service Publication, Atlanta, GA

Archambault JM, Gregory Cope W, Kwak TJ (2018) Chasing a changing climate: Reproductive and dispersal traits predict how sessile species respond to global warming. Diversity and Distributions 24:880–891

Athearn HD (1992) New records for some species of Alasmidontini. Malacology Data Net 3:90-91

Barnhart MC, Roberts AD (1997) Reproduction and fish hosts of unionids from the Ozark Uplifts. Conservation and management of freshwater mussels II pp 16-20

Bauer G (1987) Reproductive strategy of the freshwater pearl mussel *Margaritifera margaritifera*. The Journal of Animal Ecology 56:691-704

Benke AC, Cushing CE (2011) Rivers of North America. Academic Press, London, UK

Bernatchez L, Wilson CC (1998) Comparative phylogeography of Nearctic and Palearctic fishes. Molecular Ecology 7:431–452

Bogan AE (2002) Workbook and key to the freshwater bivalves of North Carolina. North Carolina Freshwater Mussel Conservation Partnership, Raleigh, NC 101 pp, 10 color plates

Bogan AE, Alderman JM (2008) Workbook and key to the freshwater bivalves of South Carolina. North Carolina State Museum of Natural Sciences, North Carolina Freshwater Bivalve Conservation Partnership

Bogan AE, Roe KJ (2008) Freshwater bivalve (Unioniformes) diversity, systematics, and evolution: status and future directions. Journal of the North American Benthological Society 27:349–369

Bogan AE, Raley ME (2012) Comparison of carolina heelsplitter (*Lasmigona decorata* (Lea, 1852)) populations (Mollusca: Bivalvia: Unionidae). Unpublished report to U.S. Fish and Wildlife Service, Asheville, NC pp 1-32

Breton S, Stewart DT, Shepardson S, Trdan RJ, Bogan AE, Chapman EG, Hoeh WR (2010) Novel protein genes in animal mtDNA: a new sex determination system in freshwater mussels (Bivalvia: Unionoida)? Molecular Biology and Evolution 28:1645-1659

Buhay JE (2009) "COI-like" sequences are becoming problematic in molecular systematic and DNA barcoding studies. Journal of Crustacean Biology 29:96-110

Campbell DC, Lydeard C (2012) The Genera of *Pleurobemini* (Bivalvia: Unionidae: *Ambleminae*). American Malacological Bulletin 30:19–38

Chong JP (2016) Genetic variation in freshwater mussels (Bivalvia: Unionidae) and its implications for species delineation and conservation. Dissertation, Iowa State University

Clarke AH (1980) The tribe Alasmidontini (Unionidae: Anodontinae), Part I*: Pegias*, *Alasmidonta* and *Arcidens*. Smithsonian Contributions to Zoology pp 1-101

Clarke AH (1985) The tribe Alasmidontini (Unionidae: Anodontinae), Part II: *Lasmigona* and *Simpsonaias*. Smithsonian Contributions to Zoology pp 1-75

Clement M, Posada DC, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657-9

Corcoran MF (1981) Geographic variation and evolutionary relationships of two species of bullhead catfishes (Siluriformes: Ictaluridae) in the Southeastern United States. Dissertation, Duke University

Cyr F, Paquet A, Martel AL, Angers B (2007) Cryptic lineages and hybridization in freshwater mussels of the genus *Pyganodon* (Unionidae) in northeastern North America. Canadian Journal of Zoology 12:1216-27

Dillon RT (2000) The ecology of freshwater molluscs. Cambridge University Press, UK

Dudding J, Hart M, Khan J, Robertson CR, Lopez R, Randklev CR (2019). Host fish associations for two highly imperiled mussel species from the southwestern United States: *Cyclonaias necki* (Guadalupe Orb) and *Fusconaia mitchelli* (False Spike). Freshwater Mollusk Biology and Conservation 22:12-19

Dudgeon D, Arthington AH, Gessner MO, Kawabata ZI, Knowler DJ, Lévêque C, Naiman RJ, Prieur-Richard AH, Soto D, Stiassny ML, Sullivan CA (2006) Freshwater biodiversity: importance, threats, status, and conservation challenges. Biological reviews 81:163-82

Eads CB, Bringolf RB, Greiner RD, Bogan AE, Levine JF (2010) Fish hosts of the Carolina Heelsplitter (*Lasmigona decorata*), a federally-endangered freshwater mussel (Bivalvia: Unionidae). American Malacological Bulletin 28:151–158

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular ecology resources 10:564–567

Ferguson CD, Blum MJ, Raymer ML, Eackles MS, Krane DE (2013) Population structure, multiple paternity, and long-distance transport of spermatozoa in the freshwater mussel *Lampsilis cardium* (Bivalvia: Unionidae). Freshwater Science 32:267-82

Foley NK, Ayuso RA (2012) Gold deposits of the Carolina Slate Belt, southeastern United States- Age and origin of the major gold producers. U.S. Geological Survey Open-File Report 2012-1179 pp 26

Folmer O, Black M, Hoeh W, Lutz R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3:294-299

Fuller SL (1974) Clams and mussels (Mollusca: Bivalvia). Pollution Ecology of Freshwater Invertebrates 215-273

Galbraith HS, Wozney KM, Smith CM, Zanatta DT, Wilson C (2011) Isolation and characterization of microsatellite loci in the freshwater mussel *Lasmigona costata* (Bivalvia: Unionoida). Conservation Genetics Resources 3:9-11

Goose Creek Watershed Management Plan (2009) Goose creek watershed management plan version 1. Charlotte-Mecklenburg Storm Water Services Water Quality Program Staff

Goudreau SE, Neves RJ, Sheehan RJ (1993) Effects of wastewater treatment plant effluents on freshwater mollusks in the upper Clinch River, Virginia, USA. Hydrobiologia 252:211–230

Grabowski TB, Isely JJ (2007) Effects of flow fluctuations on the spawning habitat of a riverine fish. Southeastern Naturalist 6:471-9

Haag WR (2009) A hierarchical classification of freshwater mussel diversity in North America. Journal of Biogeography 37:12–26

Haag WR (2012) North american freshwater mussels: natural history, ecology, and conservation. Cambridge University Press, UK

Haag WR, Williams JD (2014) Biodiversity on the brink: an assessment of conservation strategies for North American freshwater mussels. Hydrobiologia 735:45–60

Harcourt AH, Coppeto SA, Parks SA (2002) Rarity, specialization and extinction in primates. Journal of Biogeography 29:445-56

Hinzmann M, Lopes-lima M, Teixeira A, Varandas S, Sousa R, Lopes A, Machado J (2013) Reproductive cycle and strategy of *Anodonta anatina* (L., 1758): Notes on hermaphroditism. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 319:378-390

Hoellein TJ, Zarnoch CB, Bruesewitz DA, DeMartini J (2017) Contributions of freshwater mussels (Unionidae) to nutrient cycling in an urban river: filtration, recycling, storage, and removal. Biogeochemistry 135:307-24

Hocutt CH, Jenkins RE, Stauffer Jr (1986) Zoogeography of the fishes of the central Appalachians and central Atlantic Coastal Plain. The Zoogeography of North American Freshwater Fishes pp 161-211

Hoftyzer E, Ackerman JD, Morris TJ, Mackie GL (2008) Genetic and environmental implications of reintroducing laboratory-raised unionid mussels to the wild. Canadian Journal of Fisheries and Aquatic Sciences 65:1217–1229

Huelsenback JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 7:754-755

Inoue K, McQueen AL, Harris JL, Berg DJ (2014). Molecular phylogenetics and morphological variation reveal recent speciation in freshwater mussels of the genera *Arcidens* and *Arkansia* (Bivalvia: Unionidae). Biological Journal of the Linnean Society 112:535-545

Jenkins RE, Burkhead NM (1994) Freshwater fishes of Virginia: Bethesda. American Fisheries Society, FL

Johnson RI (1970) The systematics and zoogeography of the Unionidae (Mollusca: Bivalvia) of the southern atlantic slope region. Bulletin of the Museum of Comparative Zoology, MA

Keferel EP (1991) A status survey for the Carolina heelsplitter (*Lasmigona decorata*), a freshwater mussels endemic to the Carolinas. Report prepared for US Fish and Wildlife Service and North Carolina Wild Resources Commission pp 1-29

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-4

King TL, Eackles MS, Gjetvaj B, Hoeh WR (1999) Intraspecific phylogeography of *Lasmigona subviridis* (Bivalvia: Unionidae): conservation implications of range discontinuity. Molecular Ecology 8:65–78

Kozak KH, Blaine RA, Larson A (2006) Gene lineages and eastern North American paleodrainage basins: phylogeography and speciation in salamanders of the *Eurycea bislineata* species complex. Molecular Ecology 5:191–207

Lane TW, Hallerman EM, Jones JW (2019). Population genetic assessment of two critically endangered freshwater mussel species, Tennessee bean *Venustaconcha trabalis* and Cumberland bean *Venustaconcha troostensis*. Conservation Genetics 20:759-779

Lesica P, Allendorf FW (1995) When are peripheral populations valuable for conservation? Conservation Biology 9:753-60

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452

Lurman G, Walter J, Hoppeler HH (2014) Seasonal changes in the behaviour and respiration physiology of the freshwater duck mussel, *Anodonta anatina*. Journal of Experimental Biology 217:235-243

Marko P B (2002) Fossil calibration of molecular clocks and the divergence times of geminate species pairs separated by the Isthmus of Panama. Molecular Biology and Evolution 19:2005-2021

Maurakis EG, Lipscomb DL (1999) Historical relationships of atlantic slope river drainages. Virginia Journal of Science 50:1-12

McAlpine DF, Smith IM (2010) Assessment of species diversity in the atlantic maritime ecozone. NRC Research Press, Canada

Mitton JB, Koehn RK (1985) Shell shape variation in the blue mussel, *Mytilus edulis* L., and its association with enzyme heterozygosity. Journal of Experimental Marine Biology and Ecology 90:73–80

Neves RJ, Bogan AE, Williams JD, Ahlstedt SA, Hartfield PD (1997) Status of aquatic mollusks in the southeastern United States: a downward spiral of diversity. In: Benz GW and Collins DE (ed) Aquatic Fauna in Peril: The Southeastern Perspective. Special Publication No. 1, Southeast Aquatic Research Institute, Lenz Design and Communications, Decatur, GA, pp 43-86

Ortmann AE (1913) The Alleghenian Divide, and its influence upon the freshwater fauna. Proceedings of the American Philosophical Society 210:287-390

Oswald KJ, Rohde FC, Arndt RG, Grady JM, Quattro JM (2009) Evolutionarily independent genes and genomes and insights on genetic structure, evolution and conservation of the collis group of darters. Animal Conservation 12:374-83

Perkins MA, Johnson NA, Gangloff MM (2017) Molecular systematics of the critically-endangered North American spinymussels (Unionidae: *Elliptio* and *Pleurobema*) and description of *Parvaspina* gen. nov. Conservation Genetics 18:745–757

Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, Joppa LN, Raven PH, Roberts CM, Sexton JO (2014) The biodiversity of species and their rates of extinction, distribution, and protection. Science 344:1246752

Purvis A, Gittleman JL, Cowlishaw G, Mace GM (2000) Predicting extinction risk in declining species. Proceedings of the Royal Society of London. Series B: Biological Sciences 267:1947-52

Rambaut A, (2014) FigTree 1.4. 2 software. Institute of Evolutionary Biology, University Edinburgh

Régnier C, Fontaine B, Bouchet P (2009) Not knowing, not recording, not listing: numerous unnoticed mollusk extinctions. Conservation Biology 3:1214–1221

Roghair C, Coates K, Krause C, Dolloff CA (2017) Assessment of fish and macroinvertebrates in a piedmont stream near a historic gold mine, uwharrie national forest, NC. U.S. Department of Agriculture Forest Service Publication, Blacksburg, VA

Rondel CL (2019) Estimating population distribution and abundance using occupancy and detection models. Dissertation, Appalachian State University

Schwalb AN, Poos MS, Ackerman JD (2011) Movement of logperch—the obligate host fish for endangered snuffbox mussels: implications for mussel dispersal. Aquatic Sciences 73:223–231

Scott MW, Begley MT, Krebs RA, Zanatta DT (2014) Mitochondrial DNA variation in the eastern pondmussel, *Ligumia nasuta* (Bivalvia: Unionoida), in the Great Lakes Region. Freshwater Mollusk Biology and Conservation 17:60-68

Sepkoski Jr JJ, Rex MA (1974) Distribution of freshwater mussels: Coastal rivers as biogeographic islands. Systematic Biology 23:165-88

Serb JM, Lydeard C (2003) Complete mtDNA sequence of the North American freshwater mussel, *Lampsilis ornata* (Unionidae): an examination of the evolution and phylogenetic utility of mitochondrial genome organization in Bivalvia (Mollusca). Molecular Biology and Evolution 20:1854-66

Smith DR, Villella RF, Lemarié DP (2003) Application of adaptive cluster sampling to low-density populations of freshwater mussels. Environmental and Ecological Statistics 10:7-15

Spooner DE, Vaughn CC, Galbraith HS (2012) Species traits and environmental conditions govern the relationship between biodiversity effects across trophic levels. Oecologia 168:533–548

Starnes WC, Etnier DA (1986) Drainage evolution and fish biogeography of the Tennessee and Cumberland Rivers drainage realm. The Zoogeography of North American freshwater Fishes pp 325-361

Strayer DL, Dudgeon D (2010) Freshwater biodiversity conservation: recent progress and future challenges. Journal of the North American Benthological Society 29:344–358

Therriault TW, Docker MF, Orlova MI, Heath DD, MacIsaac HJ (2004) Molecular resolution of the family Dreissenidae (Mollusca: Bivalvia) with emphasis on Ponto-Caspian species, including first report of *Mytilopsis leucophaeata* in the Black Sea basin. Molecular Phylogenetics and Evolution 30:479-89

Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) W-IQ-TREE: a fast-online phylogenetic tool for maximum likelihood analysis. Nucleotide acids research 44:232-235

USFWS (2002) Endangered and threatened wildlife and plants: designation of critical habitat for the carolina heelsplitter. Federal Register 67:44502-44522

USFWS (2012) Carolina heelsplitter (*Lasmigona decorata*) 5-year review: summary and evaluation. Federal Register 71:42871- 42872

Van der Schalie H (1966) Hermaphroditism among North American freshwater mussels. Malacologia 5:77-78

Vaughn CC, Hakenkamp CC (2001) The functional role of burrowing bivalves in freshwater ecosystems. Freshwater Biology 46:1431–1446

Wacker S, Fossøy F, Larsen BM, Brandsegg H, Sivertsgård R, Karlsson S (2019) Downstream transport and seasonal variation in freshwater pearl mussel (*Margaritifera margaritifera*) eDNA concentration. Environmental DNA 1:64-73

Waller DL, Lasee BA (1997) External morphology of spermatozoa and spermatozeugmata of the freshwater mussel *Truncilla truncata* (Mollusca: Bivalvia: Unionidae). American Midland Naturalist 138:220

Ward S, Augspurger T, Dwyer FJ, Kane C, Ingersoll CG (2007) Risk assessment of water quality in three North Carolina, USA, streams supporting federally endangered freshwater mussels (Unionidae). Environmental Toxicology and Chemistry 6:2075-85

Warren ML, Burr BM, Walsh SJ, Bart HL, Cashner RC, Etnier DA, Freeman BJ, Kuhajda BR, Mayden RL, Robison HW, Ross ST (2000) Diversity, distribution, and conservation status of the native freshwater fishes of the southern United States. Fisheries 25:7-31

Watters GT (1992) Unionids, Fishes, and the Species-Area Curve. Journal of Biogeography 19:481

Watters GT, O'Dee SH (1998) Metamorphosis of freshwater mussel glochidia (Bivalvia: Unionidae) on amphibians and exotic fishes. The American Midland Naturalist 139:49–57

Watters TG, Hoggarth MA, Standbery DH (2009) The Freshwater Mussels of Ohio. The Ohio State University Press, Columbus, OH

Williams JD, Warren ML, Cummings KS, Harris JL and Neves RJ. (1993) Conservation status of freshwater mussels of the United States and Canada. Fisheries 18: 6-22

Zimmerman GF, de Szalay FA (2007) Influence of unionid mussels (Mollusca: Unionidae) on sediment stability: an artificial stream study. Fundamental and Applied Limnology 168:299–306

Tables and Figures

Table 1. All documented extant populations of *Lasmigona decorata*. Streams represented in this study are underlined. Streams represented in this study were pooled by both basin and population for analyses.

Basin	Population	Streams within population
Pee Dee	Goose Creek/Duck Creek	Goose Creek, Duck Creek
Pee Dee	Lynches River	Lynches River, Flat Creek
Santee	Sixmile Creek	Sixmile Creek
Santee	Waxhaw Creek	Waxhaw Creek
Santee	Cane Creek/Gills Creek	Cane Creek, Gills Creek
Santee	Fishing Creek Sub-basin	Fishing Creek
Santee	Rocky Creek Sub-basin	Hooper Branch, Bull Run Creek, UT Bull Run Creek, Beaverdam Creek, Custer Branch
Saluda	Redbank Creek	Redbank Creek
Saluda	Halfway Swamp Creek	Halfway Swamp Creek
Savannah	Stevens Creek Sub-basin	Little Stevens Creek, Mountain Creek, Sleepy Creek, Beaverdam Creek, Rocky Creek, Turkey Creek
Savannah	Cuffytown Creek	Cuffytown Creek

Table 2. Sampling location and haplotype number for *Lasmigona decorata* sequences analyzed in this study (n=93). Sampling locations were grouped by both basin and population for statistical analysis. * Indicates samples collected from hatchery facilities. Geographic information is provided, GPS coordinates and additional metadata is available upon request.

Basin	State	Sub-basin	Locality	Hap	Sample ID
Santee	SC	Fishing	Fishing Creek	1	TS4
Santee	NC	Sixmile	Sixmile Creek	1	MG8II2017.2*
Santee	NC	Sixmile	Sixmile Creek	1	MG8II2017.3*
Santee	NC	Sixmile	Sixmile Creek	1	MG8II2017.4*
Santee	NC	Sixmile	Sixmile Creek	1	MG8II2017.5*
Santee	NC	Sixmile	Sixmile Creek	1	VF20X17.1*
Santee	NC	Sixmile	Sixmile Creek	1	VF20X17.3*
Santee	NC	Sixmile	Sixmile Creek	1	VF20X17.9*
Santee	NC	Sixmile	Sixmile Creek	1	VF20X17.10*
Santee	NC	Sixmile	Sixmile Creek	1	VF20X17.11*
Santee	NC	Sixmile	Sixmile Creek	1	VF20X17.12*
Santee	NC	Sixmile	Sixmile Creek	1	VF20X17.14*
Santee	NC	Sixmile	Sixmile Creek	1	VF27IV2018.10*
Santee	NC	Sixmile	Sixmile Creek	1	TF47.2*
Santee	SC	Rocky	UT Bull Run Creek	3	TS1
Santee	SC	Rocky	Beaverdam Creek	3	MG160418.tws.1
Santee	SC	Rocky	Beaverdam Creek	3	TS2
Santee	SC	Rocky	Bull Run Creek	3	TS3

Basin	State	Sub-basin	Locality	Hap	Sample ID
Santee	SC	Rocky	Beaverdam Creek	3	TS5
Santee	SC	Rocky	UT Bull Run Creek	3	TS6
Santee	SC	Rocky	Bull Run Creek	3	TS7
Santee	SC	Rocky	Custer Branch	3	VF13III2017.4*
Pee Dee	NC	Goose/Duck	Goose Creek	2	MG8II2017.7*
Pee Dee	NC	Goose/Duck	Goose Creek	2	MG8II2017.8*
Pee Dee	NC	Goose/Duck	Goose Creek	2	MG8II2017.9*
Pee Dee	NC	Goose/Duck	Goose Creek	2	MG8II2017.10*
Pee Dee	NC	Goose/Duck	Goose Creek	2	MG8II2017.11*
Pee Dee	NC	Goose/Duck	Goose Creek	2	MG8II2017.12*
Pee Dee	NC	Goose/Duck	Goose Creek	2	VF13III2017.2*
Pee Dee	NC	Goose/Duck	Goose Creek	2	VF13III2017.3*
Pee Dee	NC	Goose/Duck	Goose Creek	2	VF27IV2018.3*
Pee Dee	NC	Goose/Duck	Goose Creek	2	VF27IV2018.4*
Pee Dee	NC	Goose/Duck	Goose Creek	2	VF27IV2018.5*
Pee Dee	NC	Goose/Duck	Goose Creek	2	VF27IV2018.6*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF28*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF30*
Pee Dee	NC	Goose/Duck	Duck Creek	2	TF32*
Pee Dee	NC	Goose/Duck	Duck Creek	2	TF34*
Pee Dee	NC	Goose/Duck	Duck Creek	2	TF36*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF38*

Basin	State	Sub-basin	Locality	Hap	Sample ID
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF39*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF41*
Pee Dee	NC	Goose/Duck	Duck Creek	2	TF42*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF43*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF46.2*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF48.2*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF51*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF52*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF54*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF55*
Pee Dee	NC	Goose/Duck	Goose Creek	2	TF56*
Pee Dee	SC	Lynches	Flat Creek	1	TS8
Pee Dee	SC	Lynches	Lynches River	1	TS9
Pee Dee	SC	Lynches	Lynches River	1	TS10
Pee Dee	SC	Lynches	Lynches River	1	TS11
Pee Dee	SC	Lynches	Lynches River	1	TS12
Pee Dee	SC	Lynches	Lynches River	1	VF13VI2017.1
Pee Dee	SC	Lynches	Lynches River	3	VF13VI2017.2
Pee Dee	SC	Lynches	Lynches River	1	VF13VI2017.17
Pee Dee	SC	Lynches	Lynches River	3	VF13VI2017.18
Pee Dee	SC	Lynches	Lynches River	1	VF13VI2017.19
Savannah	SC	Stevens	Turkey Creek	3	JW2III2017.1*

Basin	State	Sub-basin	Locality	Hap	Sample ID
Savannah	SC	Stevens	Mountain Creek	3	JW2III2017.2*
Savannah	SC	Stevens	Turkey Creek	3	JW2III2017.3*
Savannah	SC	Stevens	Mountain Creek	1	VF14VI2017.2
Savannah	SC	Stevens	Mountain Creek	1	VF3IV2018.1*
Savannah	SC	Stevens	Turkey Creek	1	VF3IV2018.2*
Savannah	SC	Stevens	Turkey Creek	1	VF3IV2018.3*
Savannah	SC	Stevens	Mountain Creek	1	VF3IV2018.4*
Savannah	SC	Stevens	Mountain Creek	1	VF3IV2018.5*
Savannah	SC	Stevens	Turkey Creek	1	VF3IV2018.6*
Savannah	SC	Stevens	Sleepy Creek	1	VF10V2018.1
Savannah	SC	Stevens	Mountain Creek	1	VF10V2018.4
Savannah	SC	Stevens	Mountain Creek	1	VF10V2018.5
Savannah	SC	Stevens	Mountain Creek	1	VF10V2018.6
Savannah	SC	Stevens	Mountain Creek	1	VF10V2018.8
Savannah	SC	Stevens	Mountain Creek	1	VF10V2018.9
Savannah	SC	Stevens	Mountain Creek	1	VF10V2018.11
Savannah	SC	Stevens	Little Stevens Creek	1	VF10V2018.12
Savannah	SC	Stevens	Little Stevens Creek	1	VF10V2018.13
Savannah	SC	Stevens	Little Stevens Creek	1	VF10V2018.14
Savannah	SC	Stevens	Little Stevens Creek	1	VF10V2018.16
Savannah	SC	Stevens	Little Stevens Creek	1	VF10V2018.17
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.1

Basin	State	Sub-basin	Locality	Hap	Sample ID
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.2
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.3
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.4
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.5
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.6
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.7
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.8
Savannah	SC	Stevens	Sleepy Creek	1	VF20III2019.2.9
Savannah	SC	Stevens	Mountain Creek	1	VF20III2019.3.1

Table 3. River basin, population names, specific collection localities and geographic coordinates for *Lasmigona decorata* populations represented in this study. Sampling locations were grouped by basin and population for statistical analysis. Additional metadata is available upon request.

Basin	**Sub-basin**	**Stream**	**Locality**
Santee	Fishing Creek	Fishing Creek	Millen Rd., Chester, NC
Santee	Sixmile Creek	Sixmile Creek	Marvin Rd., Union, NC
Santee	Rocky Creek	Beaverdam Creek	Hunter Rd., Chester, SC
Santee	Rocky Creek	Bullrun Creek	Peden Bridge Rd., Chester, SC
Santee	Rocky Creek	UT Bullrun Creek	Pleasant Grove Rd., Chester, SC
Santee	Rocky Creek	Custer Branch	Pleasant Grove Rd., Chester, SC
Pee Dee	Goose/Duck Creek	Duck Creek	Duck Creek Ln., Union, NC
Pee Dee	Goose/Duck Creek	Goose Creek	NC 601, Union, NC
Pee Dee	Goose/Duck Creek	Goose Creek	NC 601, Union, NC
Pee Dee	Goose/Duck Creek	Goose Creek	NC 601, Union, NC
Pee Dee	Lynches River	Lynches River	SC 265 at Lucas Drive, Lancaster, SC
Pee Dee	Lynches River	Lynches River	Mack Raley Rd., Lancaster, SC

Basin	Sub-basin	Stream	Locality
Pee Dee	Lynches River	Lynches River	SC 265 at Lucas Dr., Lancaster, SC
Savannah	Stevens Creek	Sleepy Creek	Faulkner Mountain Rd., Edgefield, SC
Savannah	Stevens Creek	Sleepy Creek	Sleepy Creek Rd., Edgefield, SC
Savannah	Stevens Creek	Steven's Creek	Little Steven's Creek Baptist Church, Edgefield, SC
Savannah	Stevens Creek	Mountain Creek	Timmerman Rd., Edgefield, SC
Savannah	Stevens Creek	Mountain Creek	Sheppard Rd., Edgefield, SC
Savannah	Stevens Creek	Mountain Creek	SC 378 at Long Cane Rd., Edgefield, SC

Table 4. Taxa, accession numbers, and authors for sequences that were utilized for outgroups in this analysis. Dashes indicate that there was no representative for that species at that locus.

Species	COI	NDI	28s	Reference
Alasmidonta heterodon	AF093840.1[1]	MG905826.1[2]	---	[1]King et al. 1999 [2]Aunins et al. 2018
Alasmidonta marginata	AF156502.1[3]	GU085335.1[4]	AF400688.1[5]	[3]Graf and O'Foighill 2000 [4]Boyer et al. 2011 [5]Graf 2002
Alasmidonta raveneliana	VF27IV18.13[6]	VF27IV18.13[6]	VF12III18.2[6]	[6]Gangloff et al. unpublished data
Alasmidonta triangulata	MG199612.1[7]	MG199739.1[7]	---	[7]Smith et al. 2017
Alasmidonta undulata	MK308197.1[8]	HM849205.1[9]	---	[8]Aguilar et al. 2018 [9]Breton et al. 2011
Alasmidonta varicosa	MK308233.1[10]	EF466103.1[11]	---	[10]Aguilar et al. 2018

Species	COI	NDI	28s	Reference
Alasmidonta varicosa				[11]Kneeland and Rhymer 2007
Alasmidonta viridis	VF27IV18.11[12]	---	VF27IV18.11[12]	[12]Gangloff et al. unpublished data
Amblema plicata	AUM15242[13] DQ648121.1[14]	AUM15242[13] MH633585.1[15]	AUM15242[13] MK036071.1[16]	[13]Gangloff et al. unpublished data [14]Elderkin et al. 2006 [15]Johnson et al. 2018 [16]Smith et al. 2019
Anodonta cygnea	MK034160.1[17]	MG385135.1[18]	MF414395.1[19]	[17]Bolotov et al. 2019 [18]Burzynski and Soroka 2017 [19]Froufe et al. 2017
Anodonta implicata	MK308353.1[20]	EF466101.1[21]	---	[20]Aguilar et al. 2018 [21]Kneeland and Rhymer 2007

Species	COI	NDI	28s	Reference
Anodontoides denigrata	MG199641.1[22]	MG199768.1[22]	---	[22]Smith et al. 2017
Anodontoides ferussacianus	MG199687.1[23]	MG199814.1[23]	---	[23]Smith et al. 2017
Lasmigona alabamensis	MG29X16.1.08[24] MG29X16.1.09[24] MG29X16.1.10[24]	MG29X16.1.08[24] MG29X16.1.09[24] MG29X16.1.10[24]	MG29X16.1.08[24] MG29X16.1.09[24] MG29X16.1.10[24]	[24]Gangloff et al. unpublished data [24]Gangloff et al. unpublished data [24]Gangloff et al. unpublished data
Lasmigona complanata	AF093845.1[25] --- --- --- --- ---	GU085349.1[26] GU085348.1[26] HM849226.1[27] HM849225.1[27] HM849224.1[27] HM849223.1 [27]	--- --- --- --- --- ---	[25]King et al. 1999 [26]Boyer et al. 2011 [27]Breton and Hoeh 2010

Species	COI	NDI	28s	Reference
Lasmigona complanata	---	HM849222.1[27]	---	
	---	HM849220.1[27]	---	
Lasmigona compressa	HM849079.1[28]	HM852930.1[31]	DQ191414.1[32]	[28]Breton and Hoeh 2010
	HM849080.1[28]	HM852929.1[31]	---	[29]Graf and Foighil 1999
	HM849081.1[28]	HM852928.1[31]	---	[30]King et al. 1999
	HM849084.1[28]	HM849233.1[28]	---	[31]Boyer et al. 2011
	HM849085.1[28]	HM849232.1[28]	---	[32]Graf and Cummings 2006
	HM856638.1[28]	HM849231.1[28]	---	
	AF156503.1[29]	HM849230.1[28]	---	
	AF093846.1[30]	HM849229.1[28]	---	
	AF093847.1[30]	---	---	
	NC 015481.1[28]	---	---	
Lasmigona costata	HM849086.1[33]	HM852931.1[36]	---	[33] Breton and Hoeh 2010
	HM849087.1[33]	GU085352.1[36]	---	[34] King et al. 1999
	HM849088.1 [33]	GU085351.1[36]	---	[35]Hewitt et al. 2018

Species	COI	NDI	28s	Reference
Lasmigona costata	HM849089.1[33]	GU085350.1[36]	---	[36]Boyer et al. 2011
	AF093848.1[34]	HM849237.1[33]	---	[37]Metzger et al. 2018
	KU985185.1[35]	HM849236.1[33]	---	
	KU985186.1[35]	HM849235.1[33]	---	
	KU985187.1[35]	HM849234.1[33]	---	
	KU985188.1[35]	---	---	
	KU985189.1[35]	---	---	
	KU985190.1[35]	---	---	
	KU985191.1[35]	---	---	
	KU985192.1[35]	---	---	
	KU985193.1[35]	---	---	
	KU985195.1[35]	---	---	
	KU985196.1[35]	---	---	
	KU985197.1[35]	---	---	
	KU985198.1[35]	---	---	

Species	COI	NDI	28s	Reference
Lasmigona costata	KU985199.1[35]	---	---	
	KU985200.1[35]	---	---	
	KU985201.1[35]	---	---	
	GU085295.1[36]	---	---	
	GU085296.1[36]	---	---	
	MH012240.1[37]	---	---	
Lasmigona decorata	AF093849.1[38]	---	---	[38]King et al. 1999
Lasmigona etowaensis	MG199611.1[39]	MG199778.1[39]	---	[39]Smith et al. 2017
	MG199651.1[39]	MG199738.1[39]	---	
Lasmigona holstonia	AY655001.1 [40]	AY655103.1[40]	AUM931.1[41]	[40]Campbell et al. 2005
	AUM931.1[41]	---	AUM931.3[41]	[41]Gangloff et al. unpublished
	AUM931.3[41]	---	AUM928.1[41]	data
Lasmigona subviridis	HM849090.1[42]	HM849240.1[42]	1XII2014.1.5[44]	[42] Breton et al. 2010
	HM849091.1[42]	HM849239.1[42]	---	

Species	COI	NDI	28s	Reference
Lasmigona subviridis	HM849092.1[42]	HM849238.1[42]	---	[43] King et al. 1999
	HM856640.1 [42]	---	---	[44]Gangloff et al. unpublished
	AF091330.1[43]	---	---	data
	AF093850.1[43]	---	---	
	AF093851.1[43]	---	---	
	TF1[44]	---	---	
	TF2[44]	---	---	
	TF3[44]	---	---	
	TF4[44]	---	---	
	TF5[44]	---	---	
	TF9[44]	---	---	
	TF10[44]	---	---	
	8.17.4[44]	---	---	
	8.17.6[44]	---	---	
	8.17.7[44]	---	---	

Species	COI	NDI	28s	Reference
Lasmigona subviridis	1X112014.1.1[44]	---	---	
	1X112014.1.2[44]	---	---	
	1X112014.1.3[44]	---	---	
	1X112014.1.4[44]	---	---	
	1XII2014.1.5[44]	---	---	
	6VI2015.1[44]	---	---	
	23V2011.1.1[44]	---	---	
	RWVDIX1985.1.1[44]	---	---	
Margaritifera margaritifera	KX056490.1[45]	EF466105.1[46]	AM779649.1[47]	[45]Valila et al. 2016 [46]Kneeland and Rhymer 2007 [47]Taylor et al. 2007
Pyganodon cataracta	JX101491.1[48]	EF446102.1[49]	---	[48]Stanton et al. 2012 [49]Kneeland and Rhymer 2007
Pyganodon grandis	AUM24446[50]	AUM24446[50]	AUM24446[50]	[50]Gangloff et al. unpublished data

Species	COI	NDI	28s	Reference
Quadrula quadrula	KX853981.1[51]	MH633595.1[52]	MK036133.1[53]	[51]Mathias et al. 2016 [52] Johnson et al. 2018 [53]Smith et al. 2019
Simpsonaias ambigua	KX822666.1[54]	---	KX822622.1[54]	[54]Lopes et al. 2016
Strophitus radiatus	MG199722.1[55]	MG199849.1[55]	---	[55]Smith et al. 2017
Strophitus undulates	TF21[56]	TF21[56]	DQ191415.1[57]	[56]Gangloff et al. unpublished data [57]Graf and Cummings 2006
Utterbackia imbecillis	HM849172.1[58]	GU085384.1[59]	---	[58]Breton et al. 2011 [59]Boyer et al. 2011

Table 5. Among- and within- basin genetic distances for *Lasmigona decorata*. Pairwise genetic distances (x100) were calculated using the maximum composite likelihood method. Numbers along the diagonal (bold) represent within-basin divergence rates.

	Santee	Pee Dee	Savannah
Santee	**0.10**		
Pee Dee	*0.24*	**0.12**	
Savannah	*0.08*	*0.19*	**0.04**

Table 6. Among- and within-population genetic distances for *Lasmigona decorata*. Pairwise genetic distances (x100) were calculated using the maximum composite likelihood method. Numbers along the diagonal (bold) represent within-population divergence rates. Values represented with an asterisk (*) had a divergence value of zero. Data were pooled at the sub-basin scale for Rocky and Stevens Creeks.

	1	2	3	4	5	6
1. Goose/Duck Creek	*					
2. Lynches River	*0.26*	**0.08**				
3. Sixmile Creek	*0.21*	*0.04*	*			
4. Fishing Creek	*0.21*	*0.04*	*	*		
5. Rocky Creek	*0.43*	*0.17*	*0.21*	*0.21*	*	
6. Stevens Creek	*0.23*	*0.05*	*0.02*	*0.02*	*0.19*	**0.04**

Table 7. Among and within haplotype genetic distances for *Lasmigona* decorata. Pairwise genetic distances (x100) were calculated using the maximum composite likelihood method. Values next to basin names indicate the dominant haplotype detected in the basin. Numbers along the diagonal (bold) represent within-basin haplotype divergence rates.

	Haplotype 1	Haplotype 2	Haplotype 3
1. Savannah	**0.20**		
2. Pee Dee	*0.21*	**0.39**	
3. Santee	*0.21*	*0.43*	**0.21**

Table 8. Genetic diversity indices and test of neutrality for *Lasmigona decorata* among (A) basins and (B) populations. All neutrality tests were non-significant ($p > 0.05$). N= number of individuals, Hd= haplotype diversity, Pi= nucleotide diversity. Data were pooled at the sub-basin scale for Rocky and Stevens Creeks.

A. Basin

	N	**Hd**	**Pi**	**No. of Haplotypes**	**Tajima's D**	**Fu's Fs**
Santee	22	0.48485	0.00095	2	1.33425	1.39229
Pee Dee	47	0.51249	0.00110	3	0.45329	0.60479
Savannah	32	0.17540	0.00035	2	-0.44826	-0.01937

B. Population

	N	**Hd**	**Pi**	**No. of Haplotypes**	**Tajima's D**	**Fu's Fs**
Goose/Duck Creek	29	0.0000	0.0000	1	0.0000	N/A
Lynches River	10	0.3556	0.0007	2	0.0150	0.4167
Fishing Creek	1	0.0000	0.0000	1	0.0000	N/A
Rocky Creek	8	0.0000	0.0000	1	0.0000	N/A
Sixmile Creek	13	0.0000	0.0000	1	0.0000	N/A
Stevens Creek	32	0.17540	0.0004	2	-0.4483	-0.0194

Table 9. Pairwise Fst values measuring genetic differentiation between basins supporting *Lasmigona decorata* populations. All values are statistically significant (P<0.05).

Basin	Santee	Pee Dee
Santee		
Pee Dee	0.46279	
Savannah	0.16953	0.47682

Table 10. Pairwise Fst values measuring genetic differentiation between *Lasmigona decorata* populations. Values with asterisk are statistically significant (P<0.05). Data were pooled at the sub-basin scale for Rocky and Stevens Creeks.

Population	1	2	3	4	5
1. Goose/Duck Creek					
2. Lynches River	0.923*				
3. Fishing Creek	1*	-0.778			
4. Rocky Creek	1*	0.755*	1		
5. Sixmile Creek	1*	0.148	0.000	1*	
6. Stevens Creek	0.916*	-0.014	-0.871	0.850*	0.015

Table 11. Intra-specific genetic distances for all *Lasmigona* taxa represented in study. Pairwise genetic distances (x100) were calculated using the maximum composite likelihood method.

	COI	***NDI***
L. alabamensis	0.301	0
L. complanata	N/A	0.341
L. compressa	0	0.050
L. costata	1.809	1.381
L. decorata	0.143	0
L. etowaensis	1.077	0.197
L. holstonia	0	N/A
L. subviridis	0.036	0

Table 12. Mean inter-specific genetic distances for all *Lasmigona* taxa represented in study. Pairwise genetic distances (x100) were calculated using the maximum composite likelihood method. Values below the diagonal (bold) represent variation at the *COI* locus, values above the diagonal (italics) represent variation at the *NDI* locus.

	1	2	3	4	5	6	7	8
1. L. alabamensis		*2.5*	*19.6*	*11.9*	*19.7*	*20.3*	*21.3*	*20.6*
2. L. complanata	**1.8**		*19.0*	*12.1*	*18.9*	*19.7*	*20.1*	*18.9*
3. L. compressa	**13.4**	**14.0**		*18.8*	*4.3*	*16.4*	*17.0*	*4.7*
4. L. costata	**11.8**	**10.9**	**17.4**		*19.7*	*21.6*	*21.2*	*20.1*
5. L. decorata	**15.2**	**15.9**	**4.2**	**18.0**		*17.3*	*17.9*	*4.7*
6. L. etowaensis	**18.4**	**18.1**	**17.1**	**21.6**	**19.4**		*1.9*	*16.1*
7. L. holstonia	**19.2**	**18.4**	**17.5**	**23.0**	**19.1**	**8.5**		*16.7*
8. L. subviridis	**11.9**	**12.6**	**3.8**	**16.5**	**5.2**	**18.0**	**16.4**	

Table 13. Summary of phylogenetic hypotheses and outcomes for single-gene and multi-gene analyses in this study.

Hypothesis	**COI**	**NDI**	**COI/NDI**	**COI/NDI/28S**
Monophyletic *Lasmigona*	Polyphyletic- 3 deeply divergent clades	Unresolved	Polyphyletic- 3 deeply divergent clades Moderate support (BPP = 90) for inclusion of *L. compressa*, *L. subviridis* and *L. decorata* in *Alasmidonta*	Polyphyletic- 3 deeply divergent clades Strong support (BPP = 96) for inclusion of *L. compressa*, *L. subviridis* and *L. decorata* in *Alasmidonta*
Closest sister taxon	Low support (BPP = 69) for *L. compressa*	Unresolved	Moderate support (BPP = 85) for L. compressa	Moderate support (BPP = 78) for *L. compressa*
Members of clade	*L. subviridis* and *L. compressa* (BPP = 100)	*L. subviridis* and *L. compressa* (BPP = 100)	*L. subviridis* and *L. compressa* (BPP = 100)	*L. subviridis* and *L. compressa* (BPP = 100)
Monophyly of other Anodontine taxa in tree (e.g., Alasmidonta)	Polyphyletic- 1 deeply divergent clade 2 distant taxa (*A. heterodon*, *A. viridis*) from main *Alasmidonta* group	Unresolved	Polyphyletic- 1 deeply divergent clade (BPP = 1) Strong support (BPP = 99) for inclusion of *A. heterodon* with *L. compressa*, *L. subviridis* and *L. decorata* Strong support (BPP = 99) for highly divergent *A. viridis* species from main *Alasmidonta* group	Polyphyletic- 1 deeply divergent clade (BPP = 1) Strong support (BPP = 97) for inclusion of *A. heterodon* with *L. compressa*, *L. subviridis* and *L. decorate* Weak support (BPP = 59) for highly divergent *A. viridis* species

Figure 1. Distribution of *Lasmigona decorata COI* haplotypes among river basins. Haplotype two is unique to the Pee Dee Basin. Haplotypes one and three are present in all three basins.

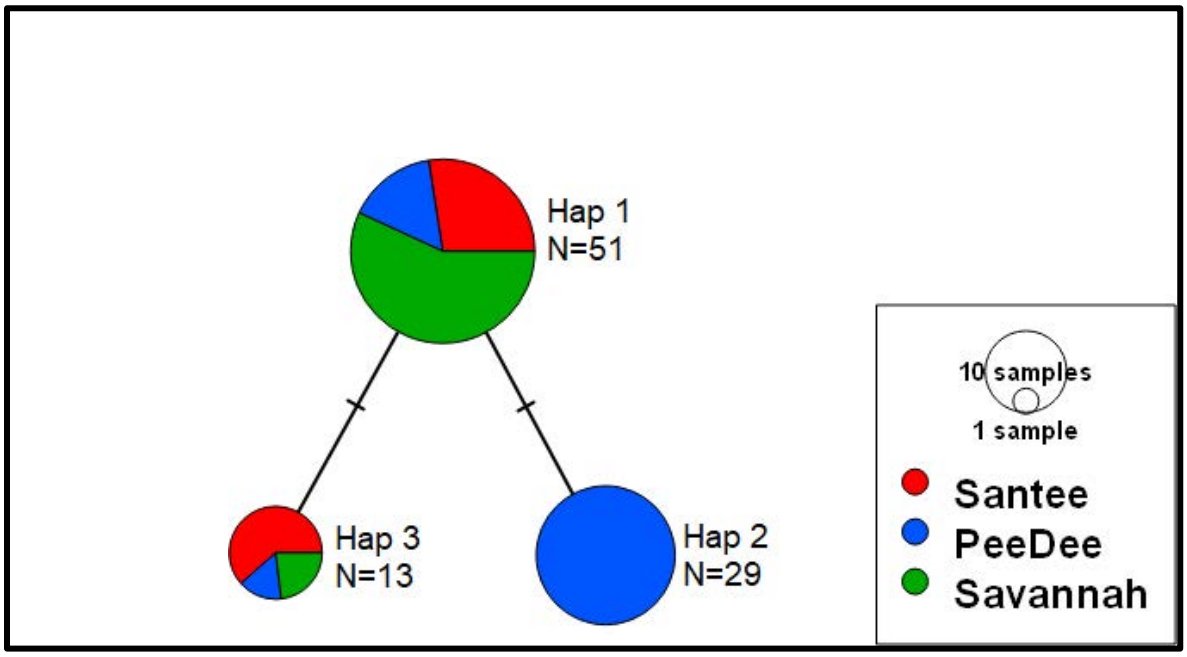


Figure 2. Distribution of the three observed *Lasmigona decorata* haplotypes within sub-basins among populations. Haplotype two is unique to the Goose/Duck creek (Pee Dee Basin) population. The Stevens Creek and Lynches River populations share haplotypes one and three.

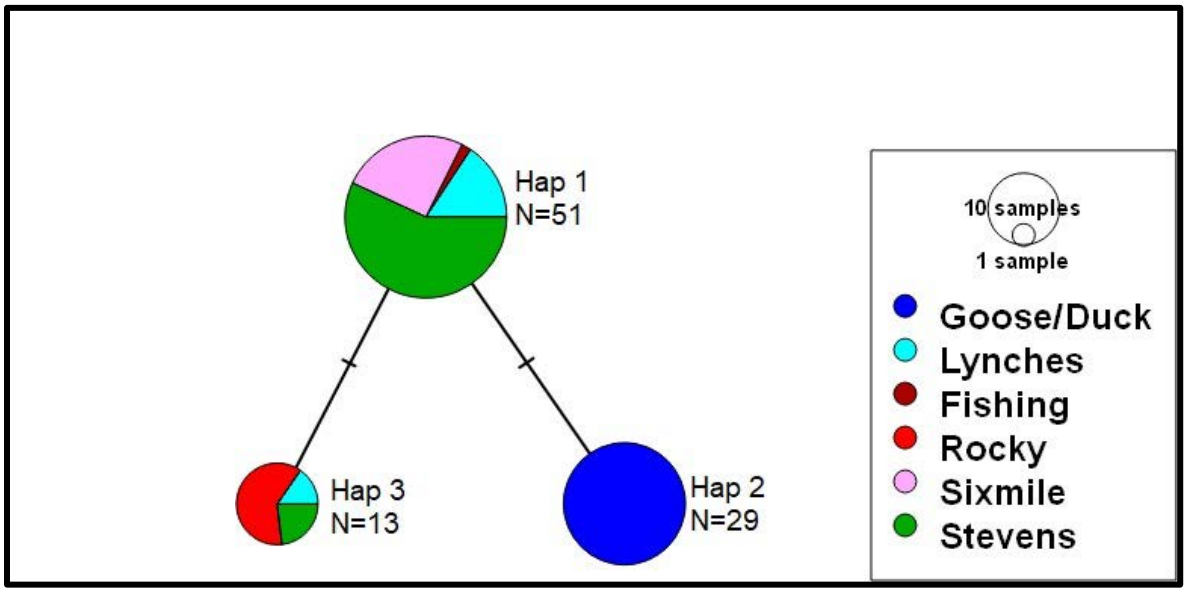


Figure 3. Bayesian analysis of *COI* dataset. Values on node represent bootstrap support. Scale bar indicates nucleotide substitutions per site. Red clades indicate polyphyletic and paraphyletic *Lasmigona* species. Green clades indicate monophyletic and polyphyletic *Alasmidonta* species. Genera included in analysis are *Amblema, Quadrula, Lasmigona, Alasmidonta, Pyganodon, Simpsonaias, Anodonta, Utterbackia, Strophitus, Anodontoides* and *Margaritifera*.

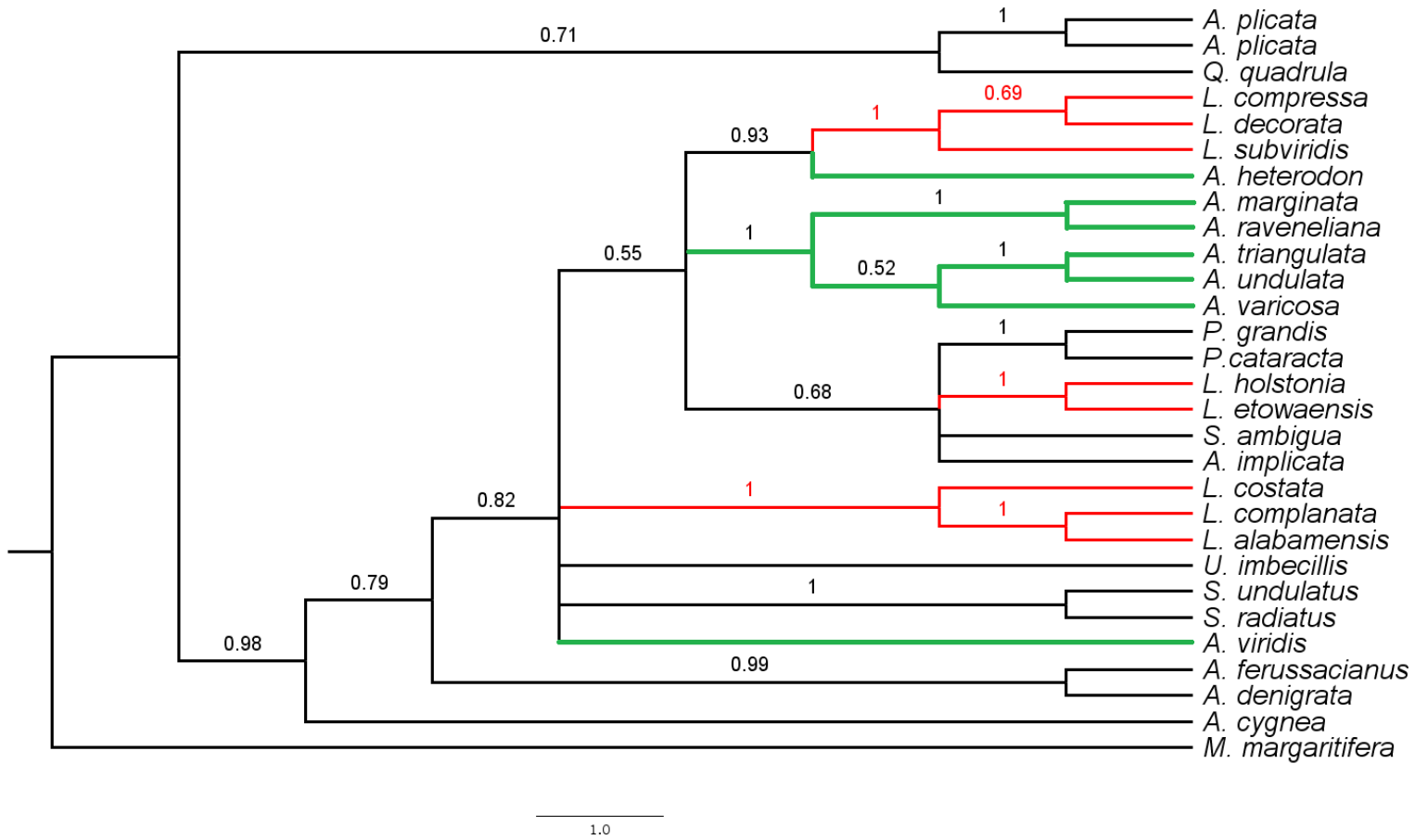


Figure 4. Bayesian analysis of *NDI* dataset. Values on node represent bootstrap support. Scale bar indicates nucleotide substitutions per site. Red clades indicate polyphyletic and paraphyletic *Lasmigona* species. Genera included in the analysis are *Strophitus, Anodontoides, Lasmigona, Amblema, Alasmidonta, Pygandon, Utterbackia, Anodonta, Quadrula* and *Margaritifera*.

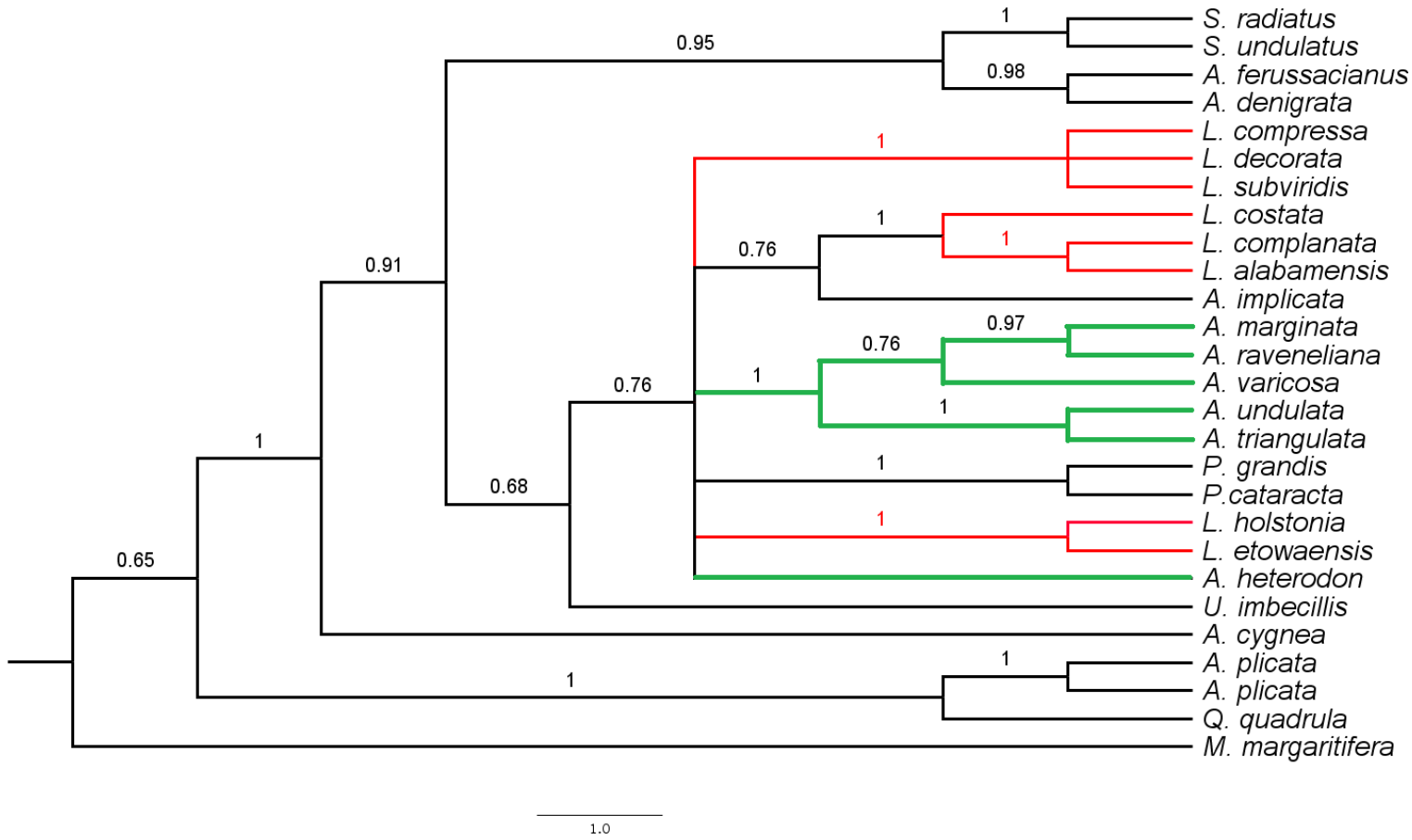


Figure 5. Bayesian analysis of concatenated *COI* and *NDI* dataset. Values on node represent bootstrap support. Scale bar indicates nucleotide substitutions per site. Red clades indicate polyphyletic and paraphyletic *Lasmigona* species. Green clades indicate monophyletic and polyphyletic *Alasmidonta* species. Genera included in analysis are *Lasmigona*, *Alasmidonta, Amblema, Quadrula, Pyganodon, Simpsonaias, Anodonta, Anodontoides, Utterbackia*, *Strophitus* and *Margaritifera*.

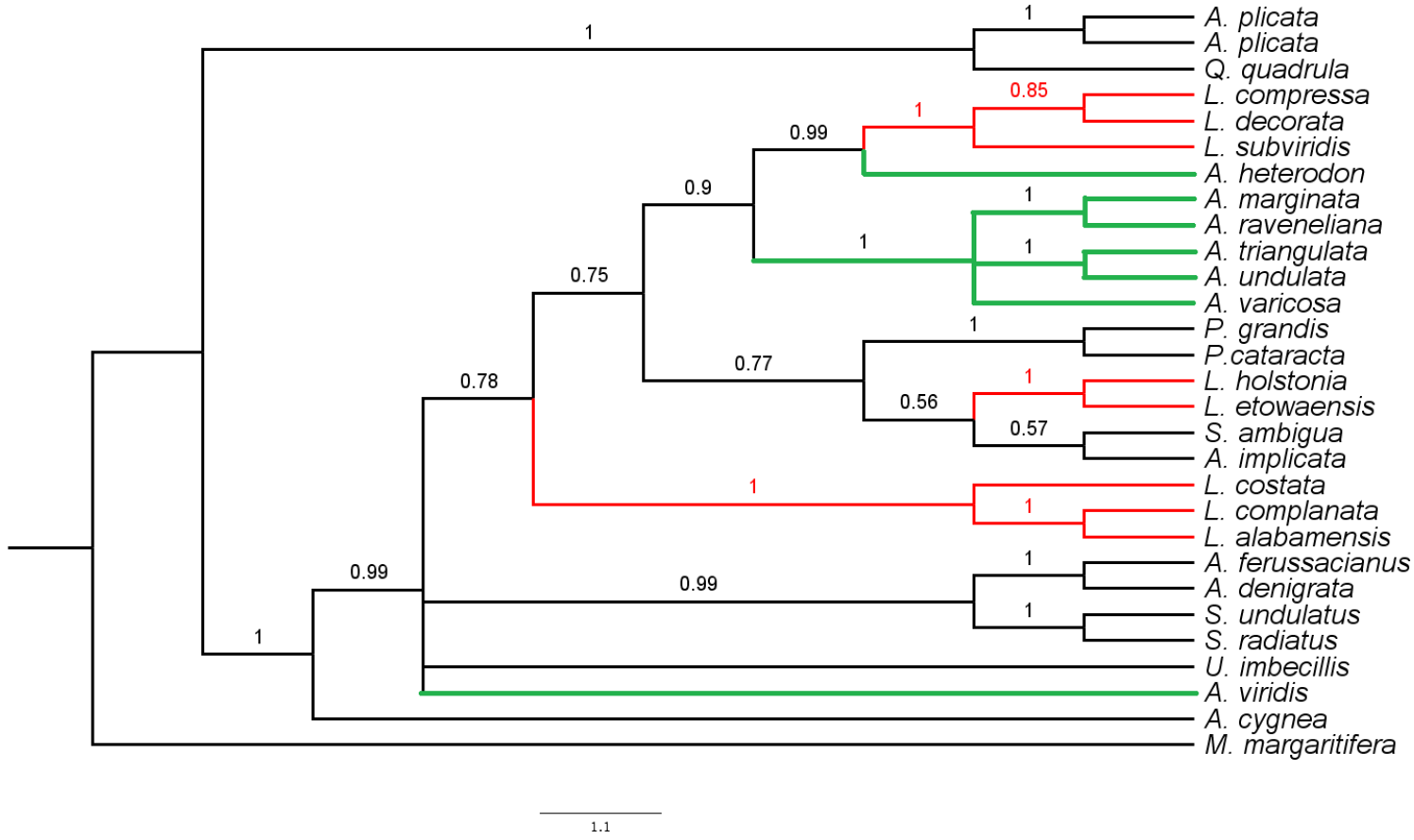


Figure 6. Bayesian analysis of concatenated *28s, COI* and *NDI* dataset. Values on node represent bootstrap support. Scale bar indicates nucleotide substitutions per site. Red clades indicate polyphyletic and paraphyletic *Lasmigona* species. Green clades indicate monophyletic and polyphyletic *Alasmidonta* species. Genera included in analysis are *Amblema, Quadrula, Lasmigona, Alasmidonta, Pyganodon, Simpsonaias, Anodonta, Anodontoides, Strophitus, Utterbackia* and *Margaritifera*.

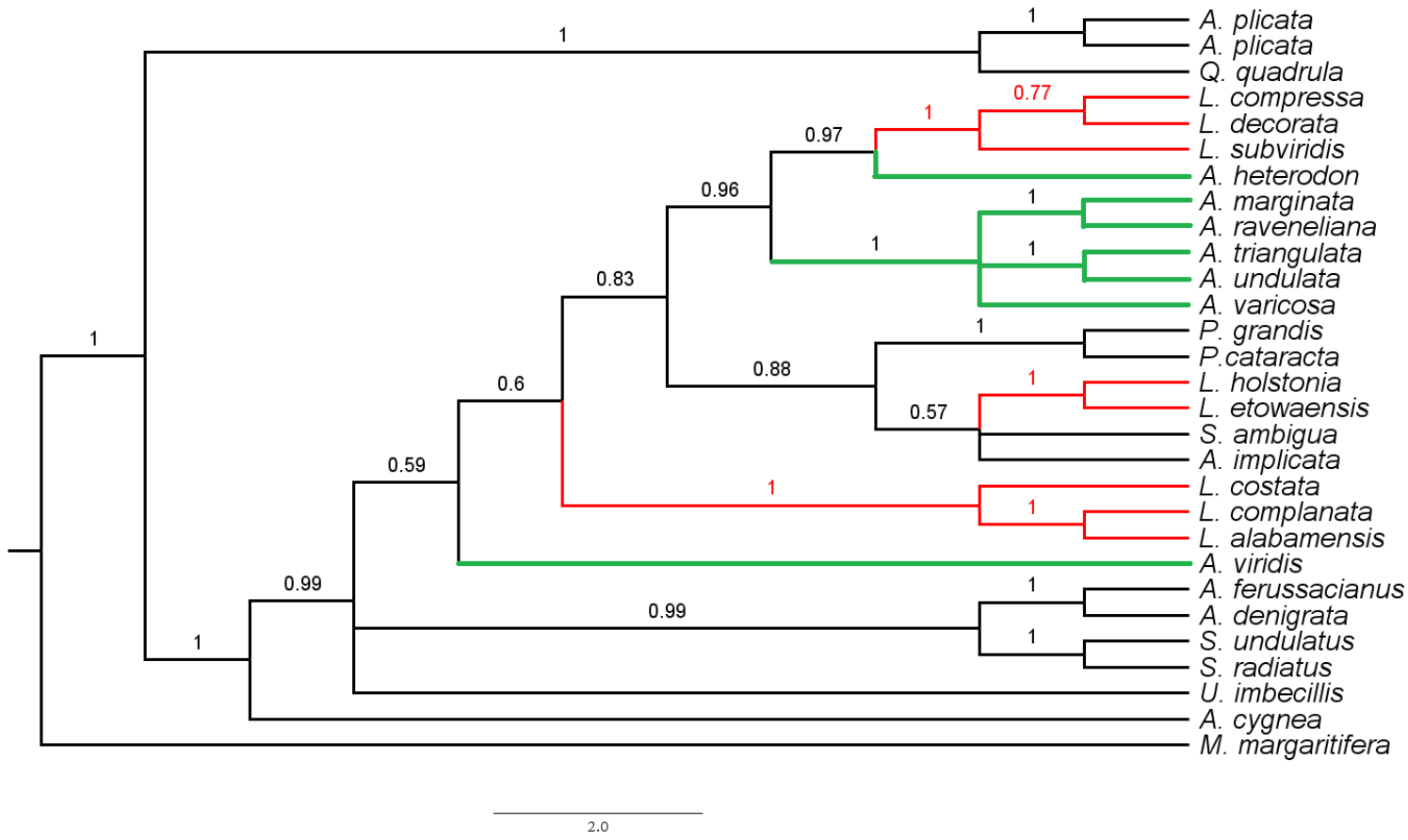


Figure 7. Minimum spanning haplotype network of *COI* dataset for *Lasmigona* taxa. Circles represent haplotypes. Size of circles indicates how many individuals are represented in that haplotype. One dash indicates a one nucleotide difference. M= number of mutations separating taxa.

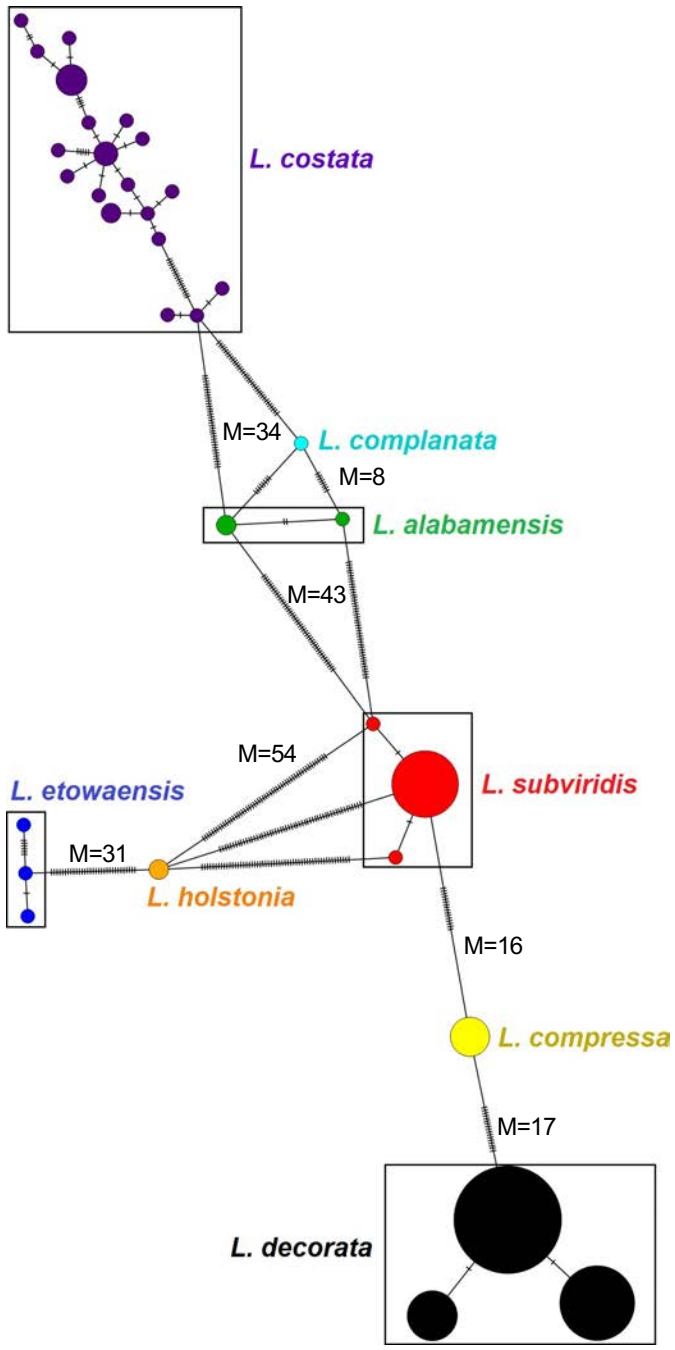
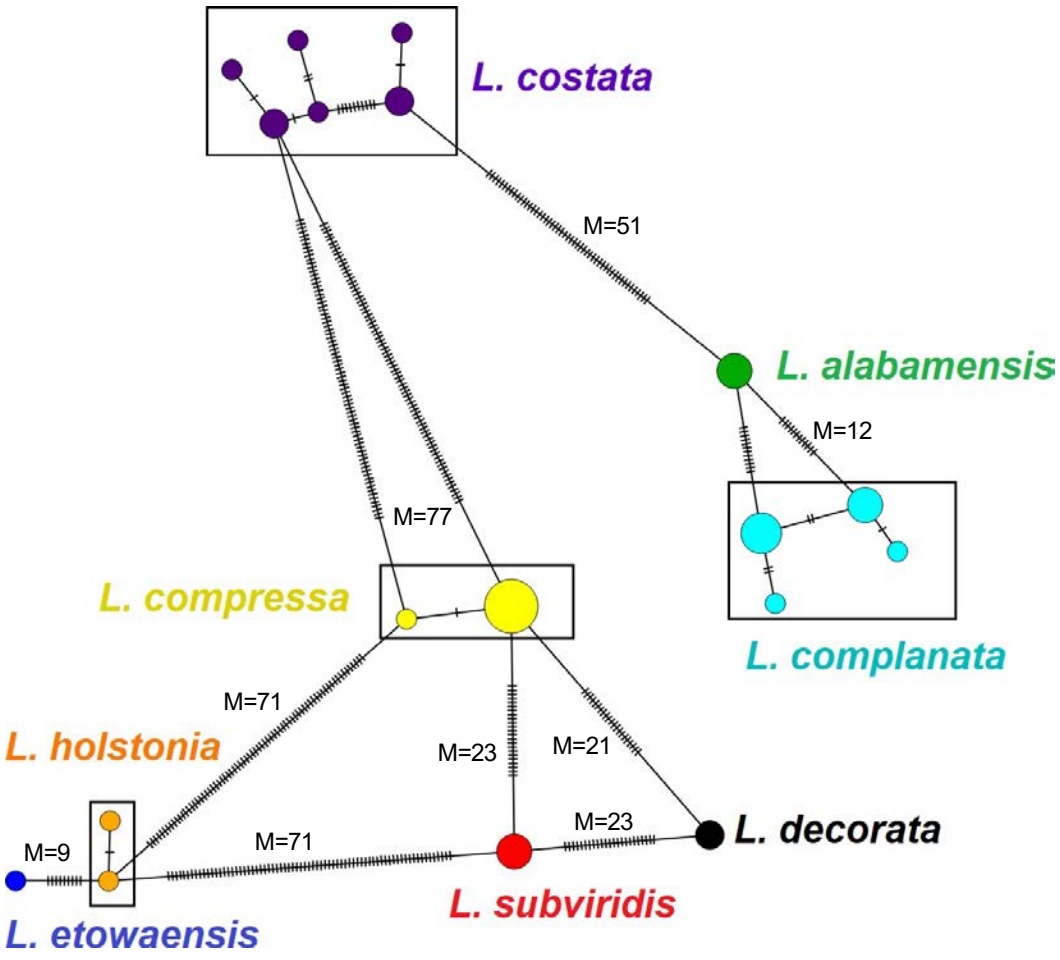


Figure 8. Minimum spanning haplotype network of *NDI* dataset for *Lasmigona* taxa. Circles represent haplotypes. Size of circles indicate how many individuals are represented in that haplotype. One dash indicates a one nucleotide difference. M= number of mutations separating taxa.



Vita

Victoria Clark Fowler was born in Spartanburg, South Carolina to Mitchell and Julie Fowler in 1994. She graduated from Rutherford Early College High School and Isothermal Community College in May 2012. She was awarded a Bachelor of Science degree from Appalachian State University in May 2016. She is currently employed by TranSystems as an Environmental Scientist in Raleigh, North Carolina.