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PHYLOGENY OF THE CRITICALLY ENDANGERED NORTH AMERICAN SPINYMUSSELS (UNIONIDAE: *ELLIPTIO* AND *PLEUROBEMA*)

A Thesis
by
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Abstract

PHYLOGENY OF THE CRITICALLY ENDANGERED NORTH AMERICAN SPINYMUSSELS (UNIONIDAE: *ELLIPTIO* AND *PLEUROBEMA*)

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Despite being common in numerous marine bivalve lineages, lateral spines are uncommon among freshwater bivalves. The North American freshwater mussel fauna includes three taxa that commonly exhibit spines: *Elliptio spinosa, Elliptio steinstansana,* and *Pleurobema collina*. All three taxa are endemic to the Southeastern US, critically endangered, and protected by the US Endangered Species Act. Currently, these species are recognized in two genera and the group is a source of considerable taxonomic confusion within the unionid tribe Pleurobemini (*Elliptio* and *Pleurobema*). Because freshwater mussels exhibit phenotypically plastic shell morphology, morphologically-based diagnoses are often problematic. I sequenced two mtDNA gene fragments (*ND1* and *COI*) and a fragment of the nuclear *ITS-1* locus from >70 specimens using standard Sanger techniques. Bayesian phylogenetic reconstructions suggest that the spinymussels do not comprise a monophyletic group. *Elliptio steinstansana* is sister to *P. collina* and these taxa form a monophyletic clade that appears to have diverged from its nearest ancestor (possibly an ancestral *Elliptio* or *Pleurobema* lineage) in the late Miocene, ~6 mya. Additionally, *E. spinosa* forms a monophyletic clade that diverged from members of the core *Elliptio* lineage

in the mid Pliocene, >1.5 million years before multiple radiations within the *Elliptio* clade. Furthermore, *E. spinosa* is highly divergent from the other spinymussels, suggesting that spines, while extremely rare in freshwater mussels worldwide, have evolved separately in two distinct bivalve lineages endemic to this region. These findings suggest a need to revise the taxonomy of this highly imperiled mussel group.

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I wouldn't have been able to maintain my sanity without the help of my friends and family. Thanks to my mother and sister, Carol and Colby, for all the support during this strange time. A special thanks to Laura Boggess for helping me keep it in perspective and get away from it all when I needed to. Thank you, Carra Parker and Elyse Russing, for commiseration and years of support and whisky drinks. Thanks to everyone in the ACRL and Siefferman Lab (past and present) for being weirdos with me.

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Foreword

The research outlined in this thesis will be submitted to the peer-reviewed journal *Molecular Phylogenetics and Evolution*. The body of this thesis has been prepared according to the style and formatting requirements for publication in this journal.

1. Introduction

Global biodiversity is in a state of rapid decline (Barnosky et al., 2011; Pereira et al., 2010) and freshwater ecosystems are among the world's most-threatened biomes (Dudgeon et al., 2006). Losses of freshwater biodiversity are estimated to currently occur at a much higher rate than terrestrial or marine ecosystems and are commonly attributed to severe habitat fragmentation and degradation caused by rapidly-growing human populations (Humphries and Winemiller, 2009; Sala, 2000; Smith, 2003; Strayer and Dudgeon, 2010). Continued loss of freshwater biodiversity is expected to drastically impair ecosystem services and human health, therefore conservation of freshwater diversity and ecosystem function is a major priority for management agencies operating from local to global scales (Abell et al., 2008; Loreau et al., 2001; Rapport et al., 1998).

Freshwater invertebrate communities exhibit high patch-scale biodiversity and freshwater mollusks (Bivalvia and Gastropoda) are among the most imperiled invertebrate groups globally (Strayer and Dudgeon, 2010). Currently, the total number of threatened freshwater mollusk taxa exceeds that of all other freshwater faunal groups combined (Régnier et al., 2009). Freshwater mussels (Bivalvia: Unionoida) are second only to freshwater snails as the most globally threatened freshwater group (Haag, 2012; Lydeard et al., 2004; Neves et al., 1997). North America has the highest number of endemic freshwater mussel taxa in the world, with ~300 described species (Bogan, 2007; Haag ,2009) and ~65% of North American mussel taxa are currently protected under state or federal legislation due

to drastic declines in population size and numbers (Haag and Williams, 2014; Strayer et al., 2004). These declines are widely attributed to degradation of lotic habitats although effects are inordinately widespread suggesting an array of causes that strongly affect mussels (Haag and Williams, 2014). These declines in habitat quality have resulted in the recent extinction of at least 35 species (Strayer and Dudgeon, 2010).

The North American spinymussels (Unionidae: *Elliptio spinosa, Elliptio steinstansana, and Pleurobema collina;* Fig. 1) are unique among freshwater mussels in that they are often characterized by the presence of conspicuous external spines. All three taxa are endemic to Southeastern Atlantic Slope (SEAS) rivers in North America. The Altamaha spinymussel (*E. spinosa;* Lea, 1836) is found only in the Altamaha River basin in Georgia, the Tar River spinymussel (*E. steinstansana*; Johnson and Clarke, 1983) is found only in the Tar and Neuse River basins in North Carolina, and the James River spinymussel (*Pleurobema collina*; Conrad, 1837) is found in the James and Roanoke river basins in Virginia and North Carolina. All three species have experienced substantial range and population declines. Populations frequently persist in isolated tributaries, exhibit low recruitment and appear highly sensitive to habitat degradation and other human-mediated disturbances (Fleming ,1995; McCormick, 2012; Petty, 2005; USFWS, 1985, 1988, 2008, 2009, 2011, 2014; Wisniewski et al., 2005). All three spinymussel taxa are listed as endangered under the US Endangered Species Act (USFWS, 1985, 1988, 2011).

External spines and other forms of ornamentation or armament are common in marine mollusks but are rare in freshwaters due to decreased Ca^{2+} availability and increased energy

costs associated with shell production (Kalff, 2002; Mackie and Filipance, 1983; Palmer, 1992). The utility of shell ornamentation is well-documented in both marine and freshwater gastropods and is believed to be a deterrent to predation in most cases (Appleton and Palmer, 1988; Bourdeau, 2009; Covich, 2010; Vermeij, 1977). In marine bivalves, shell ornamentation is likely driven by environmental pressures more than predation risk, and a variety of shell projections (e.g. spines, pustules, corrugations, etc.) are thought to aid burrowing ability and mitigation of sediment scouring around the shell (Bottjer and Carter, 1980; Stanley, 1981). In freshwater bivalves, the adaptive function of shell projections is understudied and open to conjecture (see Watters, 1992), however it seems likely that the lateral spines exhibited by spinymussels evolved to facilitate stabilization in the shifting-sand channels common in large, coastal plain streams in southeastern North America.

Previous characterizations of the spinymussels have utilized a suite of external morphological characteristics and placed taxa within the phenotypically-plastic genera *Pleurobema* and *Elliptio* (Haag, 2009; Turgeon, 1998). The presence and number of spines, for example, is often used as a diagnostic tool to distinguish *E. steinstansana* and *P. collina* from co-occurring *Elliptio* species, which often exhibit similar general shell morphology. Spine number and morphology are both variable within and among populations and age classes of both *E. steinstansana* and *P. collina* (R. Hoch, pers. comm. 2013; Petty, 2005). Taxonomic uncertainty has resulted in the assignment of *P. collina* within 4 genera over the past 50 y (Turgeon, 1998); conversely, *E. steinstansana* was designated as an *Elliptio* with only limited discussion of morphological and life-history traits (Johnson and Clarke, 1983).

Similarly, placement of *E. spinosa* may be problematic as it is the only known *Elliptio* taxon with conspicuous spines (*E. steinstansana* notwithstanding).

The current placement of *E. steinstansana* and *P. collina* in separate genera is unconvincing. *Elliptio steinstansana* and *P. collina* share remarkably similar life histories: both are tachytictic (short-term brooders), utilize analogous cyprinid fish hosts, and release unique leech-like conglutinates in the late-spring and early-summer months (Bogan, 2002; Boss and Clench, 1967; Eads and Levine, 2009; Hove and Neves, 1994; Johnson and Boss, 1984; Johnson and Clarke, 1983; Levine et al., 2011). Additionally, *P. collina* is the only currently recognized member of *Pleurobema* found on the Atlantic Slope; all other *Pleurobema* taxa are restricted to Gulf of Mexico drainages (e.g., Apalachicola, Mobile, and Mississippi drainages; Bogan, 2002; Williams et al., 2008). These life-history and biogeographic traits suggest that the spinymussels may comprise an evolutionarily distinct lineage and indicate a need to re-evaluate their phylogenetic placement.

The majority of spinymussel research to-date has focused almost exclusively on distribution/detection and propagation efforts (e.g., determining fish hosts and early life-history attributes; Eads and Levine, 2009; Hove and Neves, 1994; Johnson et al., 2012; Levine et al., 2011). No prior studies have attempted to explicitly address species boundaries or phylogenetic placement among spinymussel taxa. Moreover, few studies have generated genetic data for spinymussels. Petty (2005) conducted a genetic characterization of four *P. collina* populations and found evidence of range-wide genetic bottlenecking but did not address phylogenetic questions. Bogan et al. (2003), Campbell et al. (2005), and Campbell

and Lydeard (2012) used genetic data to examine deeper phylogenetic associations within the Pleurobemini and Unionidae. All three studies noted that *E. steinstansana* and *P. collina* specimens grouped outside of the primary *Elliptio* and *Pleurobema* clades. However, no research has yet examined the phylogenetic placement of spinymussels within Pleurobemini or species boundaries among these taxa.

Analysis of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) sequences has been shown to reliably resolve phylogenetic relationships and establish consistent species boundaries for numerous faunal groups (Hebert et al., 2003; review in Valentini et al., 2009) including many North American freshwater mussel taxa (e.g. Campbell and Lydeard, 2012; Inoue et al., 2014; Jones 2006). The use of mtDNA and nDNA markers to reconstruct phylogenies and delimit species is not without its limitations (review in Taylor and Harris, 2012) but remains an important tool for quantifying divergence rates and determining evolutionary relationships in order to practically manage threatened species and populations. If used appropriately, these markers comprise a suite of relatively cost-effective and widely-comparable metrics that often lead to broad-scale insights about the evolutionary history and divergence rates among taxa while identifying key conservation units or barriers to gene flow.

The purpose of this study was to determine the taxonomic placement of the spinymussels. I hypothesized that due to the rarity of spines in freshwater mussels globally, it is likely that the spinymussels form a monophyletic clade that is evolutionarily distinct from all other North American unionid taxa. In order to test this hypothesis, I sequenced three loci

(two mtDNA and one nDNA) and conducted robust phylogenetic reconstructions of the spinymussels and closely-related taxa. This is the first study to generate range-wide genetic data for the spinymussels as well as to have complete taxon sampling. My study will resolve the taxonomic position of these taxa within Unionidae and provide resource agencies with data to refine species concepts and management strategies for these critically endangered animals.

2. Materials and methods

2.1. Tissue collection and DNA extraction

Tissue samples were collected between 2003-2013 from multiple populations across four states (WV, VA, NC, and GA) in the Southeastern USA (Table 1, Fig. 2). *Elliptio steinstansana* samples (n=15) were collected from 2 populations in NC (Little Fishing Creek and Little River) during surveys conducted in 2013 and 2014 as well as from wild-caught broodstock currently housed in the North Carolina Wildlife Resources Commission (NCWRC) Marion Center for Aquaculture (MCAC) in Marion, NC. Tissue samples were collected non-lethally from all individuals using sterile buccal swabs (Isohelix SK-1 swabs, Boca Scientific Inc., Boca Raton, FL.) and frozen at -20°C until extraction. Total genomic DNA was isolated and purified using a Qiagen DNeasy Blood and Tissue Kit (Qiagen Sciences Inc., Valencia, CA) following manufacturer protocols. *Elliptio spinosa* samples (n=8) were collected from one population in GA during surveys in 2013. Samples were obtained using tissue swabs and isolated using a Gentra Purgene DNA extraction kit (Qiagen

Sciences Inc., Valencia, CA) following manufacturer protocols. *Pleurobema collina* samples (n=80) were collected from 4 populations in NC, VA, and WV in 2003-2004 as part of a separate study funded by USFWS and the VA Transportation Research Council (Petty, 2005). Tissue samples were collected non-lethally via mantle snips (20-30 mg) and preserved in 95% ethanol prior to extraction. Total genomic DNA was isolated and purified using a Gentra Purgene DNA extraction kit (Qiagen Sciences Inc., Valencia, CA) following manufacturer protocols. DNA concentration and quality was determined for all samples using a NanoDrop 2000 nano-spectrophotometer (Thermo Scientific, Waltham, MA). All DNA samples were stored long term at -20°C in Appalachian State University (Boone, NC) facilities.

Regions of the mtDNA cytochrome oxidase subunit I (*COI*) and NADH dehydrogenase 1 (*ND1*) genes as well as the nDNA internal transcribed spacer region 1 (*ITS-1*) were amplified for all available spinymussel specimens. For mtDNA, I used *COI* primers from Campbell and Lydeard (2012) and *ND1* primers adapted from Serb et al. 2003. For *ITS-1*, I used primers described in King et al. (1999). PCR amplifications for mtDNA were carried out under the following conditions: 12.5 µL GoTaq® Green Master Mix 2X (Promega Corporation, Madison, WI), 0.4 µL each primer (0.5 µM), 10-50 ng/µL DNA template, and nuclease-free water to a final volume of 25 µL. PCR amplifications for *ITS-1* were performed following conditions outlined in King et al. (1999). Reactions for each locus were conducted on a Bio-Rad MyCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using established protocols (Campbell and Lydeard, 2012). PCR product

quality was visually inspected on a 1% agarose gel stained with ethidium bromide. Products were purified with ExoSAP and sequenced off-site by Retrogen, Inc. (Sand Diego, CA) and the University of Georgia Genomics Facility (Athens, GA) with an ABI 3730 DNA Analyzer and ABI Big Dye Terminator Kits (Life Technologies, Grand Island, NY).

2.2. Sequence analyses

Sequences were compiled, edited, and aligned using Geneious R7 (Biomatters Ltd., Aukland, New Zealand). Sequences were checked for quality and the presence of stop-codons and mitochondrially-derived nuclear DNA fragments (numts) following recommendations in Buhay (2009). The concatenated mtDNA dataset was composed of 562 bp *ND1* fragments and 583 bp *COI* fragments for a total length of 1145 bp. The *ITS-1* dataset was composed of a 542 bp fragment and was analyzed separately from the mtDNA data due to a lack of available outgroup taxa sequences. Genetic divergence among the spinymussel species and outgroups was estimated using the maximum composite likelihood method in MEGA v6 (Tamura et al., 2013) and estimated number of haplotypes, mean nucleotide diversity (π) and mean number of base pair differences (k) were calculated for spinymussel species using DNASP v5.10.1 (Librado and Rozas, 2009).

2.3. Phylogenetic analyses

Bayesian algorithms were implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Redundant haplotypes were removed for phylogenetic analyses. I included an additional 34 GenBank sequences representing numerous species within the tribe Pleurobemini for which *ND1* and *COI* data were currently available (Table 1). I used *Amblema plicata* as an outgroup to root the phylogenetic trees. For the *ITS-1* dataset, I included an additional 23 GenBank sequences and used *Uniomerus declivus* to root the trees. I implemented jModelTest 2.1.4 to select the best-fit model of nucleotide substitution (Darriba et al., 2012). Two selection criteria (Akaike Information Criterion with finite population correction and Bayesian Information Criterion) identified the best-fit substitution model (within 95 % CI) as general time-reversible with a proportion of invariable sites and gamma-distributed rate variation across sites for mtDNA (gamma shape = 1.05) and the Hasegawa, Kishino, and Yano five-parameter model with gamma-distributed rate variation (gamma shape = 0.23) was selected for *ITS-1*. The *ITS-1* dataset contained multiple gaps, so for the analyses I considered gaps to represent a fifth nucleotide state (Campbell and Lydeard, 2012; Inoue et al., 2014).

In order to test the hypothesis that the three species of spinymussels form a monophyletic clade, I conducted uniform and constrained phylogenetic reconstructions using Bayesian Inference analysis by Metropolis-coupled Markov Chain Monte Carlo (MCMC) on the mtDNA and nDNA datasets. For both conditions, I conducted Bayesian analyses 3 times for $1x10^6$ iterations each with sampling every 1000 generations. For each run, general

parameters were kept constant and two independent reconstructions began with random trees and were run using one cold chain and three heated chains (temp=0.2) simultaneously. Split frequencies at the conclusion of each run were <0.01. I assessed burn-in using the program Tracer v1.6 (Rambaut and Drummond, 2013) and considered the iterations as stable once likelihood values became consistent; the first 10% of trees were then discarded as burn-in. The remaining trees were used to construct a 50% majority consensus topology and estimate posterior probabilities. I then compared likelihood estimates for constrained and uniform conditions using AICM model comparisons (500 bootstrap replications) in Tracer v1.6 (Baele et al., 2012).

2.4. Divergence time estimation

To estimate divergence time for spinymussel taxa I used the mtDNA dataset and a molecular clock method implemented in BEAST v1.7 (Drummond et al., 2012). I used an UPGMA starting tree with the GTR+I+G model and empirical base frequencies. A constant-size coalescent model and strict molecular clock were used. To calibrate the clock, I used known *COI* substitution rates of 0.67 to 1.21% per million years obtained from other bivalve groups (Marko, 2002; Inoue et al., 2014). I ran the analysis for $1x10^7$ iterations with sampling every 1000 generations and burn-in was assessed using Tracer v1.6.

3. Results

3.1. Sampling and sequence diversity

I sampled a total of 8 specimens of *E. spinosa*, 20 specimens of *E. steinstansana*, and 81 specimens of *P. collina*. I was unable to generate sequences for some specimens. The final concatenated mtDNA dataset consisted of 8 *E. spinosa*, 15 *E. steinstansana*, and 52 *P. collina*. The *ITS-1* dataset consisted of 5 *E. spinosa*, 11 *E. steinstansana*, and GenBank sequences representing 7 *P. collina* haplotypes.

From the concatenated mtDNA dataset, I obtained 3 haplotypes for *E. spinosa* (k = 2.50, π = 0.00216), 4 haplotypes for *E. steinstansana* (k = 2.19, π = 0.00161), and 3 haplotypes for *P. collina* (k = 0.382, π = 0.00033). For *E. steinstansana*, a single haplotype represented 73% of the sampled population. For *P. collina*, *COI* was fixed across all specimens sampled and a single haplotype (JSM haplotype 1) representing 71% of thesamples was found in all four populations. From the *ITS-1* dataset, I obtained 1 haplotype for *E. spinosa* (k = 0.00, π = 0.00), 4 haplotypes for *E. steinstansana* (k = 1.818, π = 0.00405), and the truncated alignment resulted in 7 *P. collina* GenBank haplotypes. For *E. steinstansana*, one *ITS-1* haplotype represented 72% of the specimens; the remaining haplotypes were singletons with a private haplotype exhibited by the Little River (Neuse drainage) specimen.

At mtDNA loci, genetic distances were high and inter-specific pairwise differences ranged from 0.013 to 0.138 (Table 2). *ITS-1* distances were not congruent with mtDNA and ranged from 0.013 to 0.132 (Table 3).

3.2. Phylogenetic reconstructions

Uniform and constrained phylogenetic analyses of mtDNA returned similar mean log likelihood estimates (-7854.13 and -7863.14, respectively) and AICM scores supported uniform parameters over constrained (15857.64 and 15881.18, respectively). Analysis of *ITS-1* reconstructions under uniform and constrained conditions returned similar mean log likelihood estimates (-1907.87 and -1909.44, respectively) and AICM scores supported uniform conditions over constrained (3987.98 and 4000.07, respectively). mtDNA and nDNA datasets revealed two divergent monophyletic spinymussel clades and incongruent topologies (Figs. 3 and 4). *Elliptio steinstansana* and *P. collina* formed reciprocally monophyletic sister clades highly divergent from other *Elliptio* and *Pleurobema* taxa and are more closely-related to *Fusconaia* on mtDNA loci and *Elliptio* on the *ITS-1* locus. ASM formed a monophyletic clade with a weak affinity to *Elliptio* on mtDNA loci but was more divergent at the *ITS-1* locus. The mtDNA divergence estimations suggested that *E. steinstansana* and *P. collina* diverged from other Pleurobemini in the late-Pliocene or early Miocene, 6.19 mya (95% CI: 4.49-7.21 mya), and appear to have radiated in the mid-Pleistocene, 0.69 mya (95% CI: 0.32-1.07 mya; Fig. 5). Additionally, *E. spinosa* diverged from extant members of the core *Elliptio* group in the late-Pleistocene to mid-Pliocene, 3.76 mya (95% CI: 2.71-4.86 mya), while major speciation events occurred within the *Elliptio* clade during the Pleistocene 1.87 mya (95% CI: 0.43-2.41 mya).

4. Discussion

My results illustrate both the utility and challenges of using multiple mitochondrial and nuclear loci to infer informative evolutionary relationships. Despite incongruent results from the mtDNA and nDNA loci, my study is the first to provide compelling support for the recognition of two unique monophyletic spinymussel clades. Furthermore, my study provides divergence time estimates that demonstrate the spinymussels represent divergent evolutionary lineages. Additionally, my results suggest that the presence of spines represents a convergent morphological characteristic. Because the spinymussels are highly threatened and currently listed as critically endangered, my findings have significant management and taxonomic implications.

4.1. Phylogenetic analyses

Phylogenetic analyses revealed topologies similar to those in previous studies (e.g. Campbell et al., 2005; Campbell and Lydeard, 2012), with representatives of most of the major genera within Pleurobemini (e.g., *Fusconaia*, *Pleurobema*, *Pleuronaia*) forming monophyletic clades. Phylogenetic analyses at mtDNA and nDNA loci did not return congruent topologies, likely as a result of incomplete lineage sorting and/or insufficient taxon sampling. Nonetheless, both datasets provided significant evidence for two monophyletic spinymussel clades composed of A) *E. steinstansana* and *P. collina*, and B) *E. spinosa*.

4.1.1. The James River and Tar River spinymussels

The first and more ancient clade, composed of *E. steinstansana* and sister taxon *P. collina*, is highly divergent from known species of *Pleurobema* and *Elliptio* (Tables 2 and 3) with an estimated divergence time in the late Miocene (Fig 5); almost one million years after the divergence of *Pleurobema* and two million years before the divergence of the core *Elliptio* clade. Earlier studies have shown *E. steinstansana* and *P. collina* grouped with putatively monotypic genera (e.g. *Hemistena lata*, *Elliptio (Eurynaia) dilatata)* likely because of the limited analytical resolution provided by the single specimens used in these studies (e.g. Campbell et al., 2005; Campbell and Lydeard, 2012). Inconsistencies between my mtDNA phylogenies and those of other studies (e.g. Campbell and Lydeard, 2012) can likely be attributed to the increased sample size (n=75) of spinymussels providing greater phylogeographic resolution and thus more resolved topologies. Additionally, the results of the *ITS-1* dataset, while incongruent with those of the mtDNA topologies, place *E. steinstansana* and *P. collina* as sister taxa within a well-resolved monophyletic clade divergent from the *Elliptio* and *Pleurobema* groups (Fig. 4).

My results are consistent with additional morphologic, life-history, and biogeographic evidence that suggest *E. steinstansana* and *P. collina* form a unique group. First, these two species are the only known freshwater mussels (aside from *E. spinosa*) characterized by external spine structures. Second, *E. steinstansana* and *P. collina* exhibit extremely similar breeding characteristics, including a unique leech-like conglutinate for larval dispersal and similar cyprinid host fish (e.g. Eads and Levine, 2009; Johnson and Boss, 1984; Johnson and

Clarke, 1983; Levine et al., 2011). Third, this group has an extremely limited range, occurring in only four adjacent drainage basins in the SEAS (Fig. 2). The results of my analyses together with this additional support offer considerable evidence to suggest that *E. steinstansana* and *P. collina* are extant members of a divergent ancestral lineage and warrant recognition as a unique genus.

4.1.2. The Altamaha spinymussel

Phylogenetic analyses place all *E. spinosa* sequences within a well-supported monophyletic spinymussel clade. The concatenated mtDNA topology suggests the *E. spinosa* clade exhibits some affinity for the major *Elliptio* clade (Fig. 3), however it is possible that *E. spinosa* is not a true member of *Elliptio*. BEAST analysis suggests the core *Elliptio* clade is recent, with origins in a divergence event beginning 1.87 mya (95% CI 1.39 – 2.41 mya) with estimated divergence times occurring well within the Pleistocene (Fig. 5). Additionally, divergence estimates suggest *E. spinosa* diverged from ancestors of the major *Elliptio* group within the Pliocene, an estimated 1.89 million years before the beginning of the *Elliptio* radiation. Interestingly, the *ITS-1* dataset produced an incongruent topology also suggesting that *E. spinosa* is divergent but placing the taxon closer to *Fusconaia*. This is likely due to incomplete lineage sorting within the *ITS-1* topology (e.g., *Pleurobema* and *Elliptio*), and many of the species-level relationships in this dataset show low divergence rates and resolution at this locus. Additionally, incomplete taxon sampling within other clades (e.g. *Elliptio*) may have inhibited accurate assignment of this taxon.

Freshwater mussel species richness within the Altamaha River basin is modest (16 recognized species) however the region is well known for its relatively high proportion of endemics (7 recognized species), four of which (including *E. spinosa*) are putative members of *Elliptio* (Wisniewski et al., 2005). High levels of endemism are often representative of prolonged geographic isolation resulting in divergent evolutionary lineages with narrow geographic ranges. My results suggest an intriguing scenario for highly divergent lineages within Altamaha *Elliptio*. It's possible that a widespread ancestor of all spinymussels occurred in Miocene SEAS drainages and a vicariant event sometime within the early Pliocene isolated this lineage. In this scenario, endemic members of the extant core *Elliptio* group were represented in the prehistoric Altamaha by a different ancestor, likely widespread throughout the SEAS. This group may have been isolated by a separate event (or series of events) in the early-mid Pleistocene and evolved other unique phenotypic characteristics. For example, some members of *Elliptio* endemic to the Altamaha basin share a number of uncommon plesiomorphic traits (e.g. shell microstructure; Kat 1983). My data suggest that at least two vicariant events within the Altamaha region have resulted in the genetically and morphologically unique *E. spinosa* as well as regionally endemic members of the core *Elliptio* group.

4.2. Evidence for convergent spine morphology

In freshwater mussels, external shell morphology is often convergent and, although poorly studied, is believed to be driven by environmental factors such as stream size,

substrate composition, and other hydrological conditions (Haag, 2012). Additionally, many freshwater mussel species exhibit a variety of shell projections (e.g. the presence of corrugations, pustules, or spines) that are likely a function of environmental pressures but whose adaptive function remains speculative. This study is the first to illustrate that the three known species of mussels characterized by spines represent two unique evolutionary lineages. Contrary to my predictions, my analyses suggest that the presence of spines represents a convergent morphological characteristic.

Spines and other shell projections are thought to serve a variety of purposes in freshwater gastropods, most notably as a defense mechanism against snail-eating fish and crustaceans, where shell projections serve as a tactile deterrent or decrease vulnerability to crushing (review in Covich, 2010). In freshwater bivalves, the presence of spines is extremely rare and the adaptive function of this characteristic remains speculative. It is likely that in the case of the spinymussels, a combination of environmental and predation pressures have produced two unique lineages that exhibit similar external morphologies.

The mechanisms that drive the formation of shell layers in freshwater bivalves are relatively well-studied (review in Checa, 2000). The complex process behind spine development is yet to be fully-described but appears to be similar in all three spinymussel species. Briefly, the process of spine formation begins early in the biomineralization of the shell, as the periostracal groove within the mantle secretes two layers of periostracum. In spinymussels, the periostracal groove forms an open “loop” as the periostracum is extruded from the shell, resulting in open folds that eventually fuse to form spines as the animal ages

but remain hollow throughout the animal's life (R. Hoch NCWRC, pers. comm. 2014, Fig. 6). Furthermore, this process appears to be conserved among both clades of spinymussels, as well some diverse marine bivalve groups (e.g. *Pitar spp., Arcinella spp.;* pers. obs. 2014), therefore the evolutionary mechanism driving spine formation in these groups is likely not unique. Rather, spines are likely a unique response of these freshwater mussel species to environmental and predatory pressures.

Shell ornamentation in bivalves has been shown to reduce substrate scouring around the shell and increase stability in shifting substrates such as sand and cobble (e.g. Watters, 1992). A combination of mitigated scouring and increased anchoring could effectively limit the exposure of the animal during high stream flows by reducing the amount of scour (i.e., maintaining sediment composition around the body) as well as anchoring the organism within the stable area (i.e. maintaining depth within the sediment). Additionally, shell projections may be a form of defense exhibited by spinymussels. Predation of freshwater mussels by stream-dwelling mammals (e.g. muskrats, *Ondatra zibethicus*) is well-documented (Neves and Odom, 1989), and Atlantic sturgeon (*Acipenser oxyrhynchus oxyrhyncus*), a known predator of other mollusks, are documented from the mainstem reaches of the spinymussel's range; however sturgeon populations have drastically declined in past decades (Pikitch et al., 2005) and these predation pressures may no longer be present.

4.3. Management implications and recommendations

My results describe previously unknown evolutionary relationships and suggest that taxonomic revision is necessary for the spinymussels. The mtDNA and *ITS-1* phylogenetic reconstructions suggest that *E. steinstansana* and *P. collina* form a monophyletic clade and that placement of these taxa within *Elliptio* or *Pleurobema* is inaccurate. These findings illustrate that the clade comprised of *E. steinstansana* and *P. collina* is more evolutionarily distinct than previously recognized and likely warrants recognition as a unique genus. I propose the name *Parvaspina* (small-spined) for this genus. This proposed taxonomic revision should have no effect on the conservation status of these taxa as both are critically endangered. Additionally, sequence analyses suggest that a major genetic bottleneck has depleted diversity across the range of both *E. steinstansana* and *P. collina*, however this is expected given the isolation and limited range of both taxa. Calculations of genetic distance revealed very low divergence (0.013) between *E. steinstansana* and *P. collina* for both mtDNA (Table 2) and nuclear (Table 3) loci. Additionally, divergence time estimates for these species are fairly recent (0.69 mya, 95% CI: 0.32-1.07 mya; Fig. 5). These figures are more consistent with intraspecific (i.e. population) rather than interspecific divergence estimates (see Stoekle and Thaler, 2014) and suggest that, at the broad resolution provided by this data, *E. steinstansana* and *P. collina* are extremely closely related. In order to assess the population structure and establish accurate boundaries for these species, more informative higher resolution genomic data is recommended.

These results also suggest that *E. spinosa* forms a divergent monophyletic clade and is likely not a true member of the core *Elliptio* group. I suggest the name *Canthyria* previously proposed by Swainson (1840) for this unionid group. Again, this proposed taxonomic revision should have no effect on the conservation status of this taxon as it is currently critically endangered.

5. Conclusions

Numerous taxa exhibit plastic, unique, or subtle morphologic characteristics that inhibit accurate classification. The ability of researchers to correctly classify and describe the evolutionary relationships among threatened species is vital to their conservation. This study is the first to illustrate that the critically endangered freshwater spinymussels comprise two unique evolutionary lineages. Furthermore, these lineages are endemic to the SEAS and highly divergent from currently recognized freshwater mussel genera. The results of this study lay the foundation for more refined and focused conservation activities for these highly-imperiled and unique species.

References

Abell, R., Thieme, M., Revenga, C., Bryer, M., Kottelat, M., Bogutskaya, N., Coad, B., Mandrak, N., Balderas, S.C., Bussing, W., Stiassny, M., Skelton, P., Allen, G., Unmack, P., Naseka, A., Ng, R., Sindorf, N., Robertson, J., Armijo, E., Higgins, J., Heibel, T., Wikramanayake, E., Olson, D., Lopez, H., Reis, R., Lundberg, J., Perez, M.S., Petry, P., 2008. Freshwater ecoregions of the world: a new map of biogeographic units for freshwater biodiversity conservation. BioScience 58, 403–414.

Appleton, R., Palmer, A., 1988. Water-borne stimuli released by predatory crabs and damaged prey induce more preator-resistant shells in a marine gastropod. Proceedings of the National Academy of Sciences of the United States of America 85, 4387–4391.

Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M.A., Alekseyenko, A. V., 2012. Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. Molecular Biology and Evolution 29, 2157–67. doi:10.1093/molbev/mss084

Barnosky, A.D., Matzke, N., Tomiya, S., Wogan, G.O.U., Swartz, B., Quental, T.B., Marshall, C., McGuire, J.L., Lindsey, E.L., Maguire, K.C., Mersey, B., Ferrer, E.A., 2011. Has the Earth's sixth mass extinction already arrived? Nature 471, 51–7. doi:10.1038/nature09678

Bogan, A., 2002. Workbook and key to the freshwater bivalves of North Carolina. North Carolina Museum of Natural Sciences, 1-105.

Bogan, A.E., 2007. Global diversity of freshwater mussels (Mollusca, Bivalvia) in freshwater. Hydrobiologia 595, 139–147. doi:10.1007/s10750-007-9011-7

Bogan, A., Serb, J., Lydeard, C., 2003. Determination of the systematic position and relationships of the *Elliptio* complex (Mollusca: Bivalvia: Unionidae) with distributions in North and South Carolina. A final report submitted to USGS (Contract 01HQAG0185).

Boss, K.J., Clench, W.J., 1967. Notes on *Pleurobema collina* (Conrad) from the James River, Virginia. Occasional Papers on Mollusks 3, 45–52.

Bottjer, D., Carter, J., 1980. Functional and Phylogenetic Significance of Projecting Periostracal Structures in the Bivalvia (Mollusca). Journal of Paleontology 54, 200–216.

Bourdeau, P.E., 2010. An inducible morphological defense is a passive by-product of behaviour in a marine snail. Proceedings of the Royal Society B 277, 455–62. doi:10.1098/rspb.2009.1295

Buhay, J.E., 2009. "COI-like" sequences are becoming problematic in molecular systematic and DNA barcoding studies. Journal of Crustacean Biology 29, 96–110. doi:10.1651/08-3020.1

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

Campbell, D.C., Johnson, P.D., Williams, J.D., Rindsberg, A.K., Serb, J.M., Small, K.K., Lydeard, C., 2008. Identification of "extinct" freshwater mussel species using DNA barcoding. Molecular Ecology Resources 8, 711–724. doi:10.1111/j.1755-0998.2008.02108.x

Campbell, D.C., Serb, J.M., Buhay, J.E., Roe, K.J., Minton, R.L., Lydeard, C., 2005. Phylogeny of North American amblemines (Bivalvia, Unionoida): prodigious polyphyly proves pervasive across genera. Invertebrate Biology 124, 131–164. doi:10.1111/j.1744-7410.2005.00015.x

Checa, A., 2000. A new model for periostracum and shell formation in Unionidae (Bivalvia, Mollusca). Tissue and Cell 32, 405–16. doi:10.1054/tice.2000.0129

Covich, A.P., 2010. Winning the biodiversity arms race among freshwater gastropods: competition and coexistence through shell variability and predator avoidance. Hydrobiologia 653, 191–215. doi:10.1007/s10750-010-0354-0

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics, and parallel computing. Nature Methods 9, 772.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular Biology and Evolution 29 1969-1973.

Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.-I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.-H., Soto, D., Stiassny, M.L.J., Sullivan, C.A., 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biological Reviews 81, 163–82. doi:10.1017/S1464793105006950

Eads, C., Levine, J., 2009. Propagation and culture of the Carolina heelsplitter (*Lasmigona decorata*) and Tar River spinymussel (*Elliptio steinstansana*).

Fleming, W.J., Augspurger, T.P., Alderman, J.A., 1995. Freshwater mussel die-off attributed to anticholinesterase poisoning. Environmental Toxicology 14, 877-879.

Haag, W.R., 2009. A hierarchical classification of freshwater mussel diversity in North America. Journal of Biogeography 37, 12–26. doi:10.1111/j.1365-2699.2009.02191.x

Haag, W.R., 2012. North American freshwater mussels: natural history, ecology, and conservation. Cambridge University Press, New York, NY.

Haag, W.R., Williams, J.D., 2014. Biodiversity on the brink: an assessment of conservation strategies for North American freshwater mussels. Hydrobiologia 735, 45–60. doi:10.1007/s10750-013-1524-7

Hebert, P.D.N., Ratnasingham, S., DeWaard, J.R., 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society B 270, 96–99. doi:10.1098/rsbl.2003.0025

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755.

Hove, M.C., Neves, R.J., 1994. Life history of the endangered James spinymussel *Pleurobema collina* (Conrad, 1837) (Mollusca: Unionidae). American Malacological Bulletin 11, 29–40.

Humphries, P., Winemiller, K.O., 2009. Historical impacts on river fauna, shifting baselines, and challenges for restoration. BioScience 59, 673–684. doi:10.1525/bio.2009.59.8.9

Inoue, K., Mcqueen, A.L., Harris, J.L., Berg, D.J., 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.

Johnson, J.A., Wisniewski, J.M., Fritts, A.K., Bringolf, R.B., 2012. Host identification and glochidia morphology of freshwater mussels from the Altamaha River basin. Southeastern Naturalist 11, 733–746.

Johnson, R., Boss, K.J., 1984. *Fusconaia collina* (Conrad), from the James River, Virginia, an additional note. Occasional Papers on Mollusks 5, 319–320.

Johnson, R.I., Clark, A.H., 1983. A new spiny mussel, *Elliptio (Canthyria) steinstansana* (Bivalvia: Unionidae), from the Tar River, North Carolina. Occasional Papers on Mollusks 4, 289–298.

Jones, J.W., Neves, R.J., Ahlstedt, S.A., Hallerman, E.M., 2006. A holistic approach to taxonomic evaluation of two closely related endangered freshwater mussel species, the oyster mussel Epioblasma capsaeformis and tan riffleshell *Epioblasma florentina walkeri* (Bivalvia: Unionidae). Journal of Molluscan Studies 72, 267–283. doi:10.1093/mollus/eyl004

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

Levine, J., Eads, C., Greiner, R., Bogan, A., 2011. Propogation and culture of federally listed freshwater mussel species. NCDOT Report: HWY-2009-16.

Librado, P., and Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451-1452.

Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J.P., Hector, A., Hooper, D.U., Huston, M., Raffaelli, D., Schmid, B., Tilman, D., Wardle, D., 2001. Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294, 804–8. doi:10.1126/science.1064088

Lydeard, C., Cowie, R.H., Ponder, W.F., Bogan, A.E., Bouchet, P., Clark, S. a., Cummings, K.S., Frest, T.J., Gargominy, O., Herbert, D.G., Hershler, R., Perez, K.E., Roth, B., Seddon, M., Strong, E.E., Thompson, F.G., 2004. The global decline of nonmarine mollusks. BioScience 54, 321. doi:10.1641/0006-3568(2004)054[0321:TGDONM]2.0.CO;2

Mackie, G.L., Flippance, L.A., 1983. Relationships between buffering capacity of water and the size and calcium content of freshwater mollusks: relationships between buffering capacity of water and the size and calcium content of freshwater mollusks. Freshwater Invertebrate Biology 2, 48–55.

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.

McCormick, M.A., 2012. Effects of small dams on freshwater bivalve assemblages in North Carolina piedmont and coastal plain streams. Thesis. Appalachian State University, Boone, NC.

Neves, R.J., Odom, M.C., 1989. Muskrat predation on endangered freshwater mussels in Virginia. The Journal of Wildlife Management 53, 934-941.

Neves, R.J., Bogan, A.E., Williams, J.D., Ahlstedt, S.A., Hartfield, P.W., 1997. Status of aquatic mollusks in the Southeastern United States: A downward spiral of diversity, in: Benz, G.W., Collins, D.E. (Eds.), Aquatic Fauna in Peril: The Southeastern Perspective. Southeast Aquatic Research Institute, Decatur, GA, pp. 43–85.

Palmer, A., 1992. Calcification in marine molluscs: how costly is it? Proceedings of the National Academy of Sciences of the United States of America 89, 1379–82.

Pereira, H.M., Leadley, P.W., Proença, V., Alkemade, R., Scharlemann, J.P.W., Fernandez-Manjarrés, J.F., Araújo, M.B., Balvanera, P., Biggs, R., Cheung, W.W.L., Chini, L., Cooper, H.D., Gilman, E.L., Guénette, S., Hurtt, G.C., Huntington, H.P., Mace, G.M., Oberdorff, T., Revenga, C., Rodrigues, P., Scholes, R.J., Sumaila, U.R., Walpole, M., 2010. Scenarios for global biodiversity in the 21st century. Science 330, 1496–501. doi:10.1126/science.1196624

Petty, M.A., 2005. Distribution, Genetic Characterization, and Life History of the James spinymussel, *Pleurobema collina* (Bivalvia: Unionidae), in Virginia and North Carolina. Thesis. Virginia Polytechnic Institute and State University, Blacksburg, VA.

Pikitch, E.K., Doukakis, P., Lauck, L., Chakrabarty, P., Erickson, D.L., 2005. Status , trends and management of sturgeon and paddlefish fisheries. Fish and Fisheries 6, 233–265.

Rambaut, A., Drummond, A.J., 2013. Tracer v1.6. <http://beast.bio.ed.ac.uk/tracer>

Rapport, D.J., Costanza, R., McMichael, J., 1998. Assessing ecosystem health. Trends in Ecology and Evolution 13, 397–402.

Régnier, C., Fontaine, B., Bouchet, P., 2009. Not knowing, not recording, not listing: numerous unnoticed mollusk extinctions. Conservation Biology 23, 1214–21. doi:10.1111/j.1523-1739.2009.01245.x

Sala, O.E., 2000. Global Biodiversity Scenarios for the Year 2100 . Science 287, 1770–1774. doi:10.1126/science.287.5459.1770

Serb, J.M., Buhay, J.E., Lydeard, C., 2003. Molecular systematics of the North American freshwater bivalve genus *Quadrula* (Unionidae: Ambleminae) based on mitochondrial ND1 sequences. Molecular Phylogenetics and Evolution 28, 1–11. doi:10.1016/S1055-7903(03)00026-5

Smith, V.H., 2003. Eutrophication of freshwater and coastal marine ecosystems: a global problem. Environmental Science and Pollution Research International 10, 126–39.

Stanley, S.M., Paleobiology, S., Summer, N., 1981. Infaunal survival: alternative functions of shell ornamentation in the Bivalvia (Mollusca). Paleobiology 7, 384–393.

Stoeckle, M.Y., Thaler, D.S., 2014. DNA barcoding works in practice but not in (neutral) theory. PLoS ONE 9, e100755. doi:10.1371/journal.pone.0100755

Strayer, D.L., Downing, J.A., Haag, W.R., King, T.L., Layzer, J.B., Newton, T.J., Nichols, S.J., 2004. Changing perspectives on pearly mussels, North America's most imperiled animals. BioScience 54, 429. doi:10.1641/0006-3568(2004)054[0429:CPOPMN]2.0.CO;2

Strayer, D.L., Dudgeon, D., 2010. Freshwater biodiversity conservation: recent progress and future challenges. Journal of the North American Benthological Society 29, 344–358. doi:10.1899/08-171.1

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30, 2725-2729.

Taylor, H.R., Harris, W.E., 2012. An emergent science on the brink of irrelevance: a review of the past 8 years of DNA barcoding. Molecular Ecology Resources 12, 377–88. doi:10.1111/j.1755-0998.2012.03119.x

U.S. Fish and Wildlife Service, 1985. Endangered and threatened wildlife and plants; final rule listing the Tar River spiny mussel (*Elliptio* (*Canthyria*) *steinstansana*) as an endangered species. Federal Register 50(124): 26572-26575

U.S. Fish and Wildlife Service, 1988. Endangered and threatened wildlife and plants; determination of endangered status for the James spinymussel. Federal Register 53(141): 27689-27693

U.S. Fish and Wildlife Service, 2008. James spinymussel (*Pleurobema collina*) 5-year review: summary and evaluation. Prepared by USFWS Virginia Filed Office, Gloucester, VA.

U.S. Fish and Wildlife Service, 2009. Tar River spinymussel recovery action plan. Asheville, NC. <http://ecos.fws.gov/docs/action_plans/doc3109.pdf>

U.S. Fish and Wildlife Service, 2011. Endangered status for the Altamaha spinymussel and designation of critical habitat; final rule. <http://www.gpo.gov/fdsys/pkg/FR-2011-10-11/pdf/2011-25539.pdf>

U.S. Fish and Wildlife Service, 2014. Tar River spinymussel (*Elliptio steinstansana*) 5-year review: summary and evaluation. Prepared by USFWS, Southeast Region, Raleigh Ecological Services Field Office. Raleigh, NC

Valentini, A., Pompanon, F., Taberlet, P., 2009. DNA barcoding for ecologists. Trends in Ecology and Evolution 24, 110–7. doi:10.1016/j.tree.2008.09.011

Watters, G.T., 1994. Form and function of unionoidean shell sculpture and shape (Bivalvia). American Malacological Bulletin 11, 1-20.

Williams, J.D., Bogan, A.E., Garner, J.T., 2008. Freshwater mussels of Alabama and the Mobile basin in Georgia, Mississippi, and Tennessee. University of Alabama Press. Tuscaloosa, AL.

Vermeij, G.J., 1977. The Mesozoic marine revolution: evidence from snails, predators and grazers. Paleobiology 3, 245–258.

Wisniewski, J.M., Krakow, G., Albanese, B., 2005. Current Status of endemic mussels in the lower Ocmulgee and Altamaha Rivers. Proceedings of the 2005 Georgia Water Resources Conference. Athens, GA.

Table 1. Specimen collection and locality information.

Species	n	Waterbody	Basin	State	County
Elliptio spinosa	8	Altamaha River	Altamaha	GA	McIntosh
Elliptio steinstansana	14	Fishing Creek	Tar	NC	Halifax
Elliptio steinstansana	1	Little River	Neuse	NC	Johnston
Pleurobema collina	19	Dan River	Roanoke	NC	Stokes
Pleurobema collina	10	South Fork Mayo River	Roanoke	VA	Patrick
Pleurobema collina	19	South Fork Pott's Creek	James	VA	Albemarle
Pleurobema collina	4	Ward's Creek	James	WV	Monroe

Table 2. List of specimens and corresponding information used in this study (species, Genbank accession number for loci used, and references). ** denotes chimeric sequences (i.e. sequences represent more than one vouchered specimen).

Species	COI	ND1	ITS-1	Reference
*Amblema plicata***	EF033258	AY158796	-	Chapman et al. (2008), Serb et al. (2003)
*Elliptio arca***	AY654995	AY655093	DQ383437	Campbell et al. (2005), Campbell et al. (2008)
Elliptio arctata	DQ383427	JF326440	DQ383438	Campbell et al. (2008), Campbell and Lydeard (2012)
Elliptio complanata	EU448179	EU448218	-	Gangloff et al. unpubl. data AUM9757c
*Elliptio congaraea***	HQ153542	EU448226	-	Sommer et al. (2011), Gangloff et al. unpubl. data AUM9763
Elliptio crassidens	DQ383428	AY613788	DQ383439	Campbell et al. (2008), Campbell et al. (2005)
Elliptio folliculata	EU448189	EU448231	-	Gangloff et al. unpubl. data AUM9749
*Elliptio hopetonensis***	HQ828811	EU448232	-	Small et al. (2012), Gangloff et al. unpubl. data AUM9404
Elliptio icterina	EU448191	EU448236	-	Gangloff et al. unpubl. data AUM9861a
Elliptio jayensis	*pending*	*pending*	-	Gangloff et al. unpubl. Data
Elliptio nasutilus	EU448201	EU448250	-	Gangloff et al. unpubl. data AUM9745b
Elliptio producta	HQ153567	HQ153654	-	Sommer et al. (2011)
Elliptio pullata	EU377570	EU380666	-	Gangloff et al. unpubl. data
Elliptio roanokensis	*pending*	*pending*	-	Gangloff et al. unpubl. data
*Elliptio (Eurynaia) dilatata***	EU448188	AY613789	DQ383440	Gangloff et al. unpubl. data, Campbell et al. (2005)
Fusconaia cerina	-	-	DQ383441	Campbell et a. (2008)
Fusconaia cor	AY654997	AY655096	-	Campbell et al. (2005)
Fusconaia escambia	-	-	HM230350	Campbell and Lydeard (2012)
*Fusconaia flava***	HM230370	AY613793	DQ383442	Campbell and Lydeard (2012), Campbell et al. (2005)
Fusconaia masoni	HM230371	HM230415	-	Campbell and Lydeard (2012)

Fusconaia subrotunda	AY613824	AY613794	-	Campbell et al. (2005)
Hemistena lata	AY613825	AY613796	DQ383443	Campbell et al. (2005), Campbell et al. (2008)
Plectomerus dombeyanus	AY655011	AY655110	DQ383444	Campbell et al. (2005), Campbell et al. (2008)
Pleurobema beadlianum	DQ383429	DQ385873	DQ383447	Campbell et al. (2005), Campbell et al. (2008)
Pleurobema clava	AY655013	AY613802	DQ383449	Campbell et al. (2005), Campbell et al. (2008)
*Pleurobe ma decisum***	AY613832	AY655112	DQ383454	Campbell et al. (2005), Campbell et al. (2008)
Pleurobema furvum	AY613833	AY613806	-	Campbell et al. (2005)
Pleurobema georgianum	AY613834	AY613807	DQ383457	Campbell et al. (2005), Campbell et al. (2008)
Pleurobema hanleyanium	AY655016	AY655115	DQ470003	Campbell et al. (2005), Campbell et al. (2008)
*Pleurobema oviforme***	AY655017	AY613810	DQ470004	Campbell et al. (2005), Campbell et al. (2008)
Pleurobema perovatum	AY613838	AY613811	-	Campbell et al. (2005)
Pleurobema pyriforme	AY613839	AY613812	DQ383461	Campbell et al. (2005), Campbell et al. (2008)
Pleurobema strodeanum	AY613839	AY613817	-	Campbell et al. (2005)
Pleurobema rubellum	-	-	DQ383462	Campbell et al. (2008)
Pleurobema rubrum	-	-	DQ470005	Campbell et al. (2008)
Pleurobema sintoxia	-	-	DQ470006	Campbell et al. (2008)
*Pleuronaia barnesiana***	AY613822	HM230418	-	Campbell et al. (2005), Campbell and Lydeard (2012)
*Pleuronaia dolabelloides***	AY613827	AY613798	AY772175	Campbell et al. (2005)
*Pleuronaia gibberum***	DQ383432	AY613808	DQ383458	Campbell et al. (2008), Campbell et al. (2005)
Uniomerus declivus	-	-	DQ383435	Campbell et al. (2008)

Table 3. Pairwise genetic distances for major clades from concatenated mtDNA data using the maximum composite likelihood (MCL) method. All groups show a mean (μ) distance >0.10 (**bold** values) except for sister taxa *P. collina* and *E. steinstansana,* denoted by *.

	1	*2*	*3*	*4*	*5*	*6*	*7*
1. Elliptio	-						
2. Fusconaia	0.115	-					
3. Pleuronaia	0.116	0.108	-				
4. Pleurobema	0.114	0.095	0.112	-			
5. E. spinosa	0.094	0.127	0.136	0.135	-		
6. P. collina	0.121	0.108	0.134	0.122	0.136	-	
7. E. steinstansana	0.120	0.110	0.134	0.121	0.138	0.013	-
μ	**0.113**	**0.110**	**0.129**	**0.126**	**0.137**	0.013*	

Table 4. Pairwise genetic distances for major groups from *ITS-1* data using the maximum composite likelihood (MCL) method. All groups show a mean (μ) distance >0.06 (**bold** values) except for sister taxa *P. collina* and *E. steinstansana,* denoted by *.

	1	*2*	*3*	*4*	*5*	*6*	*7*
1. Elliptio	-						
2. Fusconaia	0.053	-					
3. Pleuronaia	0.063	0.026	-				
4. Pleurobema	0.036	0.022	0.039	-			
5. E. spinosa	0.060	0.058	0.058	0.047	-		
6. P. collina	0.076	0.129	0.087	0.067	0.129	-	
7. E. steinstansana	0.088	0.132	0.085	0.073	0.132	0.013	-
μ	**0.063**	**0.073**	**0.067**	**0.062**	**0.131**	0.013*	

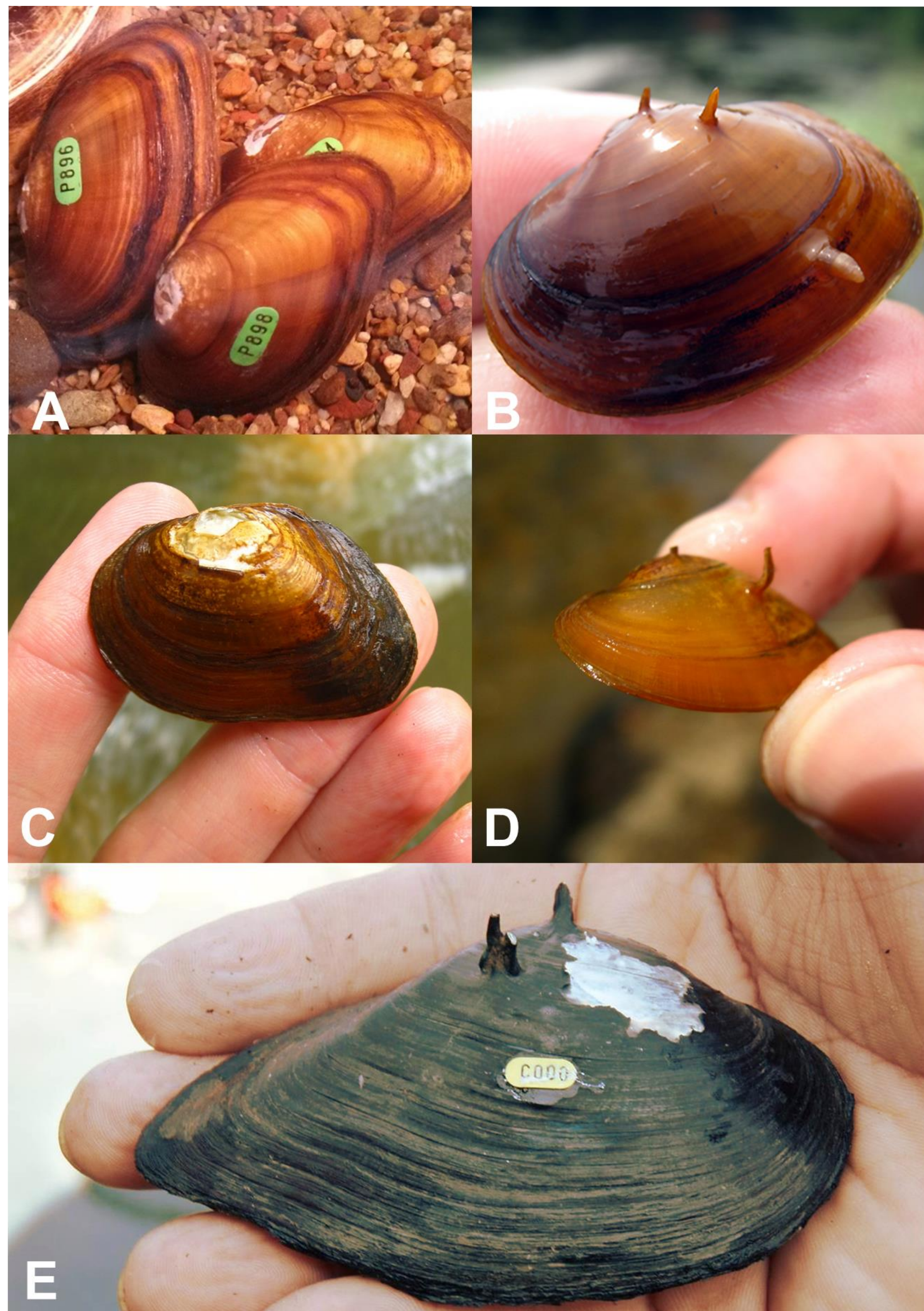

Figure 1. Focal species of this study. A) Adult broodstock *E. steinstansana*; note the absence of conspicuous lateral spines. B) Juvenile propagated *E. steinstansana*, about 1 year old. C) Adult wild *P. collina* from the South Fork Mayo River (SFMR), note that spines have been eroded in this individual. D) Juvenile *P. collina* from SFMR. E) Adult *E. spinosa* from the Altamaha River near Darien, GA. Note the presence of broken spine and. *Photo credits: A and E; authors. B, C, and D; Chris Eads.*

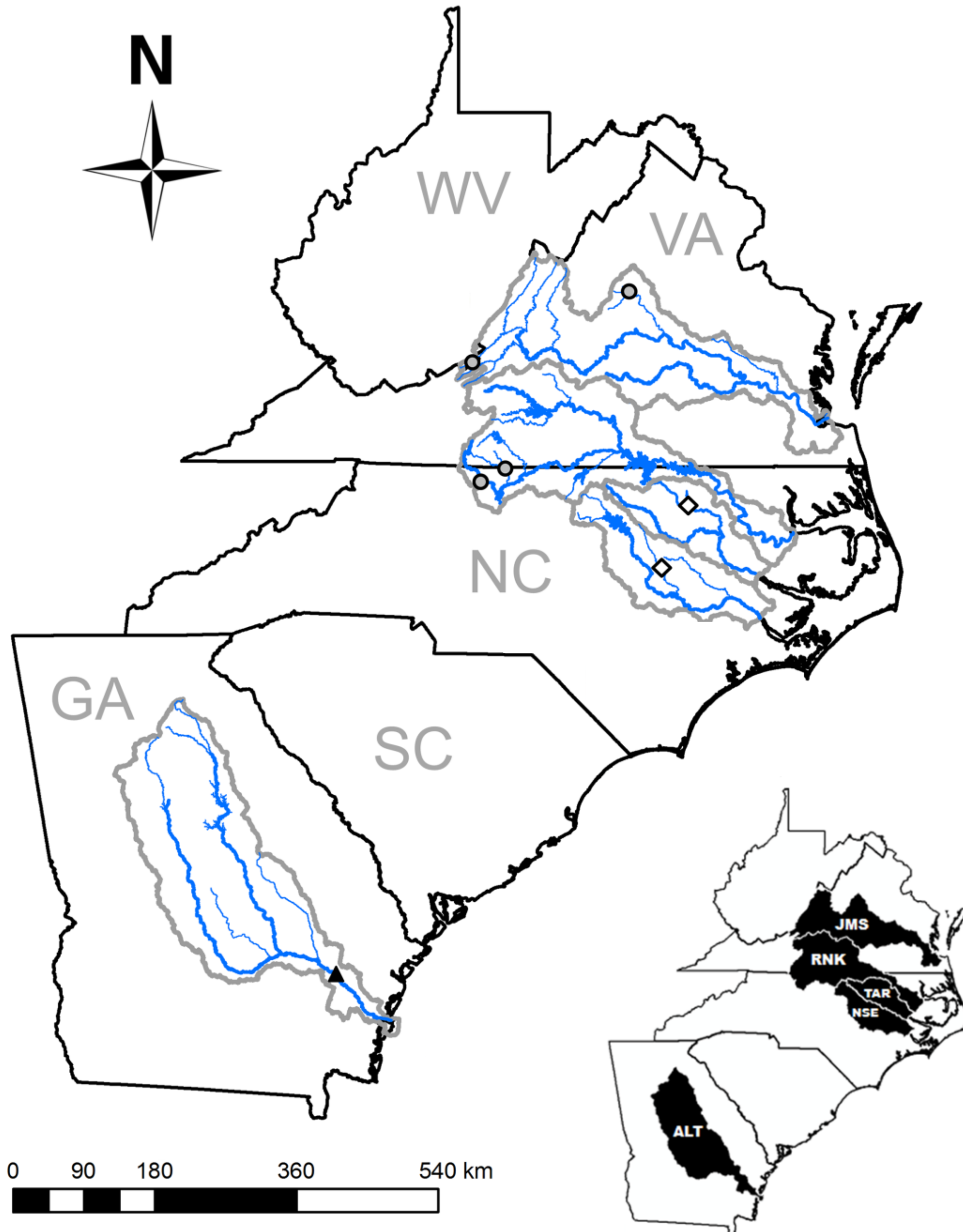

Figure 2. Collection sites in the Southeastern Unites States. Inset, river basins sampled: James (JMS), Roanoke (RNK), Tar (TAR), Neuse (NSE), and Altamaha (ALT) rivers. Shaded circles indicate collection localities for *P.collina,* open diamonds indicate *E. steinstansana,* and the closed triangle represents *E. spinosa.*

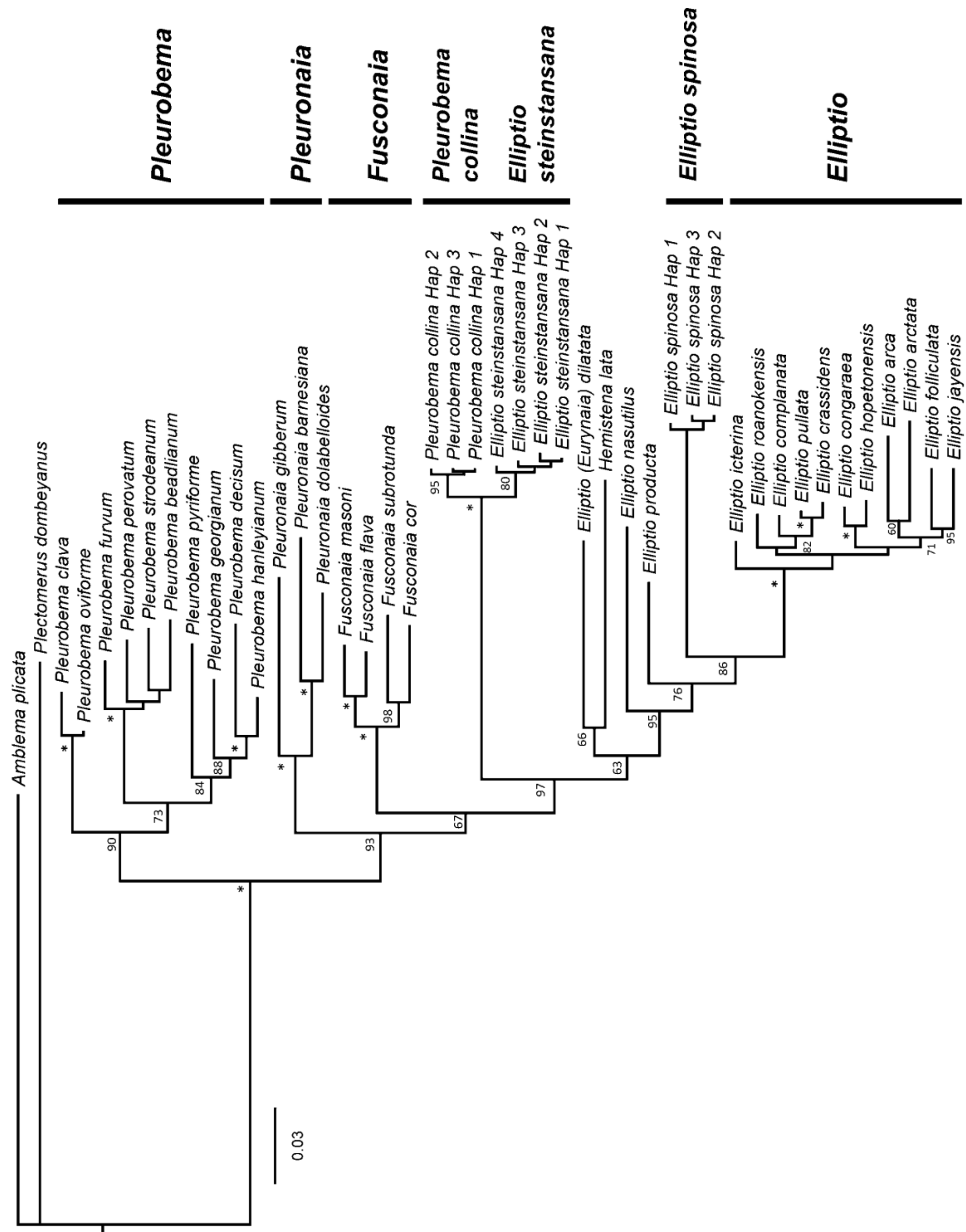

Figure 3. Phylogenetic tree from Bayesian analysis of mtDNA. Node labels indicate posterior probabilities >50%. An * indicates posterior probability >99%. Scale bar represents the number of nucleotide substitutions per site.

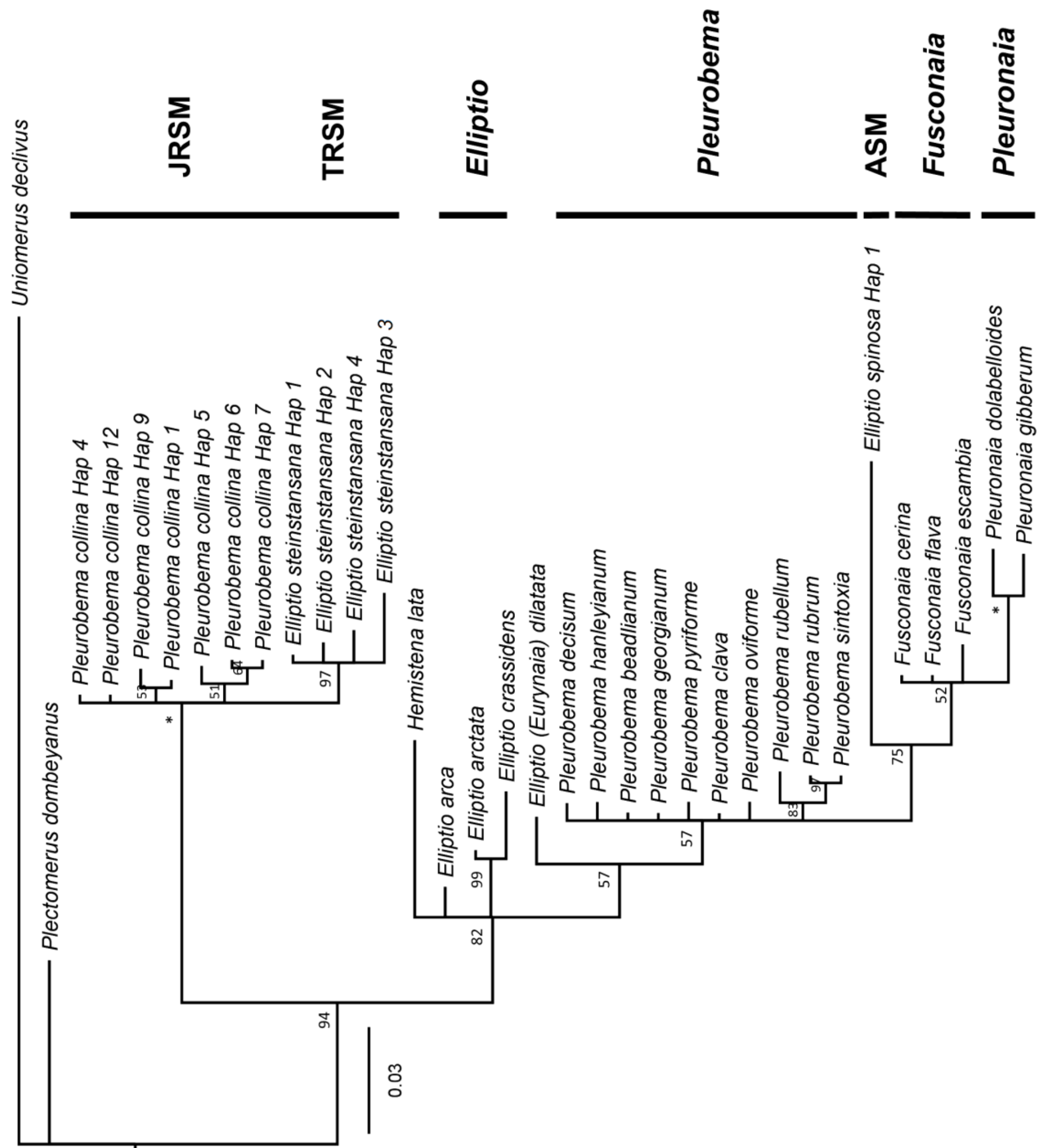

Figure 4. Phylogenetic tree from Bayesian analysis of *ITS-1* data. Node labels indicate posterior probabilities >50%. An * indicates posterior probability >99%. Scale bar represents the number of nucleotide substitutions per site.

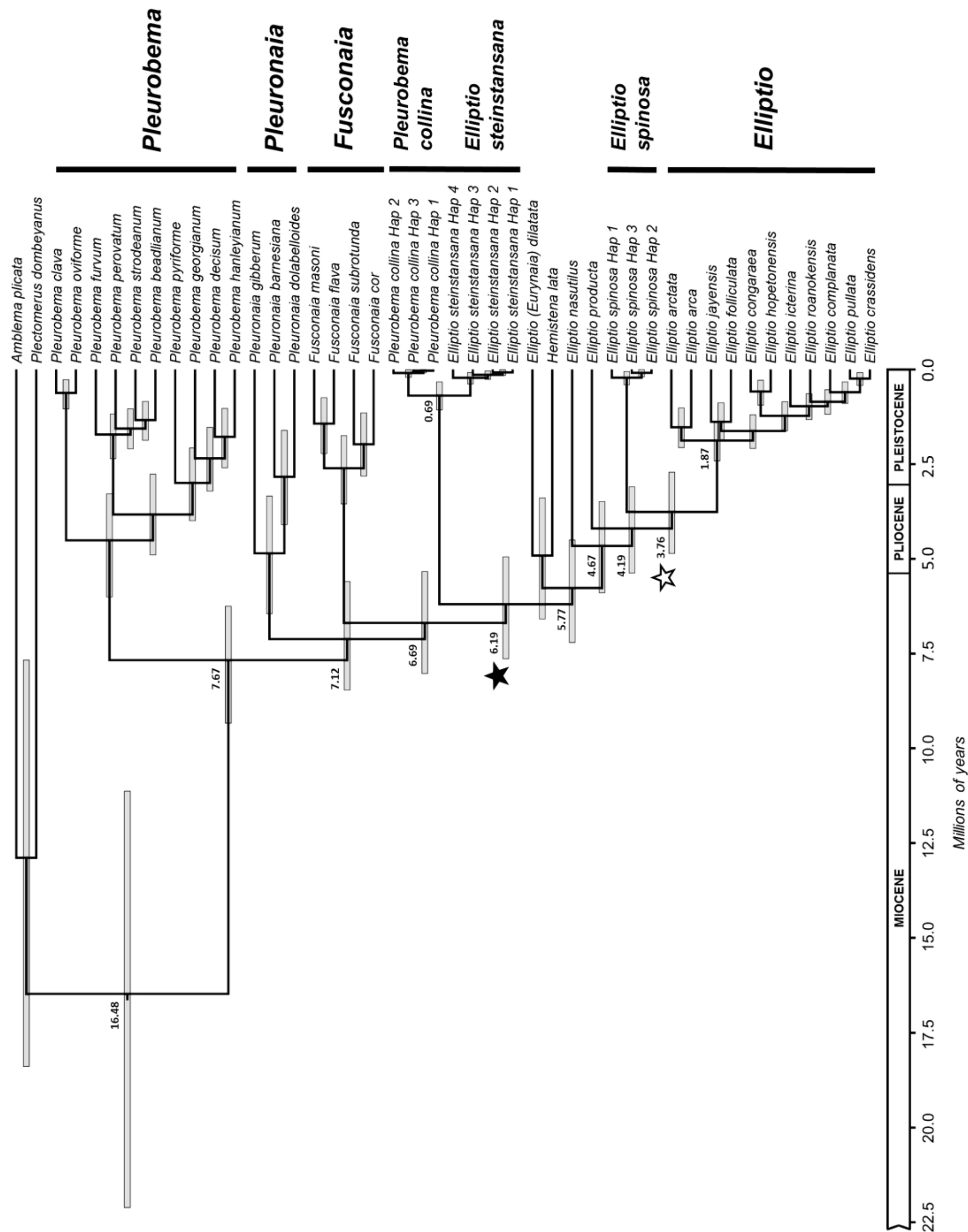

Figure 5. Maximum credibility tree from BEAST analysis of mtDNA. Time scale is in millions of years before present. Node labels represent estimated divergence time, shaded node bars represent 95% highest posterior distributions for divergences (only ancestral nodes and divergence of *P. collina* from *E. steinstansana* are labeled.). Black star indicates estimated divergence of the *P. collina* and *E. steinstansana* clade*,* open star indicates estimated divergence of *E. spinosa* clade.

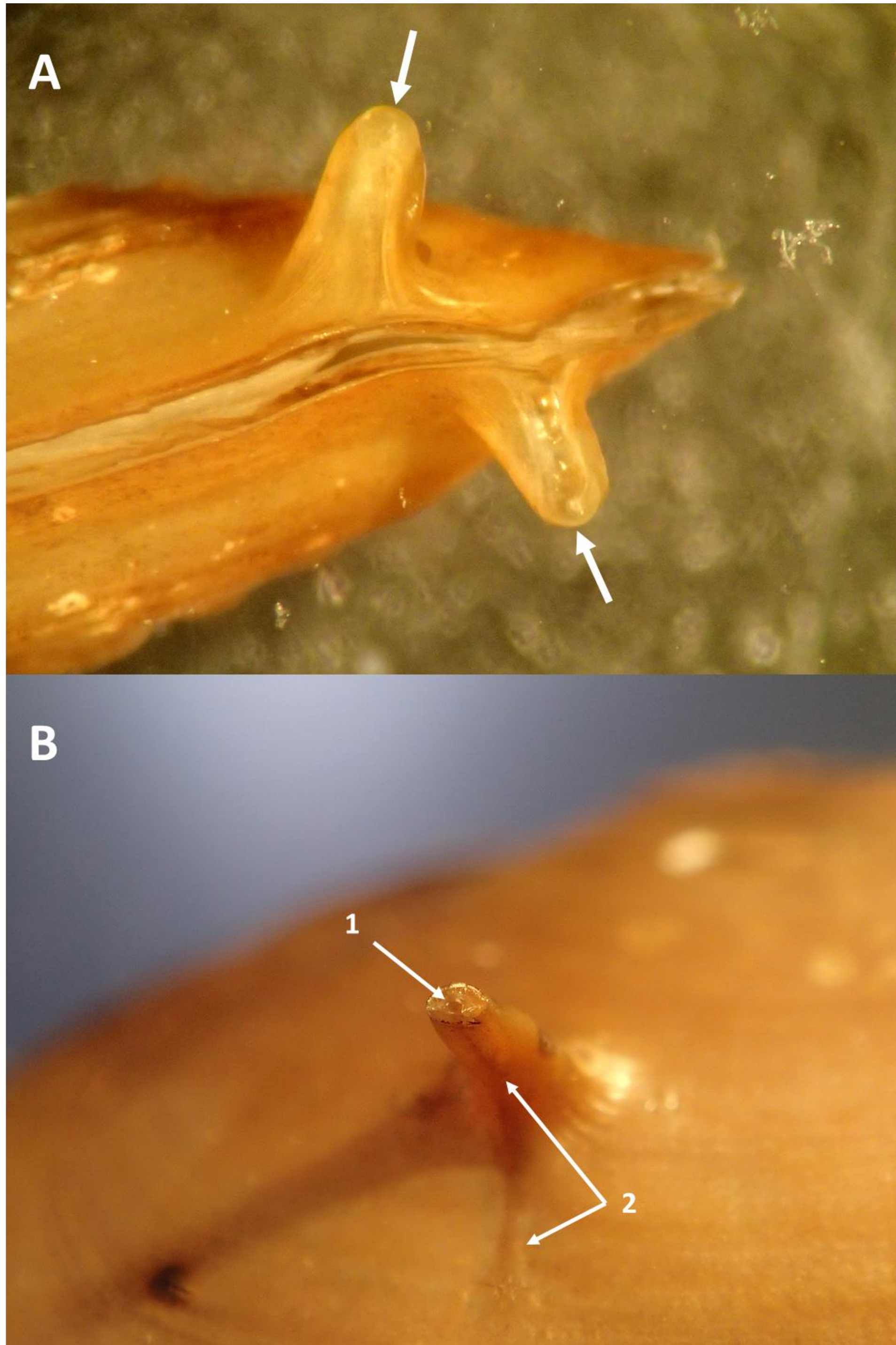

Figure 6. Development and characteristics of spines in *E. steinstansana.* A) Spine development in juvenile *E. steinstansana*, arrows indicate areas of periostracal folding that will later become fused. B) Cross sectioned spine on adult *E. steinstansana* illustrating 1) hollow central area and 2) margin of fused periostracum. *Photo credit: Rachael Hoch.*

Vita

Michael A. Perkins was born in Columbia, SC to Carol Wise and Christopher Perkins in July 1985. He has played in streams since he was a baby. He received a Bachelor of Science degree from Appalachian State University in August 2012. After completion of this here Master's degree, Michael intends to begin employment with the United States Geological Survey in Gainesville, FL.