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Molecular and morphological data reveal non-monophyly and speciation in imperiled freshwater mussels (*Anodontoides* and *Strophitus*)

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ABSTRACT

Accurate taxonomic placement is vital to conservation efforts considering many intrinsic biological characteristics of understudied species are inferred from closely related taxa. The rayed creekshell, *Anodontoides radiatus* (Conrad, 1834), exists in the Gulf of Mexico drainages from western Florida to Louisiana and has been petitioned for listing under the Endangered Species Act. We set out to resolve the evolutionary history of *A. radiatus*, primarily generic placement and species boundaries, using phylogenetic, morphometric, and geographic information. Our molecular matrix contained 3 loci: *cytochrome c oxidase subunit I, NADH dehydrogenase subunit I*, and the nuclear-encoded ribosomal *internal transcribed spacer I*. We employed maximum likelihood and Bayesian inference to estimate a phylogeny and test the monophyly of *Anodontoides* and *Strophitus*. We implemented two coalescent-based species delimitation models to test seven species models and evaluate species boundaries within *A. radiatus*. Concomitant to molecular data, we also employed linear morphometrics and geographic information to further evaluate species boundaries. Molecular and morphological evidence supports the inclusion of *A. radiatus* in the genus *Strophitus*, and we resurrect the binomial *Strophitus radiatus* to reflect their shared common ancestry. We also found strong support for polyphyly in *Strophitus* and advocate the resurrection of the genus *Pseudodontoideus* to represent *'Strophitus' connasaugaensis* and *'Strophitus' subvexus*. *Strophitus radiatus* exists in six well-supported clades that were distinguished as evolutionary independent lineages using Bayesian inference, maximum likelihood, and coalescent-based species delimitation models. Our integrative approach found evidence for as many as 4 evolutionary divergent clades within *S. radiatus*. Therefore, we formally describe two new species from the *S. radiatus* species complex (*Strophitus williamsi* and *Strophitus pascagoulaensis)* and recognize the potential for a third putative species (*Strophitus* sp. cf. *pascagoulaensis*). Our findings aid stakeholders in establishing conservation and management strategies for the members of *Anodontoides*, *Strophitus*, and *Pseudodontoideus*.

1. Introduction

Accurate taxonomic placement of rare and understudied species is central to many aspects of conservation as important biological characteristics (e.g., habitat preferences, reproductive traits) can be inferred from closely related taxa. Freshwater mussels (Bivalvia: Unionidae) represent one of the most imperiled taxonomic groups in North America with anthropogenic alterations to freshwater ecosystems leading to over 70% of the fauna considered threatened, endangered, or extinct (Williams et al., 1993; Garner et al., 2004; Haag and Williams, 2014). Several inherent characteristics of freshwater mussels exacerbate conservation concerns, such as a unique larval stage (glochidium) that requires parasitizing host fishes before metamorphosis into sessile adults (Barnhart et al., 2008). This dependency on host fish compounds sensitivity to environmental variables, as unionids are threatened by actions directly impacting them and host fish populations (Haag, 2012). Conservation of freshwater mussels is essential considering the ecosystem services they provide such as filtering benthic biomass, stabilizing substrates for erosion control, and increasing habitat heterogeneity (Zimmerman and de Szalay, 2007; Haag and Williams, 2014). Systematic research on freshwater mussels has played an integral role in the development of conservation strategies and their implementation (e.g., Campbell et al., 2008; Inoue et al., 2014; Pfeiffer et al., 2016). However, most of the systematic research on North American

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Table 1
Taxa analyzed in phylogenetic analyses with indication of samples for each gene and river drainage where specimens were collected.

Taxa (sample size)	COI & NDI	ITSI	River drainage
Alasmidonta triangulata	1	1	Apalachicola (Flint)
Anodonta couperiana	1	1	St. Johns
Anodonta hartfieldorum	1	1	Escambia
Anodonta heardi 1-2	2	2	Apalachicola
Anodonta suborbiculata	1	1	Mississippi
Anodontoides ferussacianus 1-3	3	3	Mississippi or Hudson Bay
Anodontoides ferussacianus cf. *denigrata* 1-6	6	6	Cumberland
Cristaria plicata	1	1	–
Lasmigona etowaensis 1-2	2	2	Mobile (Upper Coosa)
Pseudodontoideus connasaugaensis 1-4	8	4	Mobile (Alabama)
Pseudodontoideus subvexus 1-4	4	4	Mobile (Black Warrior)
Pseudodontoideus subvexus 5-10	10	10	Mobile (Tombigbee)
Pyganodon grandis 1	1	1	Apalachicola (Chattahoochee)
Pyganodon grandis 2	1	1	Escambia
Strophitus pascagoulaensis 1-3	10	3	Pascagoula
Strophitus radiatus 1-2	3	2	Yazoo
Strophitus radiatus 3-10	16	8	Mobile (Tombigbee)
Strophitus radiatus 11-15	11	5	Mobile (Black Warrior)
Strophitus radiatus 16-19	4	4	Apalachicola (Chattahoochee)
Strophitus radiatus 20-23	5	4	Apalachicola (Chipola)
Strophitus radiatus 24	1	1	Apalachicola (Flint)
Strophitus radiatus 25	4	1	Mobile (Alabama)
Strophitus sp. cf. *pascagoulaensis* 1	1	1	Pearl
Strophitus sp. cf. *pascagoulaensis* 2-3	3	2	Pontchartrain (Amite)
Strophitus undulatus 1-5	6	5	Colorado
Strophitus undulatus 6-7	3	2	Tennessee
Strophitus undulatus 8	2	1	Neuse
Strophitus undulatus 9	1	1	Tar
Strophitus williamsi 1-7	11	8	Escambia
Strophitus williamsi 8-21	16	14	Choctawhatchee
Utterbackia peggyae 1-2	2	2	Ochlocknee

freshwater mussels has focused on the species-rich subfamily Ambleminae, while several genera of the tribe Anodontini have received comparably less attention (Graf, 2013).

This is particularly true of the genus *Anodontoides* Baker, 1898, which consists of two species: *A. radiatus* (Conrad, 1834) and *A. ferussacianus* (Lea, 1834). *Anodontoides radiatus* occurs in several eastern Gulf of Mexico drainages including the Apalachicola, Escambia, Choctawhatchee, Mobile, Pearl, Pascagoula, and Lake Pontchartrain drainages as well as the Yazoo River, a tributary of the Mississippi River (Haag et al., 2002; Williams et al., 2008). However, the geographic distribution of *A. radiatus* remains unclear due to difficulties distinguishing it from other closely related taxa. In particular, members of the genus *Strophitus* Rafinesque, 1820 are commonly confused with *A. radiatus* due to similar external shell morphologies and high levels of intraspecific morphological variation (Frierson, 1927; van der Schalie, 1940; Clench and Turner, 1956; Stern, 1976; Vidrine, 1993; Brim Box and Williams, 2000; Williams et al., 2008, 2014). Unrecognized species-level diversity within *A. radiatus* may also be further confounding identification, as suggested by morphological variation observed throughout its range. While variant shell morphology can be diagnostic at the species-level, it can also be the product of environmental variables (Ortmann, 1920; Eagar, 1950; Inoue et al., 2013). To accurately determine species boundaries within *A. radiatus*, we aim to implement an integrative taxonomic approach combining inference from molecular, geographic, and morphological data.

In 2011, the U.S. Fish and Wildlife Service was petitioned to consider *A. radiatus* for protection under the Endangered Species Act (USFWS, 2011). Other assessments have listed the species as special concern throughout its range (Williams et al., 1993; Lydeard et al., 1999), near threatened (Cummings and Cordeiro, 2011), vulnerable (G3; NatureServe, 2016), and other elevated levels of conservation concern (Clench and Turner, 1956; Heard, 1975; Brim Box and Williams, 2000; Garner et al., 2004). The uncertain species boundaries and phylogenetic placement of *A. radiatus* make it difficult for stakeholders to accurately determine the population threats and trends necessary to design effective conservation strategies. This emphasizes the need to test current taxonomic hypotheses for members of the genera *Anodontoides* and *Strophitus*. In this study, we set out to accomplish the following objectives: 1) test the monophyly of the genera *Anodontoides* and *Strophitus*; 2) test current species hypotheses regarding the taxonomy and distribution of *A. radiatus*; 3) revise taxonomy accordingly and discuss implications for systematics and conservation.

2. Material and methods

2.1. Taxon and character sampling

To test the phylogenetic placement of *A. radiatus,* we focused our outgroup taxon sampling on the North American species in the tribe Anodontini (Table 1). We used two mitochondrial genes and one nuclear gene to estimate the phylogeny: the protein-coding mitochondrial genes *cytochrome c oxidase subunit I* (COI) and *NADH dehydrogenase subunit I* (NDI), and the nuclear-encoded ribosomal *internal transcribed spacer I* (ITSI). Tissue samples were preserved in 95% ethanol and DNA was isolated using a modified plate extraction protocol of Ivanova et al. (2006). Primers used for polymerase chain reaction (PCR) and sequencing were: COI dgLCO-1490—GGTCAACAAATCATAAAGAYATYGG and COI dgHCO-2198—TAAACTTCAGGGTGACCAAARAAYCA (Meyer, 2003); NDI Leu-uurF- TGGCAGAAAAGTGCATCAGATTAAAGC and LoGlyR—CCTGCTTGGAAGGCAAGTGTACT (Serb et al., 2003); ITSI-18S—AAAAAGCTTCCGTAGGTGAACCTGCG and ITSI-5.8S—AGCTTGCTGCGTTCTTCATCG (King et al., 1999). PCR plate amplifications were conducted using a 25 µl mixture of the following: distilled deionized water (9.5 µl), GoTaq GMM (12.5 µl) (Green Master Mix, Promega Corporation), primers (0.5 µl) and DNA template (40 ng). PCR product was sent to the Interdisciplinary Center for Biotechnology Research at the University of Florida for bi-directional sequencing on an

ABI 3730. PCR product for ITSI was handled differently than mtDNA markers, considering the possibility of multiple copies at ITSI. Initially, all individuals were sent directly for sequencing, similar to previous studies in unionids that yielded sequences that were readable without cloning (e.g., Grobler et al., 2005; Jones et al., 2006; Campbell et al., 2008; Pfeiffer et al., 2016). To attempt to sequence problematic individuals with length polymorphisms, individual copies were separated and extracted from agarose gels using QIAquick Gel Purification Kit (Qiagen), and a second PCR amplification was performed before submission for sequencing. Reliable ITSI sequences could not be obtained for some heterozygous individuals, which were subsequently excluded from phylogenetic analyses (Table 1). Geneious v 6.1.2 was used to edit and assemble chromatograms (http://www.geneious.com; Kearse et al., 2012).

2.2. Genetic analyses

Individual sequences were aligned in Mesquite v 3.1.0 (Maddison and Maddison, 2016) using MAFFT v 7.299 (Katoh and Standley, 2013). The mtDNA protein coding genes COI and NDI were aligned then translated into amino acids to ensure absence of gaps and stop codons. The ITSI alignment was performed using the default parameters in MAFFT and minor adjustments were made by eye where necessary. A concatenated alignment comprised of the two mtDNA genes (COI and NDI) was used in the program FaBox haplotype collapser (Villesen, 2007) to identify and remove redundant haplotypes prior to phylogenetic analyses.

The three-gene concatenated dataset (i.e., COI, NDI, ITSI) was analyzed using maximum likelihood (ML) in the program RAxML v 8.2.6 (Stamatakis, 2014) and Bayesian inference (BI) in the program MrBayes v 3.2.6 (Ronquist et al., 2012) using the CIPRES Science Gateway (Miller et al., 2010). RAxML analyses were conducted using 1000 tree searches and nodal support values were measured using 2000 rapid bootstraps. MrBayes analyses were conducted using 2 runs of 8 chains for $3 * 10^7$ generations sampling every 1000 trees. Partitions and substitution models for MrBayes were determined by PartitionFinder v1.1.1 (Lanfear et al., 2012) for COI, NDI, and ITSI. To determine the appropriate burn-in value, the log likelihood scores for each sampling point were analyzed using Tracer v1.6 (Rambaut et al., 2013). Markov chains were considered stationary when the log likelihood values reached a plateau. The two runs were monitored for convergence by the Potential Scale Reduction Factor (PSRF) and the average standard deviation of split frequencies.

Three topological constraint scenarios were implemented to evaluate the likelihood of alternative phylogenetic relationships in both RAxML and MrBayes: (1) *Anodontoides* constrained as monophyletic; (2) *Strophitus* constrained as monophyletic; (3) predominantly Mobile and Apalachicola clades of *A. radiatus* constrained as monophyletic. An additional negative topological constraint was performed to force *A. radiatus* from the Pearl/Amite/Pascagoula clade to be non-monophyletic. This analysis was only performed in MrBayes as negative constraints are not an option in RaxML. Bayes factors and S−H tests (Shimodaira and Hasegawa, 1999) were used to test if the topological constraints were significantly different than the optimal topology. Bayes factors were measured using twice the difference of $-ln$ likelihood ($2ln$Bf) with $2ln$Bf = 0–2 meaning not worth a mention, $2ln$Bf = 2–6 meaning positive support, $2ln$Bf = 6–10 meaning strong support, and $2ln$Bf > 10 meaning decisive support (Kass and Raftery, 1995; Grummer et al., 2013).

Uncorrected p-distances were calculated in MEGA6 (Tamura et al., 2013) to depict evolutionary divergence within *A. radiatus*. COI, NDI, and ITSI were independently analyzed (including redundant haplotypes) for within and between group genetic distances. Pairwise deletion was used for gaps and missing data. Sequences were grouped for calculation of genetic distances between drainages as follows: Pascagoula, Pearl/Amite, Escambia/Choctawhatchee, and Mobile/Apalachicola/Yazoo. These groups were determined from phylogenetic relationships depicted by concatenated ML and BI topologies. Each group was evaluated to identify diagnostic nucleotides and indels that distinguish respective clades at COI, NDI, and ITSI independently. Positions for each diagnostic nucleotide at COI and NDI were determined using the complete mtDNA genome of *Cristaria plicata* (Genbank accession: KM233451) as a reference. Nucleotide positions for ITSI were determined by using an alignment consisting of only *A. radiatus*, and we used the sequence of our only *A. radiatus* from the Pearl River as a reference (Genbank accession number MG199854). The relationship between average genetic distance (uncorrected p-distance) and two geographical distance matrices (Euclidean and stream distance) was tested using a partial Mantel test implemented in the software zt version 1.1 using 1000 permutations (Bonnet and Peer, 2002). GenAlex 6.5 (Peakall and Smouse, 2012) was used to calculate Euclidean distance and the distance measurement tool in Google Earth was used to measure stream distances between sampling localities. A haplotype network was generated for all *A. radiatus* samples from the concatenated COI and NDI dataset, and the ITSI dataset independently using a median joining network in PopART 1.7 with the default epsilon value set at 0 (Bandelt et al., 1999). Complete deletion was used for gaps and missing data.

2.3. Species delimitation

We used two coalescent-based approaches to identify independently evolving lineages similar to recent species delimitation studies (e.g., Hedin, 2015; Pfeiffer et al., 2016). The first approach employed ∗BEAST (Heled and Drummond, 2010) implemented in BEAST 2.4 (Bouckaert et al., 2014) using the CIPRES Science Gateway. Bayes factors delimitation (BFD; Grummer et al., 2013) was designed to evaluate marginal likelihoods of species hypotheses. The molecular matrix (COI, NDI, and ITSI) included all individuals representing *A. radiatus* and *S. undulatus*. The matrix was realigned and the best partitioning scheme and substitution models were evaluated using the methods described above. We executed 10^8 generations in ∗BEAST sampling every 5000th tree and removed the first 10% as a burn-in. The proportion of invariant sites was set initially to 0.1 and estimated by ∗BEAST and the gamma category count was set at 4 with shape estimated by ∗BEAST. Base frequencies were empirical or equal dependent on the substitution model. A relaxed log normal molecular clock was fixed at 1.0 for the ITSI partition and the partitions for COI and NDI were initially set at 1.0 then estimated by ∗BEAST. Yule process was used as the species tree prior paired with a piecewise linear and constant root population size model.

We tested seven species models using ∗BEAST by grouping individuals of *A. radiatus* according to geography, morphometrics, phylogenetic results, and current taxonomy (Table 1). The first three models were based on mtDNA signal and geography (Fig. 4): 1 - predominantly Apalachicola clade one species, predominantly Mobile clade one species, and all other rivers as separate species; 2 - Apalachicola/Mobile/Yazoo clade one species and all other rivers separate species; 3 - Apalachicola/Mobile/Yazoo drainages one species, Escambia/Choctawhatchee drainages one species, and remaining rivers separate species. The 4th model tested support for the concatenated optimal topology (Fig. 1) and sequence divergence (Table 3; Fig. 4): Apalachicola/Mobile/Yazoo drainages one species, Escambia/Choctawhatchee drainages one species, Pearl/Amite drainages one species, and the Pascagoula drainage as a separate species. Clades supported by our morphometric data were tested with the 5th model (Fig. 4): Apalachicola/Mobile/Yazoo drainages one species, Escambia/Choctawhatchee drainages one species, and the Pearl/Amite/Pascagoula drainages one species. The 6th model tested signal depicted by nDNA (Figs. 3 and 4): Apalachicola/Mobile/Yazoo/Escambia/Choctawhatchee drainages one species and the Pearl/Amite/Pascagoula drainages one species. The 7th model tested current taxonomy: one species

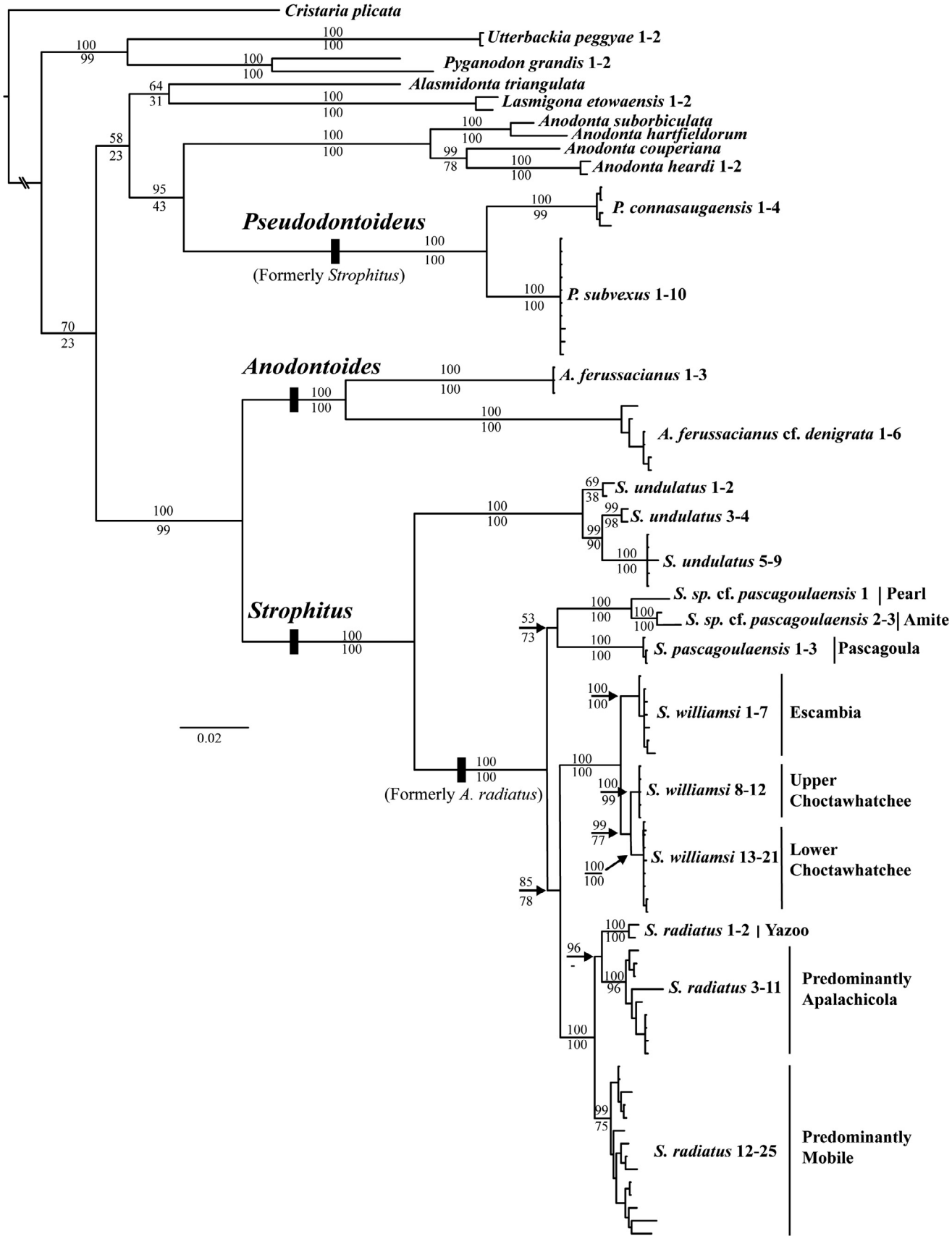

Fig. 1. Concatenated (COI, NDI, ITSI) optimal topology generated by Bayesian Inference (BI) analysis. Values above branches represent BI posterior probability and values below represent Maximum Likelihood (ML) bootstrap support. Former taxonomy is noted in brackets below branches. Additional information on the individual terminals are available in Table 1 of Smith et al. (2017).

distributed across all drainages. The marginal likelihood of each for each species model was estimated using a pairwise path-sampling/stepping stone analysis (PS/SS; Lartillot and Philippe, 2006; Xie et al., 2011). Path sampling/stepping stone marginal likelihood estimations were performed on each species model using a chain length of 10^7 and 100 path steps with a 25% burn-in value in BEAST2 (Baele et al., 2013).

The second coalescent-based species delimitation approach employed Bayesian Phylogenetics and Phylogeography v 3.2 (BPP; Rannala and Yang, 2003, 2013; Yang and Rannala, 2010; Yang, 2015) to test the effects of historical demographics on the probability of speciation. We implemented reversible-jump Markov chain Monte Carlo (rjMCMC) using 5,000,000 generations sampling every 5th generation with a burn-in of 10% for two separate analyses. We specified ∗BEAST model 1 as the topological prior for both analyses. A preliminary analysis was implemented in BPP to estimate population size (Θ) and species divergence time (τ; Rannala and Yang, 2003). The species delineation variable was set to 0 and species tree was set to 0 (A00). Demographic priors for the analysis were: historical population size [$\Theta = G(1, 10)$] and divergence time [$\tau = G(1, 10)$]. A second A00 analysis using the estimated mean Θ and τ values as priors was performed to ensure accuracy of the estimation. Step lengths were user specified and adjusted so that acceptance proportions were approximately equal to 30% based on developers' recommendations (Yang, 2015). The estimated parameters from the A00 analysis were used as priors for the A10 analysis to address the effects of historical population size and time to most recent common ancestor. The species delineation variable was set to 1 and species tree was set to 0 (Yang and Rannala, 2010; Rannala and Yang, 2013). The A10 analysis was run with algorithm 0 with a fine-tune parameter ($\varepsilon = 2$) (Yang and Rannala, 2010; Rannala and Yang, 2013).

2.4. Morphometric analyses

We conducted a morphometric analysis of external shell dimensions for all *A. radiatus* specimens used in genetic analyses along with museum specimens from all focal drainages (see Table 2 in Smith et al., 2017). Specimens were grouped according to the clades recovered in the concatenated optimal topology (Figs. 1 and 4): Apalachicola/Mobile/Yazoo, Escambia/Choctawhatchee, Pascagoula, and Pearl/Amite. Three measurements were taken for morphological analyses: maximum length (anterior to posterior), height (dorsal to ventral), and width (right to left valve) to the nearest 0.01 mm using digital calipers. Height and width were measured at the posterior end of the hinge ligament for consistent measurements. Length, height, and width values were $\log_e$-transformed. This transformation results in a scale-invariant matrix while preserving information about allometry (Jolicoeur, 1963; Strauss, 1985; Kowalewski et al., 1997). Morphological analyses were performed in R v 3.3.1. The $\log_e$-transformed variables were evaluated for normality using Shipiro-Wilk tests (Sokal and Rohlf, 1995). For bivariate relationships between morphological variables (e.g., H ~ L), differences between the estimated slopes of each clade were compared using an analysis of variance (AOV). We analyzed morphological variation between $\log_e$-transformed variables of sister clades (e.g., Apalachicola/Mobile/Yazoo ~ Escambia/Choctawhatchee) in the vegan package (Oksanen et al., 2016) using permutational multivariate analyses of variance (MANOVA). Permutational MANOVAs were performed using 10^6 iterations. A significance level of $\alpha = 0.05$ was assumed when assessing the statistical significance of all tested hypotheses.

3. Results

3.1. Taxon and character sampling

A molecular matrix was created consisting of 8 genera and 15 species aligned to 2041 nucleotides (nt). Each of the taxa in the three-gene concatenated dataset was represented with three genes: COI (avg. ≈ 642 nt), NDI (avg. ≈ 797 nt) and ITSI (592 nt including an avg. of ≈13.4% gaps). Both mtDNA protein coding genes (COI and NDI) contained no gaps or stop codons. The COI and NDI concatenated alignment (1449 nt) consisted of 141 individuals represented by 97 unique haplotypes. The final three-gene concatenated data set included 97 individuals representing outgroups and members of the *A. radiatus* species complex from the following drainages: Pascagoula, Pearl, Pontchartrain (Amite River), Mississippi (Yazoo River), Mobile, Escambia, Choctawhatchee, and Apalachicola (Table 1; Fig. 2; also see Smith et al., 2017 – Table 1).

3.2. Genetic analyses

Six partitions and nucleotide substitution models were determined by Partitionfinder for BI analyses: ITSI partition-K80 + G, COI 1st position- SYM + I, COI and NDI 2nd position- HKY + I, COI 3rd position-HKY + G; NDI 1st position- HKY + G, NDI 3rd position- GTR. RaxML used GTR + G for all partitions following recommendations of the developer (Stamatakis, 2014). Convergence of the two MrBayes runs was supported by the average PSRF value (1.0) and the mean of the standard deviation of split frequencies (0.001552). A 25% burn-in value was implemented before optimal log likelihood was reported. We present the phylogenetic reconstruction based on the concatenated three-gene matrix using BI in Fig. 1. Bayesian and ML topologies depicted nine clades within *A. radiatus*: Pearl; Amite; Pascagoula; Escambia; upper Choctawhatchee; lower Choctawhatchee; Yazoo; predominantly Mobile; and predominantly Apalachicola (Fig. 1).

Strophitus and *Anodontoides* were both resolved as paraphyletic. *Strophitus connasaugaensis* (Lea, 1858) and *S. subvexus* (Conrad, 1834) are sister to the genus *Anodonta* Lamarck, 1799. *Strophitus undulatus*, which is the type species of *Strophitus*, was well-supported to be nested within *Anodontoides*. Constraining *Anodontoides* and *Strophitus* independently to be monophyletic resulted in topologies with likelihood values significantly worse than the optimal topology (Table 2). Constraining the predominantly Mobile and Apalachicola clades of *A. radiatus* to be monophyletic resulted in marginally better topologies than the optimal topology using Bayes factors, but no significant difference was found between topologies with an S—H test (Table 2). Constraining *A. radiatus* from the Pearl/Amite and Pascagoula clades to not be monophyletic resulted in significantly better topologies than the optimal (Table 2).

A total of 83 individuals of the *A. radiatus* species complex were included in the concatenated mtDNA (COI and NDI) dataset. The average pairwise distance values (within and between groups) for COI, NDI, and ITSI are depicted in Table 3. Pairwise genetic distance values between the four groups for COI ranged from 3.5 to 7.8% and NDI values ranged from 4.8 to 8.1%. Fifty-two individuals of the *A. radiatus* species complex were included in the ITSI dataset. Pairwise genetic distance values between the four groups in ITSI ranged from 0.3 to 4.1%. The number of diagnostic nucleotides and indels (COI/NDI/ITSI) was calculated for the following four groups: Pearl/Amite (10/18/2); Pascagoula (10/14/3); Escambia/Choctawhatchee (6/9/0); Mobile/Apalachicola/Yazoo (3/7/0) (see Table 2 in Smith et al., 2017). No significant correlation was found between genetic and geographic distance throughout populations of *A. radiatus* based on a partial Mantel Test (COI: $r = -0.07$, $\alpha = 0.25$; NDI: $r = -0.056$, $\alpha = 0.31$; ITSI: $r = -0.02$, $\alpha = 0.49$). The mtDNA and nDNA haplotype networks are depicted in Fig. 3.

3.3. Species delimitation

The molecular matrix used in coalescent-based models was aligned to 2019 nt. Each taxon was represented by three genes: COI (avg. ≈ 642 nt), NDI (avg. ≈ 798 nt) and ITSI (570 nt including an avg. of ≈ 11.5% gaps). Five partitions and nucleotide substitution models were

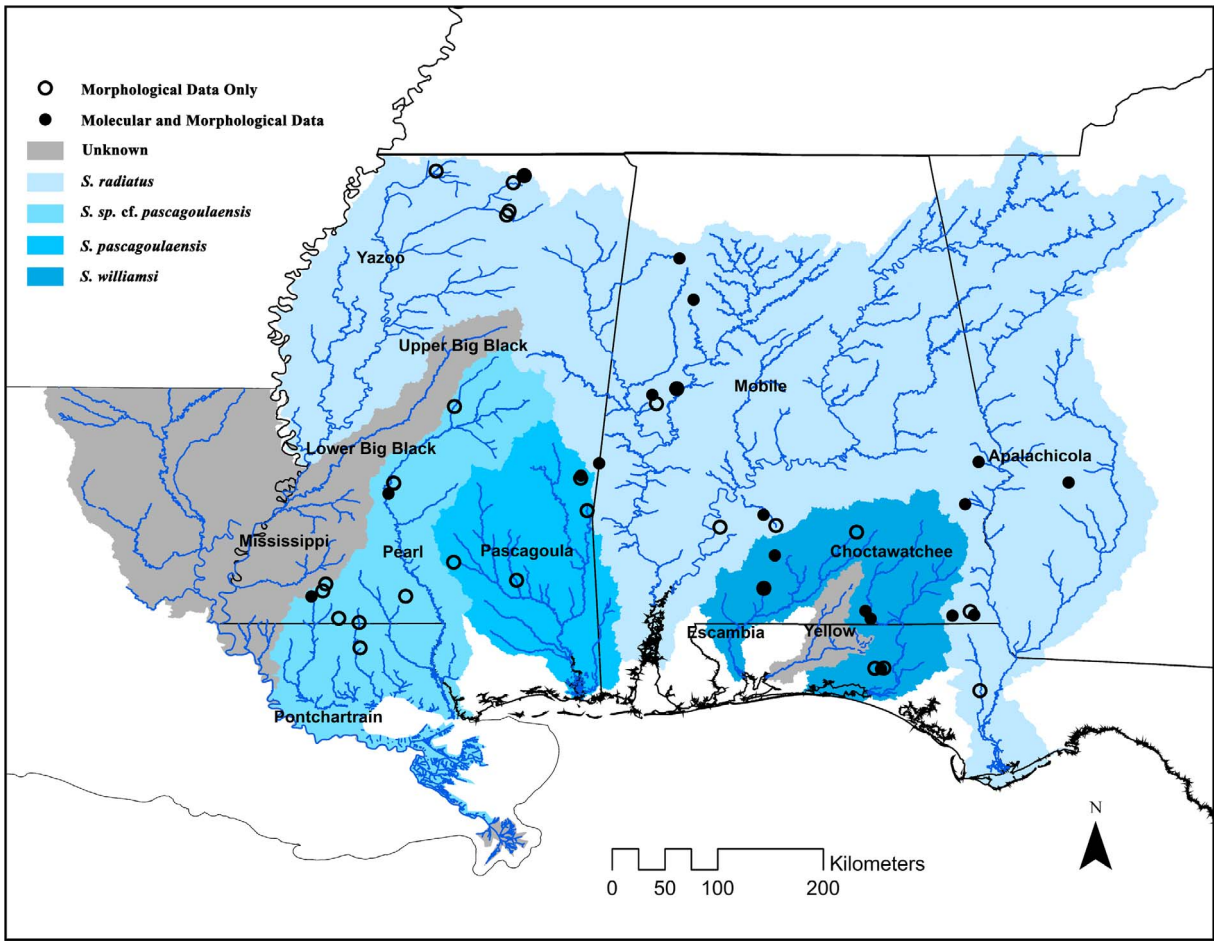

Fig. 2. Map illustrating ranges of *S. radiatus*, *S. williamsi*, *S. pascagoulaensis*, and *S. sp.* cf. *pascagoulaensis* with indication of sampling sites for individuals used in collection of molecular and morphometric data.

selected by PartitionFinder for ∗BEAST: ITSI partition-K80 + I, COI and NDI 1st position- K80 + G, COI and NDI 2nd position- HKY + I, COI 3rd position- HKY + I, NDI 3rd position- TrN + G. Path sampling/Stepping stone estimations depicted species model 1 most likely and rejected all other species models; therefore species model 1 was selected as the guide tree for BPP.

The guide tree implemented for species delimitation in the A10 BPP analysis consisted of 9 lineages: *A. radiatus* from the predominantly Apalachicola clade, predominantly Mobile clade, Escambia, Choctawhatchee, Pearl, Amite, Pascagoula and Yazoo; and *S. undulatus*.

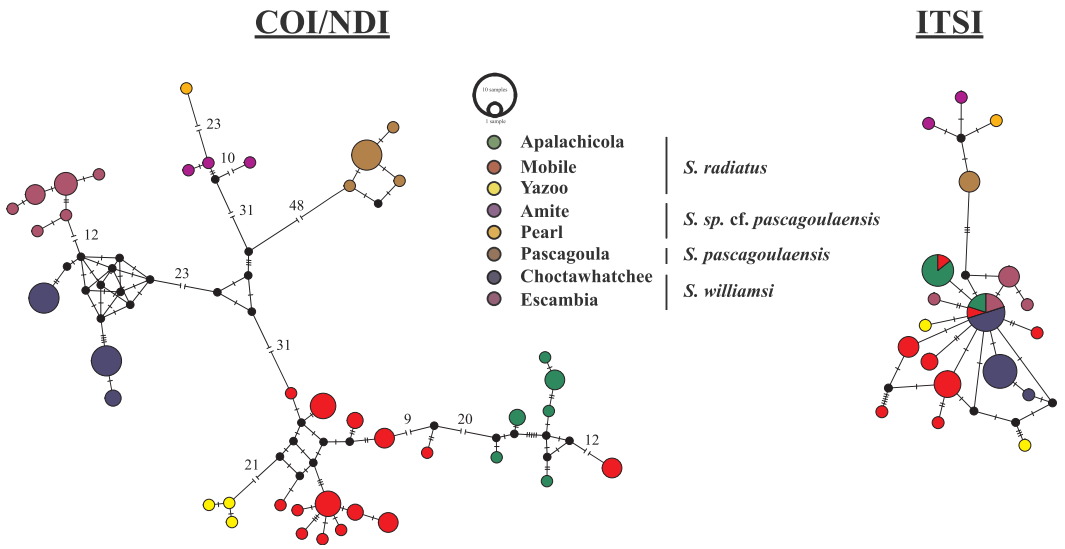

Fig. 3. Mitochondrial (COI and NDI) and ITSI haplotype networks of individuals in the *S. radiatus* species complex. Each circle represents a unique haplotype with the size relative to frequency of individuals with the haplotype. Colors represent sampled drainages. Black circles represent unsampled haplotypes. Black tick marks or numbers along each line indicate the number of nucleotide substitutions between haplotypes.

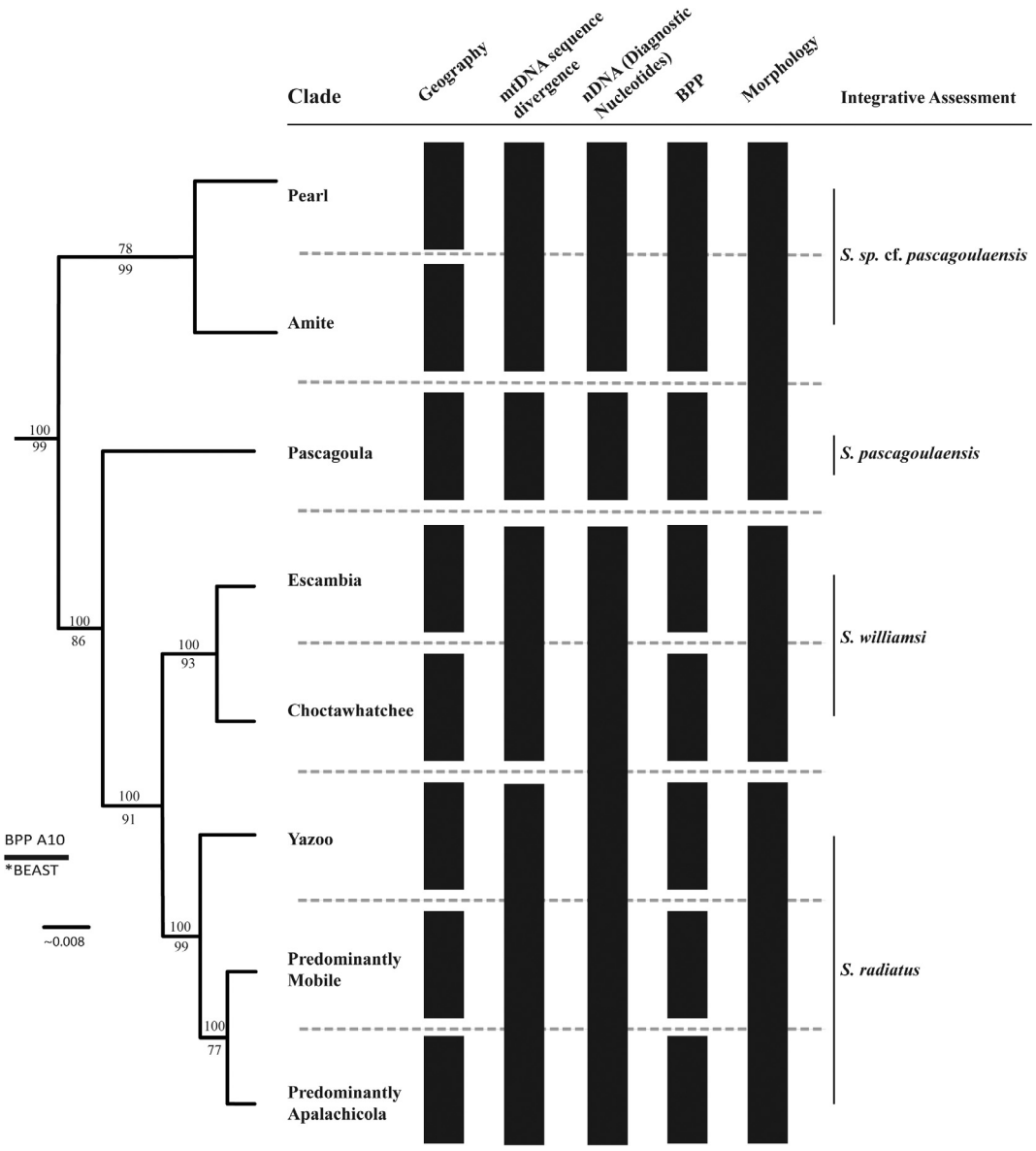

Fig. 4. Concatenated inference from geography, morphometrics, and phylogenetic analyses. The phylogeny represents the *BEAST and BPP topology and nodal support above the branch represents posterior probabilities (PP) from BPP and below represents PP from *BEAST model 1.

Table 2
Comparisons of topological constraints to the concatenated optimal topology in Bayesian (BI) and maximum likelihood (ML) analyses. Bold values represent significantly different scenarios.

Scenario	ML *ln* (best tree)	P-value	Topology	BI *ln* (HME)	2*ln*Bf	Topology
Optimal	−14021.04	–	–	−13951.07	–	–
Strophitus monophyletic	**−14151.37**	**< 0.01**	**Significantly Worse**	**−14094.27**	**286.40**	**Significantly Worse**
Anodontoides monophyletic	**−14160.00**	**< 0.01**	**Significantly Worse**	**−14018.82**	**135.50**	**Significantly Worse**
Predominantly Mobile and Apalachicola monophyletic	−14024.33	> 0.05	Not Significantly Worse	−13948.75	−4.64	Marginally Better
Pearl/Amite, and Pascagoula not monophyletic				**−13939.71**	**−22.71**	**Significantly Better**

The first A00 analysis in BPP estimated mean $\Theta \approx 0.237$ and $\tau \approx 0.0159$. The second A00 analysis using priors for historical population size [Θ = G (2, 9)] and divergence [τ = G (2, 125)] estimated mean $\Theta \approx 0.264$ and $\tau \approx 0.0159$. The limited divergence of mean Θ and τ estimates between the two independent runs justified the implementation of the priors Θ = G (2, 9) and τ = G (2, 125) in the A10 analysis. The A10 analysis using these priors strongly supported (PP = 100) seven clades of *A. radiatus* as distinct species (Choctawhatchee, Escambia, predominantly Apalachicola, predominantly Mobile, Yazoo, Pascagoula, and Pearl/Amite). Posterior probabilities (speciation probabilities) for the A10 analysis ranged from 78 to 100 (Fig. 4).

3.4. Morphological analyses

We measured a total of 142 individuals representing members of the *A. radiatus* species complex from the following clades: Pascagoula (19), Apalachicola/Mobile/Yazoo (50), Pearl/Amite (41), and Escambia/

Table 3
Interspecific and intraspecific uncorrected p-distance in the *S. radiatus* species complex. Pairwise genetic distance reported as mean (min-max). Values next to taxon names indicate the number of individuals sampled in each species. Upper Table: Lower triangle represents COI pairwise genetic distance while the upper triangle represents ND1 pairwise genetic distance between clades. Lower Table: ITSI pairwise genetic distance.

Species (Number of individuals) COI/NDI	1	Between clades 2	3	4	COI Within Clade	ND1 Within Clade
1. *S. pascagoulaensis* (10)		6.1 (5.3–7.2)	7.4 (6.7–8.1)	6.5 (5.9–7.0)	0.1 (0.0–0.2)	0.1 (0.0–0.2)
2. *S. radiatus* (42)	4.7 (4.3–5.7)		6.5 (5.1–8.0)	5.8 (4.8–6.9)	1.6 (0.0–3.0)	1.6 (0.0–3.4)
3. *S. sp.* cf. *pascagoulaensis* (4)	6.4 (5.9–7.7)	5.9 (4.5–7.8)		6.9 (5.8–8.1)	2.0 (0.0–3.9)	1.5 (0.0–2.9)
4. *S. williamsi* (27)	4.8 (4.3–5.1)	4.2 (3.5–4.9)	5.1 (4.3–6.6)		0.6 (0.0–1.1)	1.1 (0.0–2.2)
ITSI	1	2	3	4	ITSI Within Clade	
1. *S. pascagoulaensis* (3)					0	
2. *S. radiatus* (25)	2.1 (1.4–4.1)				1.0 (0–3.4)	
3. *S. sp.* cf. *pascagoulaensis* (3)	0.5 (0.3–0.6)	2.3 (1.7–4.5)			0.8 (0.6–0.9)	
4. *S. williamsi* (21)	1.4 (1.2–1.8)	1.2 (0.3–3.4)	1.9 (1.8–2.4)		0.3 (0.0–1.1)	

Choctawhatchee (32). A list of the measured individuals along with their catalog numbers and geographic locality (latitude, longitude) are available (see Table 3 in Smith et al., 2017). The AOV analyses showed no significant differences in slopes ($\alpha > 0.05$). The permutational MANOVAs between $\log_e$-transformed variables (i.e., H, W, and L) identified significant morphological differentiation between the Apalachicola/Mobile/Yazoo and Escambia/Choctawhatchee clades ($\alpha \approx 0.013$) but did not identify significant divergence between the Pascagoula and Pearl/Amite clades ($\alpha \approx 0.396$).

4. Discussion

In this study, we implement an integrative approach to resolve taxonomic relationships in the freshwater mussel genera *Anodontoides* and *Strophitus* using molecular, morphometric, and geographic data. A multilocus investigation revealed non-monophyly in *Strophitus* and *Anodontoides*, and high intraspecific divergences within *A. radiatus*. In the following sections, we describe how multiple independent lines of evidence guide our generic reclassification and recognition of four species in the *A. radiatus* species complex: *Strophitus radiatus* (Apalachicola/Mobile/Yazoo), *Strophitus williamsi* (Escambia/Choctawhatchee), *Strophitus pascagoulaensis* (Pascagoula), and *Strophitus* sp. cf. *pascagoulaensis* (Pearl/Amite). Our findings have profound implications regarding the systematics and conservation of this highly imperiled group of organisms.

4.1. Generic placement

The optimal topology (Fig. 1) resolves both *Strophitus* and *Anodontoides* as non-monophyletic, rejecting current taxonomic hypotheses. *Strophitus connasaugaensis* and *S. subvexus* are resolved sister to *Anodonta*, while *S. undulatus* is nested within *Anodontoides*. Constraining *Strophitus* (i.e., *S. undulatus, S. subvexus,* and *S. connasaugaensis*) as monophyletic resulted in topologies significantly worse than the optimal (Table 2). Our molecular data strongly supports the polyphyly of *Strophitus*, therefore we advocate the resurrection of the genus *Pseudodontoideus* (Frierson, 1927) for '*S. connasaugaensis*' and '*S. subvexus*' to distinguish the taxa from the type species of *Strophitus* (*S. undulatus*). *Pseudodontoideus* is endemic to the Mobile drainage with *P. connasaugaensis* considered restricted to eastern rivers (Alabama, Cahaba, Coosa and Tallapoosa) and *P. subvexus* confined to western rivers (Tombigbee and Black Warrior). It is unclear if these two species occur in sympatry in the Mobile drainage, but in general the distribution is poorly known and additional surveys are needed to properly assess the conservation status of these taxa (Williams et al., 2008).

The optimal topology also indicates that *Anodontoides* is paraphyletic; *S. undulatus* is nested between *A. radiatus*, and *A. ferussacianus* (type species) and *A. ferussacianus* cf. *denigrata* (Fig. 1). Reconstructions constrained to reflect current taxonomy (i.e., *Anodontoides* as monophyletic) resulted in topologies significantly worse than the optimal (Table 2). Morphological data provides additional support for including *A. radiatus* in *Strophitus*. Shells of *A. radiatus* have a small pseudocardinal tooth that is similar to the rudimentary teeth seen in *S. undulatus*, whereas *A. ferussacianus* and *A. ferussacianus* cf. *denigrata* have no dentition (i.e., edentulous) (Fig. 5). The presence of weak pseudocardinal teeth provides a useful morphological character uniting *A. radiatus* with *S. undulatus* (Simpson, 1914; Parmalee and Bogan, 1998; Watters et al., 2009). Therefore, we resurrect the binomial *Strophitus radiatus* to reflect common ancestry with *S. undulatus* and distinguish it from *A. ferussacianus* and *A. ferussacianus* cf. *denigrata*. These findings align with the work of recent researchers who advocated for the inclusion of *A. radiatus* in *Strophitus* (Stern, 1976; Vidrine, 1993). Reproductive characters are also consistent with phylogenetic relationships depicted between the two genera. *Anodontoides ferussacianus* releases glochidia in mucous strands while *S. undulatus* releases glochidia in conglutinates that resemble annelids and aquatic insect larvae (Hove, 1995; Watters, 1995, 2002, 2008). Previous research has hypothesized that conglutinate production is the synapomorphic trait distinguishing *Strophitus* from sister taxa (Simpson, 1914), however reproductive biology of *S. radiatus* is undocumented and warrants investigation.

4.2. Evaluation of species boundaries

Inference from distance and model-based methodologies and emerging coalescent-based analyses provided various levels of support for species-level relationships (Figs. 1 and 4). For example, BI and ML concatenated analyses depicted as many as nine clades in the *S. radiatus* species complex (Fig. 1). Interspecific mtDNA p-distances, diagnostic nucleotides, and geographic distribution of haplotypes support recognition of four evolutionarily independent lineages: *S. radiatus*, *S. williamsi*, *S. pascagoulaensis*, and *S. sp.* cf. *pascagoulaensis* (Fig. 3; Table 3; also see Table 2 in Smith et al., 2017). The levels of mtDNA interspecific divergence are greater than or equal to previous studies on unionids (e.g., Roe and Lydeard, 1998; Serb et al., 2003; Jones et al., 2006; Elderkin et al., 2008) and the Barcode Index Number (BIN; Ratnasingham and Hebert, 2013), which delineates species at 2.2% genetic distance using COI. There was no significant correlation between geographic and genetic distances, indicating that isolation by distance was not driving the observed mtDNA divergence among these taxa (Fig. 2; Fig. 4).

Contrastingly, ITSI data depicts two groups with limited divergence: *S. radiatus/S. williamsi* and *S. pascagoulaensis/S. sp.* cf. *pascagoulaensis* (Fig. 3; Table 3). Despite this limited divergence at ITSI, we see no haplotype sharing between major clades and no signal for hybridization at mtDNA loci (Fig. 1). Furthermore, resolving phylogenetic relationships based on one nDNA locus could be problematic given that nuclear loci are more susceptible to incomplete lineage sorting (ILS) (Funk and

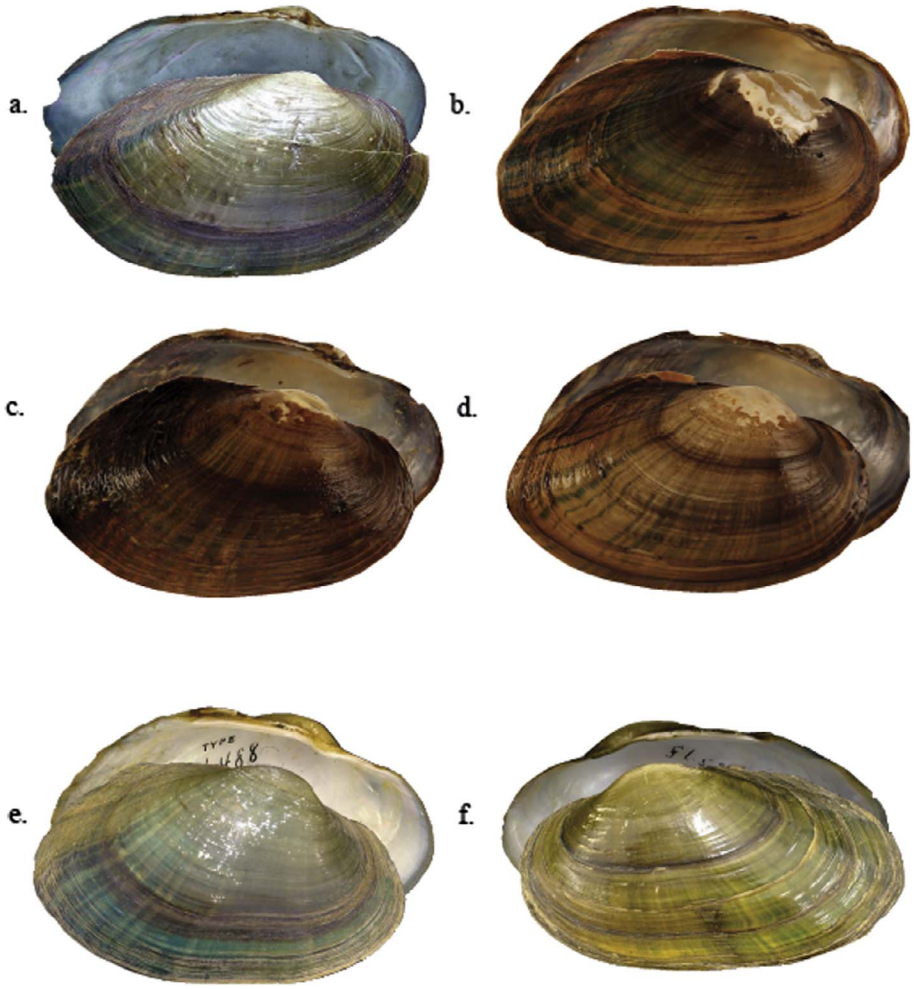

Fig. 5. Photographs of type specimens of (a) *S. radiatus* (ANSP 41147), (b) *S. williamsi* (UF439319), (c) *S. pascagoulaensis* (UF439322), (d) *S. sp.* cf. *pascagoulaensis* (UF438877), (e) *S. undulatus* (USNM86488), (f) *A. ferussacianus* (USNM86520) (Photographs of *S. radiatus*, *S. undulatus*, and *A. ferussacianus* courtesy of www.musselproject.uwsp.edu).

Omland, 2003; Wiens et al., 2010), which has been documented in studies inferring evolutionary relationships among closely related unionid species (e.g. Grobler et al., 2011; Graf et al., 2014; Chong et al., 2016). We do not see any strong geographical inconsistencies between mtDNA and nDNA topologies that would rule out ILS (Toews and Brelsford, 2012), and ILS is expected due to the slower mutation rate and larger effective population size (N_e) relative to mtDNA loci (Moore, 1995; Funk and Omland, 2003; Ballard and Whitlock, 2004; McCracken and Sorenson, 2005).

To address this phenomenon, we employed recently developed coalescent-based approaches that improve estimations of species relationships when ILS is present (Rannala and Yang, 2003; Degnan and Salter, 2005; Carstens and Knowles, 2007). We directly linked historical demographics to phylogenetic inference and tested alternate hypotheses of lineage divergence using ∗BEAST and BPP. Bayes factor delimitation calculated from PS/SS analyses in ∗BEAST strongly supported species model 1 (Table 4) as the best option for the guide topology in BPP. Multi-locus investigations using BPP exploit the stochastic nature of divergence to calculate the posterior probabilities of a speciation event by linking two key parameters: effective population size (Θ) and divergence time (τ) (Fujita et al., 2012; Camargo and Sites, 2013). Estimating values for Θ and τ in BPP (A00) improves the accuracy of species delimitation considering incorrect priors have been documented to significantly affect speciation probabilities (Yang and Rannala, 2010; Yang, 2015). Therefore, we estimated priors for Θ and τ and implemented them in the A10 analysis. Although BPP hypothesized seven well-supported evolutionarily independent lineages (Fig. 4), this approach has been demonstrated to overestimate biodiversity due to its sensitivity to recent divergence events and population structure (Barley et al., 2013; Carstens and Satler, 2013; McKay et al., 2013; Miralles and Vences, 2013; Satler et al., 2013; Hedin, 2015; Pfeiffer et al., 2016; Sukumaran and Knowles, 2017). Our findings agree with previous studies (Fujita et al., 2012; Sadowska-Deś et al., 2014; Pfeiffer et al., 2016; Sukumaran and Knowles, 2017) that have cautioned the use of

Table 4
Comparison of *S. radiatus* species models implemented in *BEAST using Bayes factor species delimitation (Grummer et al., 2013). Bold 2*ln*Bf values represent rejected species models.

*BEAST Models	PS/SS *ln*	2*ln*Bf	Reject	Species Model
1	−6221.28	–	–	All rivers (8 species)
2	−6241.76	**40.96**	**Yes**	Apalachicola/Mobile/Yazoo, all other rivers (6 species)
3	−6259.40	**76.24**	**Yes**	Apalachicola/Mobile/Yazoo, Escambia/Choctawhatchee, all other rivers (5 species)
4	−6258.89	**75.22**	**Yes**	Apalachicola/Mobile/Yazoo, Escambia/Choctawhatchee, Pearl/Amite, Pascagoula (4 species)
5	−6266.21	**89.86**	**Yes**	Apalachicola/Mobile/Yazoo, Escambia/Choctawhatchee, Pearl/Amite/Pascagoula (3 species)
6	−6312.37	**182.18**	**Yes**	Apalachicola/Mobile/Yazoo/Escambia/Choctawhatchee, Pearl/Amite/Pascagoula (2 species)
7	−6349.48	**256.40**	**Yes**	One species distributed across all drainages (1 species)

coalescent-based SDMs alone to identify species and advocate implementing a holistic approach to species delimitation.

Our optimal topology (Fig. 1) is concordant with our coalescent-based analyses (∗BEAST and BPP) with two exceptions: (1) the monophyly of the predominantly Apalachicola and Yazoo clades; and (2) the monophyly of *S. pascagoulaensis* and *S. sp.* cf. *pascagoulaensis* (Fig. 1; Fig. 4). The BI topology resolved a monophyletic Yazoo/predominantly Apalachicola clade, while coalescent-based methods depict a monophyletic Apalachicola/Mobile clade. With shared haplotypes between the Apalachicola and Mobile drainages, we expect that phylogenetic relationships are accurately depicted in coalescent-based methods. To test this hypothesis, a topological constraint was enforced to make the predominantly Apalachicola and predominantly Mobile clades monophyletic. The result of this constraint only marginally supported the coalescent-based topology (Table 2). Both BI and ML topologies also resolved a clade consisting of *S. pascagoulaensis* and *S. sp.* cf. *pascagoulaensis*, contrasting from the coalescent-based topology (Figs. 1 and 4). Therefore, we employed an additional topological constraint to test the monophyly of *S. pascagoulaensis* and *S. sp.* cf. *pascagoulaensis* and found significant support for the coalescent-based topology (Table 2; Fig. 4). Even though significant support for the coalescent-based topology was not found with both constraints, our molecular data strongly support the ability of coalescent-based SDMs to resolve historical relationships of recently diverged taxa. We emphasize the importance of their implementation when inferring taxonomic relationships, especially in the presence of ILS.

Morphometric analyses identified significant differentiation between *S. radiatus* and *S. williamsi*, but did not differentiate *S. pascagoulaensis* and *S. sp.* cf. *pascagoulaensis* (Section 3.4). These findings partially support our molecular data but also suggest diversity within this group may be cryptic. Alternatively, morphological similarity could be the result of environmental variables, a phenomenon that has been well-documented in freshwater mussels (e.g., Ortmann, 1920; Eagar, 1950) and recent studies have examined it using both molecular and morphological data (e.g., Zannatta et al., 2007; Inoue et al., 2013). This highlights the importance of implementing an integrative framework to evaluate species boundaries, particularly where morphological characteristics could be profoundly influenced by abiotic factors.

Our integrative assessment of species boundaries based on multiple independent lines of evidence resolved four well-supported evolutionary groups within the *S. radiatus* species complex (Figs. 1 and 4). These four clades exhibited a clear signal for mtDNA interspecific divergence (Table 3; Fig. 4), which is typically considered a prerequisite for formal species recognition (e.g., Cracraft, 1983; Nixon and Wheeler, 1990; Davis and Nixon, 1992; Sites and Marshall, 2004; Meyer and Paulay, 2005). Despite the high levels of mtDNA divergence, limited divergence was present at ITSI. *Strophitus pascagoulaensis* and *S. sp.* cf. *pascagoulaensis* were diagnosable at ITSI, while several individuals of *S. radiatus* and *S. williamsi* exhibited haplotype sharing. ITSI depicts little signal in recent speciation events and is routinely used in phylogenetic studies given that it is the most variable nuclear marker widely available for freshwater mussels (e.g., Jones et al., 2006; Campbell et al., 2008; Campbell and Lydeard, 2012; Inoue et al., 2014; Pfeiffer et al., 2016). Monophyly has been considered an unrealistic expectation in delimiting recently diverged species, especially when using conserved loci (Hudson and Coyne, 2002; Rannala and Yang, 2003; Hickerson et al., 2006; Knowles and Carstens, 2007; Zhang et al., 2011). Therefore, we do not delimit species using ITSI alone and follow previous studies that combine nDNA inference with mtDNA, morphological, and geographic information (e.g., Jones et al., 2006; Inoue et al., 2014; Pfeiffer et al., 2016). *Strophitus radiatus* and *S. williamsi* are diagnosable geographically, at mtDNA loci, and depict significant morphological divergence (Fig. 4). Given no strong geographical inconsistencies between mtDNA and nDNA topologies, we conclude that limited divergence at ITSI is a product of ILS rather than ongoing gene flow. However, we feel that the lack of morphological support concomitant with low sample sizes limits our ability to make definitive conclusions regarding the taxonomic relationships between the Pascagoula and Pearl/Amite clades. In light of our findings, we err on the side of caution and designate the Pearl/Amite clade as a putative species (*Strophitus* sp. cf. *pascagoulaensis*) and formally recognize three species in this complex: *Strophitus radiatus* (Apalachicola/Mobile/Yazoo), *Strophitus williamsi* (Escambia/Choctawhatchee), and *Strophitus pascagoulaensis* (Pascagoula).

4.3. Species accounts

***Strophitus radiatus* (Conrad, 1834)**

Rayed Creekshell

Synonyms: *Alasmidonta radiata*, Conrad, 1834, Amer. J. Sci. 25(2): 341, pl. 1, fig. 10.

Margaritana elliottii, Lea, 1858, Proc. Acad. Nat. Sci. 10: 138.

Margaritana elliptica, Lea, 1859, Proc. Acad. Nat. Sci. 11: 113.

Anodonta showalterii, Lea, 1860, Proc. Acad. Nat. Sci. 12: 307.

Type Material: Holotype: ANSP 41147 (Fig. 5a), length 64 mm. Conrad (1834) described the type locality as 'small streams in south Alabama.' Based on historical accounts, the type specimen is believed to be from the Black Warrior River drainage near the community of Erie, Greene County, Alabama (Wheeler, 1935).

Diagnosis: *Strophitus radiatus* resembles its sister species *S. williamsi* but most individuals were found to be less inflated (width 30–40% of length vs. 35–42%) and more elongate (height 48–61% of length vs. 52–64%) (Fig. 5; Table 3 in Smith et al., 2017). *Strophitus radiatus* is distinguished from other members of the species complex by 3 diagnostic nucleotides at COI (342:A, 570:G, 573:G) and 7 diagnostic nucleotides at ND1 (10:T, 120:T, 177:T, 188:C, 231:T, 532:A, 749:C) (Table 2 in Smith et al., 2017).

Description: Shell- Length to 84.6 mm; thin; moderately inflated; oval to elliptical outline; posterior margin rounded to bluntly pointed. Max height 48–61% of the total length. Max width 30–40% of total length. Low posterior slope and ridge; umbo broad and slightly elevated above the hinge line. Periostracum yellow-green to dark olive with variable green rays. Weak pseudocardinal tooth present, with one tooth per valve. Nacre white to bluish white.

Distribution: *Strophitus radiatus* is endemic to the Mobile and Apalachicola drainages, and the Yazoo River of the Mississippi drainage.

Remarks: Our data supports the hypothesis that historical stream capture event(s) occurred between the Apalachicola, Mobile, and Yazoo drainages (Figs. 1, 3 and 4). Previous research proposed that *S. radiatus* was introduced to the upper Yazoo River from the western tributaries of the Tombigbee River via historical stream captures and our molecular data, along with known distributions of other freshwater taxa (e.g., *Notropis ammophilus*, *N. rafinesquei*, *Etheostoma raneyi*, *E. lachneri*, *Orconectes chickasawae*) supports that hypothesis (Haag et al., 2002). The concatenated optimal topology also supports two Apalachicola/Mobile clades, one consisting predominantly of samples from the Mobile drainage (Black Warrior and Tombigbee rivers, and one Chattahoochee River individual) and the other primarily from the Apalachicola (Chattahoochee and Flint rivers, and one Alabama River individual). The limited divergence and haplotype sharing between the Mobile and Apalachicola drainages could be due to a possible historical stream capture event(s) between the Alabama and Chattahoochee rivers and warrants further investigation.

Strophitus radiatus has also been reported from the lower Mississippi and Big Black rivers (Jones et al., 2005). Sampling efforts for *S. radiatus* at historically-occupied localities in the Big Black River were unsuccessful. It is possible that accounts of *S. radiatus* in tributaries of the lower Mississippi and Big Black rivers may represent *S. undulatus*, a wide-ranging species occurring across the Mississippi Basin, along the Atlantic Slope, and in Gulf drainages west of the Mississippi River to the Guadalupe River in Texas (Howells et al., 1996; Williams et al., 2008).

Additional sampling of *S. undulatus* and *S. radiatus* in the lower Mississippi River drainage is necessary to determine whether *S. radiatus* does indeed occur in the southern Mississippi and Big Black rivers or if accounts are based on misidentified *S. undulatus* specimens.

***Strophitus williamsi* new species Smith et al.**

Flatwoods Creekshell

Holotype: UF439319 (Fig. 5b), length 47 mm, Bruce Creek on Walton Bridge Road, about 14 km SSW of Ponce de Leon (30.6142°N; 86.0128 °W), Walton County, Florida, 22 Apr. 2011.

Paratypes: UF441102, lengths 41.53–53.56 mm, 8 wet specimens, Bruce Creek on Walton Bridge Road, about 14 km SSW of Ponce de Leon (30.6142 °N; 86.0128 °W), Walton County, Florida, 22 Apr. 2011.

UF438158, lengths 27.99–39.41 mm, 10 wet specimens, Sepulga River upstream of CR29, about 11 miles WSW of Georgiana (31.58155°N; 86.91755 °W), Conecuh County, Alabama, 17 Sept. 2013.

Etymology: The specific epithet *williamsi* is in honor of James D. Williams in recognition of his contributions to the conservation and natural history of North American freshwater biodiversity.

Diagnosis: *Strophitus williamsi* resembles its sister species *S. radiatus* but the majority of individuals were more laterally compressed (width 35–42% of length vs. 30–40%) and less elongate (height 52–64% of length vs. 48–61%) compared with *S. radiatus* (Fig. 5; Table 3 in Smith et al., 2017). *Strophitus williamsi* is distinguished from other members of the species complex by 6 diagnostic nucleotides at COI (261:A, 300:G, 363:G, 411:A, 612:G, 663:A) and 9 diagnostic nucleotides at ND1 (24:C, 90:C, 123:A, 231:A, 273:T, 326:C, 423:G, 489:T, 768: C) (Table 2 in Smith et al., 2017).

Description: Shell- Length to 53.6 mm; thin; moderately inflated; oval to elliptical outline; posterior margin rounded to bluntly pointed. Max height 52–64% of total length. Max width 35–42% of total length. Low posterior slope and ridge; umbo broad and slightly elevated above the hinge line. Periostracum dark olive with variable green rays. Weak pseudocardinal tooth present, with one tooth per valve. Nacre bluish white.

Distribution: *Strophitus williamsi* is endemic to the Escambia and Choctawhatchee drainages.

Remarks: *Strophitus williamsi* has not been discovered in the Yellow River drainage and this warrants further investigation as many taxa that occur in the Escambia and Choctawhatchee drainages are also found in the Yellow River (Williams et al., 2014). The pattern of biogeography that we observed in *S. williamsi* matches a multitude of endemic freshwater mussel species to the Escambia, Yellow, and Choctawhatchee river drainages that are not found in the adjacent drainages: *Elliptio mcmichaeli* (Clench and Turner, 1956), *Fusconaia escambia* (Clench and Turner, 1956), *Hamiota australis* (Simpson, 1900), *Obovaria choctawensis* (Athearn, 1964), *Pleurobema strodeanum* (Wright, 1898), *Quadrula succissa* (Lea, 1852), and *Reginaia rotulata* (Wright, 1899) (Williams et al., 2014).

***Strophitus pascagoulaensis* new species Smith et al.**

Pascagoula Creekshell

Type Material: Holotype: UF439322 (Fig. 5c), length 38 mm, Buckatunna Creek at Causeyville Road (32.26286°N; 88.56979°W), Lauderdale County, Mississippi, 09 Sept. 2014.

Paratypes: UF438202, length 33.62–56.88 mm, 11 wet specimens, Buckatunna Creek at Causeyville Road (32.26286°N; 88.56979°W), Lauderdale County, Mississippi, 09 Sept. 2014.

Etymology: The common name, Pascagoula Creekshell, and the species name *pascagoulaensis* are to reflect the type locality.

Diagnosis: *Strophitus pascagoulaensis* resembles its putative sister taxa *S. sp.* cf. *pascagoulaensis* but is distinguished from other members of the species complex by 10 diagnostic nucleotides at COI (222:A, 267:A, 375:A, 429:C, 471:C, 498:G, 568:A, 571: C, 627: T, 657: G), 14 diagnostic nucleotides at ND1 (304:A, 318:G, 327:C, 411:C, 432:C, 466:T, 471:T, 486:G, 555:G, 565:A, 576:C, 700:A, 745:G, 750:A), and 3 diagnostic nucleotides at ITSI (52:A, 56:C, 406:G) (Table 2 in Smith et al., 2017).

Description: Shell- Length to 58.1 mm; thin; moderately inflated; oval to elliptical outline; posterior margin rounded to bluntly pointed. Max width 52–63% of total height. Max width 33–42% of total length. Low posterior slope and ridge; umbo broad and slightly elevated from hinge line. Periostracum light brown to yellow-green with variable green rays. Weak pseudocardinal tooth present, with one tooth per valve. Nacre bluish white.

Distribution: *Strophitus pascagoulaensis* is endemic to the Pascagoula, Pearl, and Lake Pontchartrain drainages.

Remarks: A formal species description for *Strophitus* sp. cf. *pascagoulaensis* (Fig. 5d) awaits further investigation to include additional specimens from the Pearl and Amite drainages. However, the putative species is extremely rare in the Pearl River drainage. *Strophitus* sp. cf. *pascagoulaensis* is listed as an imperiled species by the Mississippi Natural Heritage Program (The Nature Conservancy, 2004).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.10.018.

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