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RESEARCH ARTICLE

Generic reclassification and species boundaries in the rediscovered freshwater mussel '*Quadrula*' *mitchelli* (Simpson in Dall, 1896)

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Abstract The Central Texas endemic freshwater mussel, *Quadrula mitchelli* (Simpson in Dall, 1896), had been presumed extinct until relict populations were recently rediscovered. To help guide ongoing and future conservation efforts focused on *Q. mitchelli* we set out to resolve several uncertainties regarding its evolutionary history, specifically its unknown generic position and untested species boundaries. We designed a molecular matrix consisting of two loci (*cytochrome c oxidase subunit I* and *internal transcribed spacer I*) and 57 terminal taxa to test the generic position of *Q. mitchelli* using Bayesian inference and maximum likelihood phylogenetic reconstruction. We also employed two Bayesian species validation methods to test five a priori species models (i.e. hypotheses of species delimitation). Our study is the first to test the generic position of *Q. mitchelli* and we found robust support for its inclusion in the genus *Fusconaia.* Accordingly, we introduce the binomial, *Fusconaia mitchelli* comb. nov., to accurately represent the systematic position of the species. We resolved *F. mitchelli* individuals in two well supported and divergent clades that were generally distinguished as distinct species using Bayesian species validation methods, although alternative hypotheses of species delineation were also supported. Despite strong evidence of genetic isolation within *F. mitchelli*, we do not advocate for species-level status of the two clades as they are allopatrically distributed and no morphological, behavioral, or ecological characters are known to distinguish them. These results are discussed in the context of the systematics, distribution, and conservation of *F. mitchelli*.

Keywords Unionidae · Species rediscovery · Species delimitation · Bayesian phylogenetics and phylogeography · *Fusconaia*

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Introduction

More human-mediated freshwater mollusk extinctions have occurred in the North American rivers draining to the Gulf of Mexico than any other region on Earth and represent greater than a third of all known modern freshwater mollusk extinctions (Regnier et al. 2009). However, in the past decade at least eight of these "extinct" mollusks have been rediscovered (Campbell et al. 2008; Ó Foighil et al. 2011; Randklev et al. 2012; Whelan et al. 2012), including *Quadrula mitchelli* (Simpson in Dall, 1896), a Central Texas endemic freshwater mussel. Historically *Q. mitchelli* was thought to be distributed across much of Central and West Texas including the Brazos, Colorado, Guadalupe, and Rio Grande drainages, but due to its nearly complete absence from decades of survey work it had been presumed extinct (Howells 1994; Howells et al. 1997; Howells 2006; Haag 2009; Howells 2010; Burlakova et al. 2011; Haag and Williams 2013). Relict populations of *Q. mitchelli* have recently been rediscovered in each drainage of its historic range, except the Rio Grande (Randklev et al. 2013b). The small and disjunct nature of the remaining populations make *Q. mitchelli* a high conservation priority and regulations at both the state and federal level are underway to mandate its conservation (Texas Register 2010; USFWS 2009). To help guide ongoing and future conservation efforts focused on *Q. mitchelli* we set out to resolve several uncertainties regarding its evolutionary history, specifically its uncertain generic position and untested species boundaries.

Previous classifications, based largely on shell characters, have allied *Q. mitchelli* with several Nearctic and Mesoamerican genera (i.e., *Barynaias, Elliptio, Nephronaias, Quadrula, Quincuncina,* and *Sphenonaias*) (Simpson 1900, 1914; Frierson 1927; Strecker 1931; Wurtz 1950; Haas 1969; Howells 2010) but no phylogenetic test of generic position has been undertaken. In recent decades, *Q. mitchelli* was placed in the genus *Quincuncina* along with *Quin. burkei, Quin. infucata,* and *Quin. kleiniana* due to the shared occurrence of shell sculpturing and brooding larvae in both the inner and outer demibranchs (i.e., tetrageny) (Wurtz 1950; Burch 1975; Coney and Taylor 1986; Turgeon et al. 1988; Howells et al. 1996). Molecular evidence presented by Lydeard et al. (2000) demonstrated that *Quincuncina* was not a valid genus and its constituent species belonged to either *Fusconaia* (i.e., *F. burkei*) or *Quadrula* (i.e., *Q. infucata* and *Q. kleiniana*). However, Lydeard et al. (2000) did not address the implications of this result as it relates to the systematic position of *Q. mitchelli*. Subsequent phylogenetic analyses focused on *Fusconaia* or *Quadrula* also did not address the systematic position of *Q. mitchelli* and it remains unresolved (Serb et al. 2003; Burlakova et al. 2012; Campbell and Lydeard 2012). Since the rediscovery of *Q. mitchelli* it has been primarily treated as a member of the genus *Quadrula* (Howells 2010; Randklev et al. 2012; Mabe and Kennedy 2013; Randklev et al. 2013a, b, c; Sowards et al. 2013; Mabe and Kennedy 2014), although two online initiatives consider it a *Fusconaia* (Graf and Cummings 2014; IUCN 2015).

Although *Fusconaia* and *Quadrula* share various traits (e.g., quadrate/subquadrate shell shape, shell sculpturing, and tetragenous brooding) these genera are distantly related and resemble each other due to convergent evolution rather than shared common ancestry (Graf and Ó Foighil 2000; Graf and Cummings 2006). The untested placement of *Q. mitchelli* in one of two ecologically and behaviorally different genera is an unacceptable foundation for conservation efforts to build on. Without a classification that reflects common ancestry, conservation biologists cannot confidently make inferences about the essentially unknown biology of *Q. mitchelli*. Our phylogenetic test of the systematic position of *Q. mitchelli* necessitates generic reclassification and we advocate for the new binomial, *Fusconaia mitchelli* comb. nov (used hereinafter). The generic reclassification of *F. mitchelli* is discussed in the context of the biology of closely related *Fusconaia* spp. and how stakeholders can leverage this information in regards to future conservations efforts.

Another fundamental problem concerning the conservation of *F. mitchelli* (and many freshwater mussels in general) is the lack of clear species boundaries. Delineation of freshwater mussel species has traditionally relied on authoritative interpretation of highly plastic shell characters and poses many problems for distinguishing molluscan species boundaries, including the "splitting" of morphologically variable species (e.g., *Anodonta cygnea* >400 synonyms) and the "lumping" of superficially similar but independently evolving lineages (i.e., cryptic species) (Knowlton 2000; Graf 2007; Graf and Cummings 2007). In the past several decades systematic malacology has turned to various molecular methods to help distinguish species boundaries (e.g., molecular clades diagnosed by fixed life history traits and phenetic distances), but the coalescent-based models developed by the burgeoning field of statistical species delimitation have yet to be implemented (Fujita et al. 2012; Carstens et al. 2013). Given the biological, economic, and political importance of accurately distinguishing species boundaries, statistical species delimitation methods are quickly becoming a fundamental research need, especially in regard to taxon-based conservation efforts (Niemiller et al. 2013; Hedin 2015). We utilized two coalescent-based species validation methods, as well as several operational criteria for measuring lineage separation (e.g. reciprocal monophyly, molecularly diagnostic, morphologically distinct), to explore the species boundaries of *F. mitchelli* with a focus on the validity of a junior synonym *Fusconaia iheringi* (Wright, 1898). Furthermore, issues regarding the taxonomic availability and validity of *Sphenonaias taumilapana* (Conrad, 1855), also considered a junior synonym of *F. mitchelli* (despite the former's earlier description), are discussed in the context of the distribution and conservation of *F. mitchelli*.

Materials and methods

Taxon and character sampling

To test the systematic position of *F. mitchelli* we designed our ingroup taxon sampling to include representative genera of each tribe in the subfamily Ambleminae, focusing on the tribes Pleurobemini and Quadrulini. The outgroup consisted of a representative of the subfamily Unioninae (Table 1). We employed two molecular markers to reconstruct the phylogeny: the nuclear-encoded ribosomal *internal transcribed spacer 1* (*ITS1*) and the mitochondrial protein-coding *cytochrome c oxidase subunit I* (*COI*). Tissue samples or non-lethal tissue swabs were preserved in 95 % ethanol and DNA was isolated using a modified

Table 1 Taxa analyzed in the phylogenetic analyses with associated metadata

Taxa	Accession (*COI* / *ITS1*)	Voucher (*COI* / *ITS1*)	Drainage	References (*COI* ; *ITS1*)
Tribe ANODONTINI				
Anodonta suborbiculata	KT285619 / KT285663	MMNS10163	Mississippi	*
Tribe AMBLEMINI				
Amblema plicata 1	KT285618 / KT285662	FLMNH441152	Sabine	*
Amblema plicata 2	AF156512 / AY294561	UMMZ 265698 / no voucher	Great Lakes	Graf and Ó Foighil (2000); Manendo et al. (2008)
Amblema neislerii	KT285617 / KT285661	FLMNH437977	Apalachicola	*
Tribe PLEUROBEMINI				
Elliptio fumata	KT285621 / KT285665	FLMNH441058	Chattahoochee	*
Elliptio crassidens	KT285622 / KT285666	FLMNH441250	Ohio	*
Elliptio dilatata	AF156506 / DQ383440	UMMZ265700 / UAUC2735	Great Lakes / Tennessee	Graf and Ó Foighil (2000); Campbell et al. (2008)
Elliptoideus sloatianus	KT285623 / KT285667	FLMNH441118	Apalachicola	*
Fusconaia askewi 1	KT285625 / KT285669	FLMNH441253	Sabine	*
Fusconaia askewi 2	KT285624 / KT285668	FLMNH441157	Sabine	*
Fusconaia askewi 3	KT285626 / KT285670	FLMNH441253	Sabine	*
Fusconaia burkei 1	KT285627 / KT285671	FLMNH441049	Choctawhatchee	*
Fusconaia burkei 2	KT285628 / KT285672	FLMNH441129	Choctawhatchee	*
Fusconaia cerina	AY613823 / DQ383441	UAUC3233 / UAUC3376	Mobile	Campbell et al. (2005); Campbell et al. (2008)
Fusconaia escambia 1	KT285630 / KT285674	FLMNH441048	Escambia	*
Fusconaia escambia 2	KT285631 / KT285675	FLMNH428548	Escambia	*
Fusconaia escambia 3	KT285632 / KT285676	FLMNH428548	Escambia	*
Fusconaia escambia 4	KT285633 / KT285677	FLMNH441031	Escambia	*
Fusconaia flava 1	AF232822 / DQ383442	UAUC146	Ohio	Lydeard et al. (2000); Campbell et al. (2008)
Fusconaia flava 2	KT285634 / KT285678	FLMNH375436	Red	*
Fusconaia flava 3	KT285635 / KT285679	FLMNH375436	Red	*
Fusconaia flava 4	KT285636 / KT285680	FLMNH375436	Red	*
Fusconaia mitchelli 1	KT285651 / KT285695	FLMNH441081	Guadalupe	*
Fusconaia mitchelli 2	KT285652 / KT285696	FLMNH441082	Guadalupe	*
Fusconaia mitchelli 3	KT285653 / KT285697	Photo voucher	Guadalupe	*
Fusconaia mitchelli 4	KT285654 / KT285698	Photo voucher	Guadalupe	*
Fusconaia mitchelli 5	KT285637 / KT285681	FLMNH438156	Brazos	*
Fusconaia mitchelli 6	KT285638 / KT285682	FLMNH438156	Brazos	*
Fusconaia mitchelli 7	KT285639 / KT285683	FLMNH438156	Brazos	*
Fusconaia mitchelli 8	KT285650 / KT285694	FLMNH438010	Colorado	*
Fusconaia mitchelli 9	KT285640 / KT285684	FLMNH438155	Colorado	*
Hemistena lata	AY613825 / DQ383443	UAUC2797	Clinch	Campbell et al. (2005)
Plethobasus cyphyus	AY613828 / DQ383445	UAUC1639 / UAUC3157	Clinch	Campbell et al. (2005); Campbell et al. (2008)
Pleurobema clava	AY655013 / DQ383449	UAUC1477	Allegheny	Campbell et al. (2005); Campbell et al. (2008)
Pleurobema collina	AY613830 / DQ383450	UAUC1074	James	Campbell et al. (2005); Campbell et al. (2008)
Pleurobema cordatum	EF619917 / DQ383451	UAUC2926 / UAUC3530	Tennessee	Genbank; Campbell et al. (2008)
Pleurobema pyriforme	KT285645 / KT285689	FLMNH441228	Flint	*
Pleurobema ridelli	KT285646 / KT285690	FLMNH441165	Sabine	*
Pleurobema sintoxia	GU085308 / DQ470006	Psin1 / UAUC1714	St. Croix / Tennessee	Boyer et al. (2011); Campbell et al. (2008)
Pleurobema strodeanum	KT285647 / KT285691	FLMNH441231	Escambia	*
Pleuronaia dolabelloides	AY6132827 /AY772175	UAUC2819 / no voucher	Duck / Tennessee	Campbell et al. (2005); Grobler et al. (2006)
Tribe QUADRULINI				
Cyclonaias tuberculata	HM230410 / HM230353	UAM1490	Tennessee	Genbank
Quadrula apiculata	KT285648 / KT285692	FLMNH441088	Colorado	*
Quadrula houstonensis	KT285649 / KT285649	FLMNH441135	Brazos	*
Quadrula mortoni	KT285655 / KT285699	FLMNH441171	Sabine	*
Quadrula petrina	KT285656 / KT285700	FLMNH441084	Guadalupe	*
Tritogonia verrucosa	KT285657 / KT285701	FLMNH441208	Colorado	*
Uniomerus declivis	KT285659 / KT285703	FLMNH438312	Sabine	*
Tribe LAMPSILINI				
Cyrtonaias tampicoensis	KT285620 / KT285664	FLMNH441144	Colorado	*
Glebula rotundata	KT285642 / KT285686	FLMNH440905	Guadalupe	*
Lampsilis teres	KT285644 / KT285688	FLMNH441218	Brazos	*
Leptodea fragilis	KT285643 / KT285687	FLMNH441212	Brazos	*
Truncilla macrodon	KT285658 / KT285702	FLMNH441137	Colorado	*
Villosa lienosa	KT285660 / KT285704	FLMNH441251	Red	*
incertae sedis Ambleminae				
Reginaia ebenus 1	AY654999 / HM230352	UAUC71 / UAM3149	Tennessee / Coosa	Campbell et al. (2005); Genbank
Reginaia ebenus 2	KT285629 / KT285673	FLMNH438113	Mobile	*
Reginaia rotulata	KT285641 / KT285685	FLMNH441101	Escambia	*

Sequences generated in this study are denoted with *

plate extraction protocol of Ivanova et al. (2006) or a Gentra PureGene Tissue Kit (Qiagen Inc.), respectively. Primers for polymerase chain reaction (PCR) and sequencing were as follows: COI dgLCO-1490—GGTCAACAAATCATAAAGAYATYGG and COI dgHCO-2198—TAAACTTCAGGGTGACCAAARAAYCA (Meyer 2003); ITS-1 18S—AAAAAGCTTCCGTAGGTGAACCTGCG and ITS-1 5.8S—AGCTTGCTGCGTTCTTCATCG (King et al. 1999). The PCR plate amplifications were conducted using 27 µl reactions with the following reagents and volumes: H_20 (14.74 µl), 5X TaqMaster PCR enhancer (5.4 µl) (5 Prime, Inc.), magnesium solution (2.7 µl @ 25 mM) (5 Prime, Inc.), dNTP (0.54 µl @ 10 µM), primers (0.54 µl @ 10 µM), *Taq* (0.54 µl @ 5 U/µl), and DNA template

(2.0 μl). Unpurified PCR product was sent to the Interdisciplinary Center for Biotechnology Research at the University of Florida for bidirectional Sanger sequencing on an ABI3730. Chromatograms were assembled and edited using Geneious v 6.1.2 (http://www.geneious.com, Kearse et al. 2012).

Genetic analyses

Sequences were aligned in Mesquite v 2.7.5 (Maddison and Maddison 2011) using ClustalW (Larkin et al. 2007). The *COI* alignment was translated into amino acids and contained no stop codons. Minor adjustments to the *ITS1* alignment were made by eye. The molecular matrix was divided into four partitions: three partitions for the protein coding *COI* (one partition per codon position) and one partition for *ITS1*. jModelTest v 2.1.4 (Darriba et al. 2012) was used to find the best fit model of nucleotide substitution for each partition according to the Akaike information criterion. Loci were analyzed independently (*COI* only and *ITS1* only) and in concatenation (*COI* + *ITS1*) using both maximum likelihood (ML) and Bayesian inference (BI), totaling six independent reconstructions. Phylogenetic analyses were conducted in RAxML v 8.0.0 (Stamatakis 2014) and MrBayes v 3.2.2 (Ronquist et al. 2012) using the CIPRES Science Gateway (Miller et al. 2010). Maximum likelihood analyses were conducted using 1000 tree searches and nodal support was measured using 2000 rapid bootstraps. Bayesian inference analyses were implemented using 2 runs of 8 chains for 24×10^6 generations sampling every 1000 trees and omitting the first 8000 as burn-in. Convergence of the two runs was monitored by the average standard deviation of split frequencies and the Potential Scale Reduction Factor (PSRF). We used a S–H test (Shimodaira and Hasegawa 1999) and Bayes factors to test if various positive and negative topological constraints were significantly different than the optimal topology (Table 2). Bayes factors were measured using two times the difference of the -*ln* likelihood (2*ln*Bf) and interpreted following the methods of Kass and Raftery (1995) as modified by Nylander et al. (2004) (i.e., 2*ln*Bf 0-2: “not worth a bare mention”, 2–6: “positive” support, 6–10: “strong” support, >10: “very strong” support).

Haplotype networks for both markers were generated independently using 95 % parsimony connection limits in TCS1.21 (Clement et al. 2000). Uncorrected p-distances were calculated in Mesquite using the default settings (i.e., estimate ambiguity differences and do not count gaps vs. non gaps as differences). The relationship between average uncorrected p-distance and geographic distance was measured using a Mantel test as implemented on the Isolation by Distance Web server using 1000 randomizations (Jensen et al. 2005). We measured contemporary river and coastal distances between the four *F. mitchelli* collection localities using the Distance Measurement Tool in Classic Google Maps.

Species delimitation

We employed *BEAST v 1.8.0 (Heled and Drummond 2010) using Bayes factors species delimitation (BFD; Grummer et al. 2013) and Bayesian phylogenetics and phylogeography v 2.2 (BPP; Rannala and Yang 2003; Yang and Rannala 2010) as species validation methods. We used a unified concept of species that identifies species as separately evolving metapopulation lineages and evaluated lineage separation by the accumulation of properties species may or may not acquire over their existence (de Queiroz 1998, 2007). The molecular matrix used for these analyses included only the *Fusconaia* individuals and markers in Table 1. *BEAST was executed using 4×10^8 generations saving every 40,000th tree and removing the first 10 % as burn-in. The substitution models determined by jModelTest caused Markov Chain Monte Carlo (MCMC) convergence problems diagnosed by the low effective sample size of various statistics summarized in the program Tracer v 1.5 (Rambaut and Drummond 2009). To obtain convergence we used the simpler HKY model for each partition but maintained the same site heterogeneity models resolved in jModelTest (following Grummer et al. 2013). An uncorrelated relaxed molecular clock was fixed at 1.0 for the *ITS1* partition and was estimated for the three *COI* partitions. Yule process was utilized as the species tree prior with a piecewise linear and constant root population size. We tested five a priori species models by partitioning samples into putative lineages based on the geography and preexisting taxonomy of *F. mitchelli*; species model 1—*F. mitchelli* present in the Guadalupe, Colorado, and Brazos drainages; species model 2—*F. mitchelli* in the Guadalupe and Colorado drainages, and an undescribed species in the Brazos drainage; species model 3—*F. mitchelli* in the Guadalupe drainage, and *F. iheringi* in the Colorado and Brazos drainages; species model 4—*F. mitchelli* in the Guadalupe and Brazos drainages, and *F. iheringi* in the Colorado drainage; species model 5—*F. mitchelli* in the Guadalupe drainage, *F. iheringi* in the Colorado drainage, and an undescribed species in the Brazos drainage (Fig. 1). The marginal likelihood of each species model was estimated using harmonic mean estimation (HME), smoothed harmonic mean estimation (sHME; Newton and Raftery (1994) as modified by Suchard et al. (2001)), Path-Sampling (PS; Lartillot and Philippe (2006)), and Stepping Stone (SS; Xie et al. (2011)). sHME was measured using 1000 bootstrap replicates in Tracer. Path sampling and SS marginal likelihood estimation were performed on each species model using a

Table 2 Statistical comparison of topological constraints and the optimal topology generated in the BI and ML concatenated analyses

Maximum likelihood					Bayesian inference		
	ln	P value	SD	Topology	*ln*	2*ln*BF	Topology
Optimal	−9324.70	–	–	–	−9333.20	–	–
"*Quadrula*"	−9416.81	<0.01	16.17	Significantly worse	−9419.21	172.02	Significantly worse
Brazos monophyletic	−9324.99	>0.05	3.079	No difference	−9325.50	−15.4	Significantly better
Negative constraints							
Guadalupe group not monophyletic					−9340.13	13.86	Significantly worse
Colorado/Brazos group not monophyletic					−9348.55	29.6	Significantly worse

"*Quadrula*" constraint requires *Fusconaia mitchelli* to be resolved in a paraphyletic *Quadrula* in respect to *Cyclonaias* and *Tritogonia*

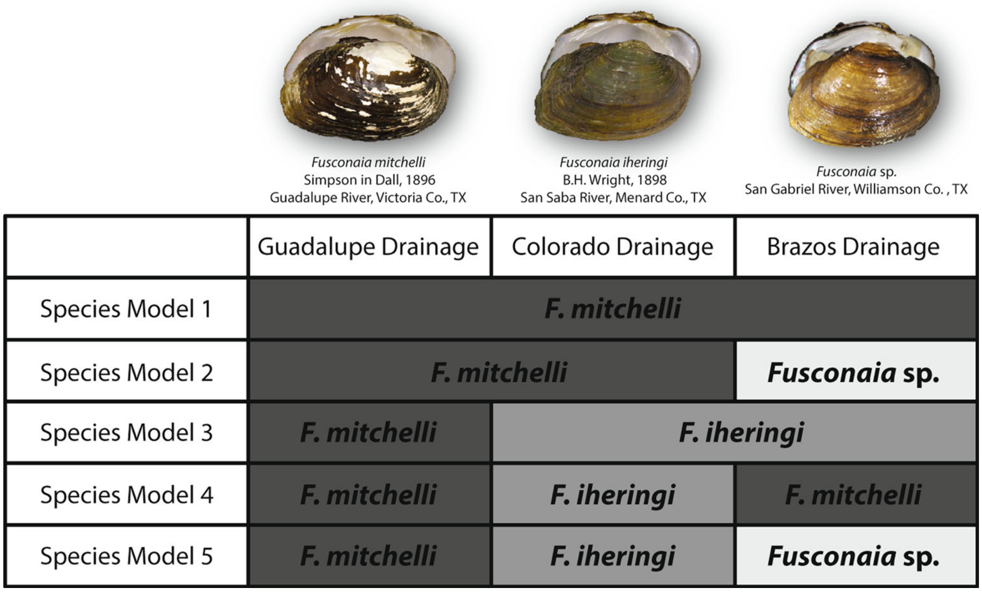

Fig. 1 Depiction of the five a priori species models used in *BEAST and the photographed types of *Fusconaia mitchelli* (USNM128364) and *Fusconaia iheringi* (USNM152171) (courtesy of www.mussel-project.uwsp.edu) and a sequenced *Fusconaia mitchelli* individual from the Brazos River drainage

chain length of 10^7 and 100 path steps in *BEAST (Baele et al. 2012, 2013).

Species delimitation using BPP was performed using 500,000 reversible-jump MCMC generations sampling every 5th generation with a burn-in of 10,000. The species delineation variable was set to 1 using algorithm 0 and a fine-tune parameter of 2. The supraspecific relationships within *Fusconaia* resolved in the *BEAST analyses were used as the guide tree. The effects of ancestral population size (θ) and root age (τ) priors on the speciation probabilities were assessed using six population demographic scenarios. Each prior was assigned a Gamma G(α,β) distribution with a prior mean = α/β, and a prior variance = α/β^2. The six population demographic priors are as follows: Scenario 1—Large ancestral population sizes ($\theta \sim$ G(1,10)) with deep divergences ($\tau \sim$ G(1,10)); Scenario 2—Large ancestral population sizes ($\theta \sim$ G(1,10)) with shallow divergences ($\tau \sim$ G(2,2000)); Scenario 3—Moderate ancestral population sizes ($\theta \sim$ G(1.5, 150)) with deep divergences ($\tau \sim$ G(1,10)); Scenario 4—Moderate ancestral population sizes ($\theta \sim$ G(1.5, 150)) with shallow divergences ($\tau \sim$ G(2,2000)); Scenario 5—Small ancestral population sizes ($\theta \sim$ G(2,2000)) with deep divergences ($\tau \sim$ G1,10)); and Scenario 6—Small ancestral population sizes ($\theta \sim$ G(2,2000)) with shallow divergences ($\tau \sim$ G(2,2000)). Each scenario was run four times utilizing different starting trees to ensure convergence of the runs and consistency among the speciation probabilities. We averaged the speciation probabilities of the four runs of each scenario and interpreted probabilities >95 % as strong evidence of cladogenesis (Leaché and Fujita 2010; Yang and Rannala 2010).

Results

We generated a molecular matrix consisting of 20 genera and 38 species represented by 57 terminals aligned to 1271 nucleotides (nt). Each of the terminal taxa are represented by both *COI* (avg = 634 nt) and *ITS1* (avg = 509 nt). The average percentage of gaps per taxon in the *ITS1* alignment was 17.1 %. The *COI* alignment had no indels or stop codons. We sequenced *F. mitchelli* individuals from the three drainages in which it has been rediscovered; Guadalupe (*Fm*1-4), Brazos (*Fm*5-7), and Colorado (*Fm*8,9), including topotypic material for *F. iheringi* and near topotypic for *F. mitchelli* (two counties upstream) (Table 1). Novel sequences generated in this study (n = 88) represent over 75 % of the total data set. Thirteen terminals are represented by previously published sequences downloaded from Genbank (https://www.ncbi.nlm.nih.gov/genbank/), eight of which are chimeric terminals (i.e., *COI* and *ITS1* sequences not generated from the same individual). We used the following nucleotide substitution models for BI: *COI* POS 1—GTR + GAMMA; *COI* POS 2—HKY; *COI* POS 3—GTR + GAMMA + I; *ITS1*—SYM + GAMMA. We used GTR + GAMMA for all partitions in the ML analysis given the models available in RAxML and the recommendations in the manual (Stamatakis 2006). Clear convergence of the two BI runs was supported by the average of the standard deviation of split frequencies (0.0019) and the average PSRF value (1.000).

The optimal topology (i.e., the one with the highest likelihood) was generated using BI and the concatenated matrix (*COI* + *ITS1*) and strongly supported *F. mitchelli* as a member of the genus *Fusconaia* (Fig. 2). Constraining *F. mitchelli* as a member of a paraphyletic *Quadrula* (in respect to *Cyclonaias* and *Tritogonia*) resulted in models that were significantly worse in both ML (SH-test ≤ 0.01) and BI ($2ln\text{BF} = 172.02$) (Table 2). *Fusconaia mitchelli* was resolved with weak support as sister to a clade

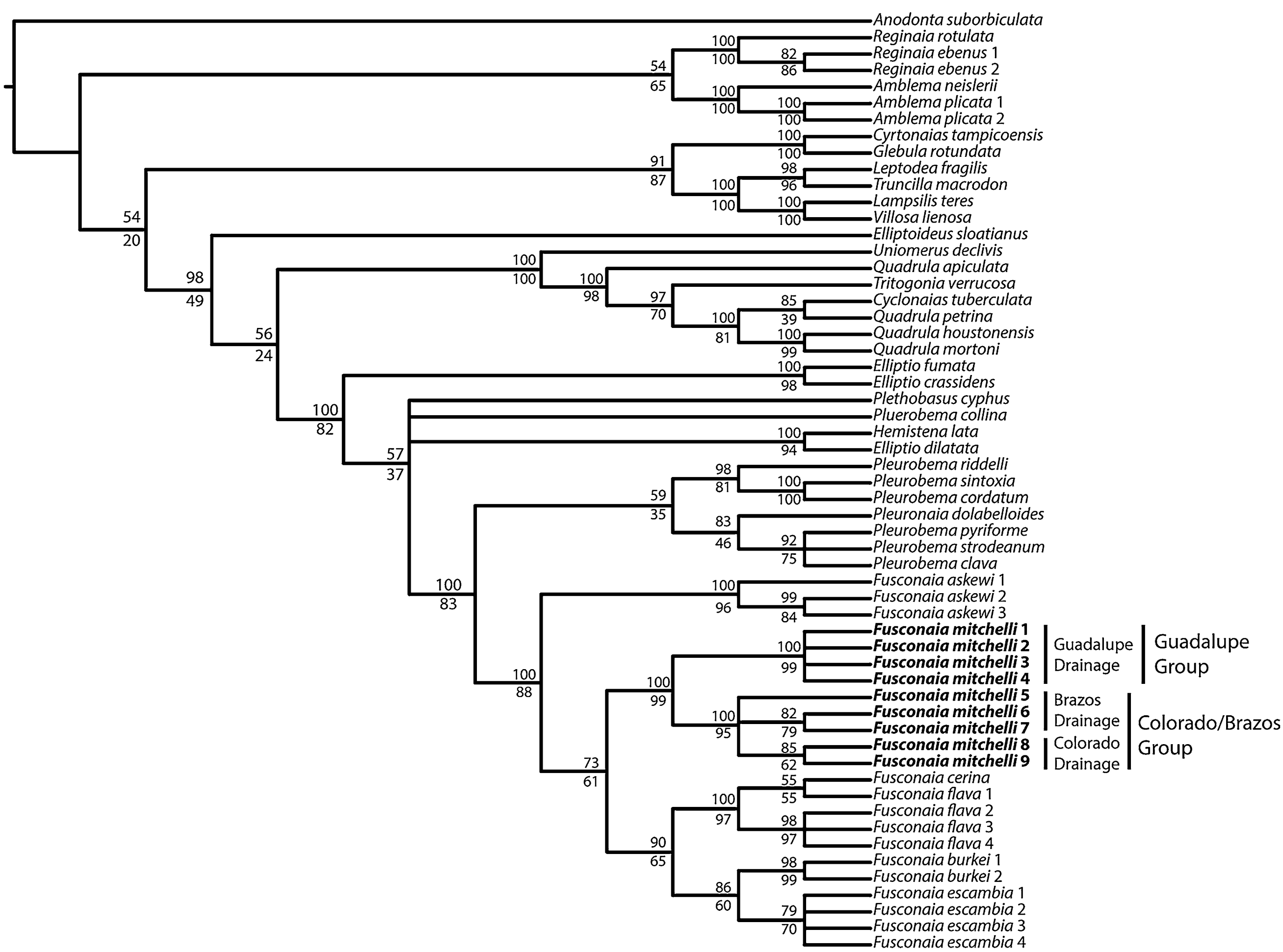

Fig. 2 Most likely topology generated in the BI concatenated analysis. Values above and below the branch lengths represent BI posterior probability (PP) and ML bootstrap support (BS), respectively

composed of mutually monophyletic *F. escambia* and *F. burkei*, and the *F. flava/cerina* species complex.

Each reconstructed phylogeny resolved *F. mitchelli* individuals in two divergent clades; the Guadalupe group (*Fm*1-4) and Colorado/Brazos group (*Fm*5-9). Bayesian posterior probabilities (PP) and ML bootstrap support (BS) values strongly supported the Guadalupe group as monophyletic in the concatenated analyses (Fig. 2) and both loci independently (Table 3). Individuals from neither the Colorado nor the Brazos were strongly supported (i.e., <90 PP and <70 BS) as geographically independent clades in any of the six analyses. The two drainages together (i.e., Colorado/Brazos group) were supported as monophyletic in each analysis except the ML *ITS1* analysis (Table 3). *ITS1* provided little resolution within *Fusconaia*: the only clades resolved with >50 PP/BS were the Guadalupe group and the Colorado/Brazos group (Table 3). The combined and *COI* analyses resolve the Guadalupe group and the Colorado/Brazos as sister to each other, while the *ITS1* analyses resolved the two clades in a polytomy with the other *Fusconaia* representatives.

Negative constraints employed on a monophyletic Guadalupe group or a monophyletic Colorado/Brazos group resulted in topologies that were significantly worse than the optimal topology (Table 2). Negative constraints were only applied in BI as this option is unavailable in RAxML.

Table 3 Comparison of nodal support and uncorrected p distances within *Fusconaia* spp. in this study

Taxa	Drainage (# of indv.)	Nodal support (PP/BS)			*COI* sequence divergence (%)			*ITS1* sequence divergence (%)		
		Combo	*COI*	*ITS1*	Min	Max	Mean	Min	Max	Mean
F. mitchelli	Colorado (2)	85/62	81/58	-/-	0.74	0.74	0.74	0	0	0
F. mitchelli	Brazos (3)	-/-	-/-	-/-	0.15	0.91	0.61	0	0	0
F. mitchelli	Guadalupe (4)	100/99	100/99	98/70	0	0.30	0.15	0	0.19	0.10
F. mitchelli	Colorado/Brazos group (5)	100/95	100/88	98/68	0.56	1.27	0.81	0	0	0
F. mitchelli	Colorado/Brazos group versus Guadalupe group (9)	100/99	100/99	-/-	1.52	2.22	1.79	0.39	0.59	0.43
F. flava	Mississippi (4)	-/-	-/-	-/-	0	1.55	.82	0	0	0
F. cerina	Mobile (1)	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. flava and *F. cerina*	Mississippi versus mobile (5)	100/97	100/95	-/-	1.33	2.43	1.65	0	0	0
F. escambia	Escambia (4)	79/70	71/71	-/-	0	0.31	0.15	0	0.21	0.10
F. burkei	Choctawhatchee (2)	98/99	97/99	-/-	0	0	0	0	0	0
F. escambia and *F. burkei*	Escambia versus Choctawhatchee (6)	86/60	71/46	-/-	0.77	1.11	0.86	0	0.21	0.07
F. askewi	Sabine (3)	100/96	100/98	-/-	0.33	0.49	0.42	0	0.19	0.13

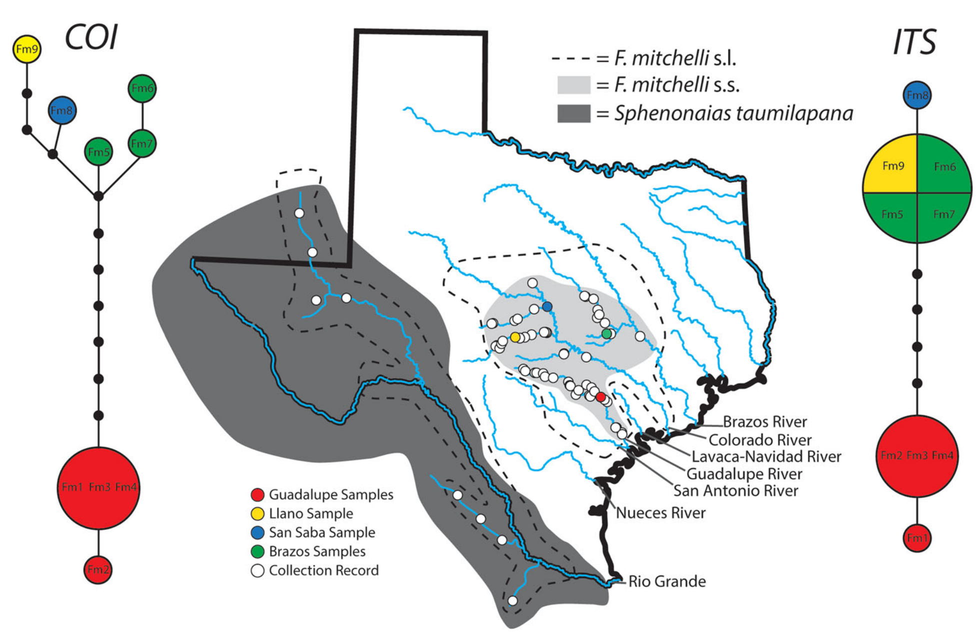

Fig. 3 Mitochondrial (*COI*) and nuclear (*ITS1*) haplotype networks of the nine *Fusconaia mitchelli* individuals. Each labeled circle represents a sampled haplotype with size relative to its observed frequency and color corresponding to genetic sampling localities. Map of Texas river systems with the approximate range of *F. mitchelli s.l.* (redrawn from Randklev et al. (2013b)), *F. mitchelli* s.s., and *S. taumilapana*

Constraining the Brazos individuals to be monophyletic resulted in topologies that were either not significantly different (ML: S–H >0.05) or significantly better (BI: −15.4 2*ln*BF) than the optimal topology (Table 2).

The *CO1* and *ITS1* haplotype networks depicted two distinct groups corresponding to individuals from the Guadalupe drainage and individuals from the Colorado and Brazos drainages (Fig. 3). The two clusters were separated by seven *COI* substitutions and five *ITS1* substitutions. Four of the five Colorado and Brazos drainage individuals shared the same *ITS1* haplotype; otherwise haplotypes were not shared between individuals in different drainages. There are 12 fixed nt differences that distinguish the Colorado/Brazos group from the Guadalupe group (*COI*: 7 nt; *ITS1*: 5 nt) (Fig. 4). There is only one fixed mitochondrial position between individuals from the Colorado and Brazos drainages (*COI* position 372). The minimum and maximum *COI* sequence divergence between the Guadalupe and Colorado/Brazos group was 1.52 and 2.22 percent, more than two times greater than the difference between allopatric and morphologically distinct *F. escambia* and *F. burkei* (Table 3). There was no significant correlation between genetic and geographic distance within *F. mitchelli* (*COI*: mantel's r = 0.18, p = 0.63; *ITS1*: mantel's r = 0.14 p = 0.59).

BFD found significant differences between the various species models but was dependent on the marginal likelihood estimation method implemented (Table 4). Harmonic mean estimation and sHME produced consistently higher estimated marginal likelihoods than PS and SS and did not discriminate any significant differences between the five species models. Path sampling and SS determined that species model 3 was the best model and was significantly better than species model 1 (2*ln*Bf: 13.52 and 13.55), species model 2 (2*ln*Bf: 11.85 and 12.01), and species model 4 (2*ln*Bf: 15.45 and 15.56). Species model 3 was only marginally better than species model 5 (2*ln*Bf: 0.54 and 0.64).

BPP scenarios 1–4 recognized the Guadalupe and Colorado/Brazos groups as distinct species consistent with species model 3. BPP scenarios 5 and 6 recognized *F. mitchelli* individuals in each drainage as distinct species, consistent with species model 5 (Fig. 5). The average speciation probability across all nodes varied from 78 (Scenario 1) to 98 (Scenario 6) and an increase in speciation probabilities was obvious in some nodes when the

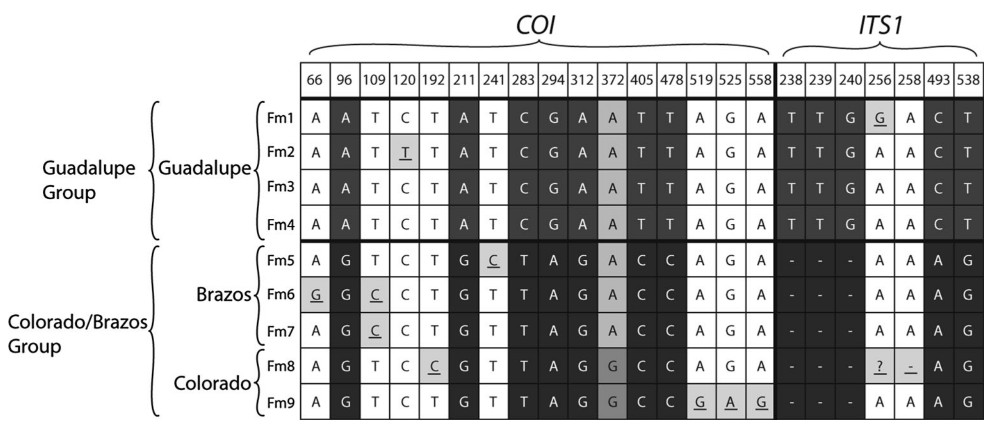

Fig. 4 Variable nucleotide positions within *Fusconaia mitchelli* s.s. Diagnostic nucleotide positions between the Guadalupe group and the Colorado/Brazos group are highlighted (*dark gray*) and in *white lettering*, diagnostic nucleotide between the Colorado and Brazos samples are highlighted (*gray*) and in *black lettering*, variable but not fixed differences are highlighted (*light gray*) with *lettering underlined*

Table 4 Comparison of the five species models in Fig. 1 using Bayes factor species delimitation (BFD; Grummer et al. 2013)

Species model	HME			sHME			PS			SS		
	ln	2*ln*BF	Reject	*ln*	2*ln*BF	Reject	*ln*	2*ln*BF	Reject	*ln*	2*ln*BF	Reject
Species model 1	−2074.270	2.60	No	−2085.251	4.57	No	−2205.441	**13.52**	Yes	−2205.369	**13.55**	Yes
Species model 2	−2073.346	0.75	No	−2083.417	0.90	No	−2204.610	**11.85**	Yes	−2204.602	**12.01**	Yes
Species model 3	−2073.581	1.22	No	−2083.767	1.60	No	**−2198.683**	**–**	**–**	**−2198.595**	–	–
Species model 4	−2073.475	1.01	No	−2083.537	1.14	No	−2206.406	**15.45**	Yes	−2206.375	**15.56**	Yes
Species model 5	−**2072.971**	–	–	−**2082.968**	–	–	−2198.951	0.54	No	−2198.916	0.64	No

Bolded "*ln*" values highlight the lowest negative log-likelihood generated using each likelihood estimation method. Bolded values under "2*ln*BF" are Bayes factors that are significantly worse than the best model under that estimation method

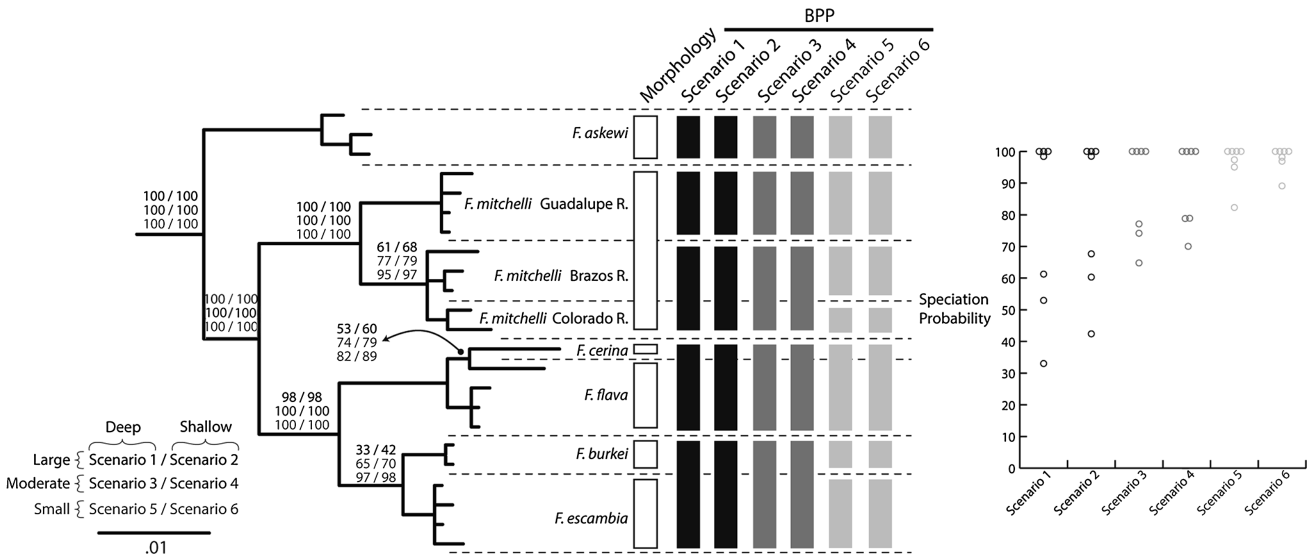

Fig. 5 Congruence and conflict in the BPP analyses among the six scenarios. Numbers above the branch lengths correspond to speciation probabilities generated in the six population demographic scenarios. Scatter plot shows the distribution of speciation probabilities generated in the six scenarios

population size prior was decreased (Fig. 5). None of the scenarios recognized *F. flava* and *F. cerina* as distinct species and only the scenarios assuming small ancestral population size (Scenarios 5 and 6) supported *F. escambia* and *F. burkei* as distinct species.

Discussion

Generic position

Our study is the first to test the generic position of *"Quadrula" mitchelli* and we find robust support for its inclusion in the genus *Fusconaia*. *Fusconaia mitchelli* is strongly supported in a clade exclusive to all other *Fusconaia* species included in the analysis (Fig. 2). Constraining *F. mitchelli* among a paraphyletic *Quadrula* (with respect to *Cyclonaias* and *Tritogonia*) resolved topologies that were significantly worse than the optimal topology (Table 2). Accordingly, we advocate for the binomial, *F. mitchelli,* to accurately represent the systematic position of the species. *Fusconaia mitchelli* is resolved as sister to a clade composed of mutually monophyletic *F. escambia* and *F. burkei,* and the *F. flava*/*cerina* species complex; however this relationship had weak support (Fig. 2). Greater sampling of *Fusconaia* spp. from the surrounding freshwater faunal regions, as well as sampling additional molecular markers, will provide better resolution of the intrageneric relationships of *Fusconaia,* which remain largely unresolved (Burdick and White 2007; Burlakova et al. 2012; Campbell and Lydeard 2012). However, the monophyly of *Fusconaia* is well supported and provides substantial predictive power in interpreting many aspects of the essentially unknown biology of *F. mitchelli*. Stakeholders can infer that *F. mitchelli* will likely brood eggs and larvae from early spring to late summer and can expect minnows (family Cyprinidae) to serve as the primary hosts (Bruenderman and Neves 1993; Haag and Warren Jr 1997, 2003; White et al. 2008; Williams et al. 2008). Equipped with a classification that reflects common ancestry, stakeholders can more effectively allocate their time and resources as it relates to characterizing life histories and establishing captive breeding programs, which are two of the most important components of conserving critically endangered mussel populations and species (Neves 1997; Haag and Williams 2013).

Species boundaries

There is strong molecular evidence for genetic isolation within *F. mitchelli*, especially between individuals from the Guadalupe drainage (*Fm*1-4 = Guadalupe group) and individuals from the Colorado and Brazos drainages (*Fm*5-9 = Colorado/Brazos group). The Guadalupe group and the Colorado/Brazos group are well supported as reciprocally monophyletic clades in five of the six phylogenetic reconstructions (Fig. 2; Table 3), do not share haplotypes (Fig. 3), and are molecularly diagnosable (Fig. 4), each of which are common molecular properties of species and have been previously used as criteria for delimiting species. However, the genetic isolation of these populations may reflect intraspecific geographic structuring rather than speciation, as these populations are allopatrically distributed, dispersal-limited, and no morphological, ecological, or behavioral characters are known to distinguish them. Recognizing that speciation is a process, we view the above properties not as absolute requirements of species but rather as attributes species may or may not acquire over their lifetime, and discuss them in the context of their operational relevance to measuring lineage separation within *F. mitchelli* (de Queiroz 2007).

Reciprocal monophyly has been criticized as being an unrealistic expectation of species delimitation because incomplete lineage sorting is common in recently diverged lineages, especially if slowly evolving nuclear loci are utilized in phylogenetic reconstruction (Hudson and Coyne 2002; Rannala and Yang 2003; Hickerson et al. 2006; Knowles and Carstens 2007; Zhang et al. 2011). Conversely, reciprocal monophyly has also been criticized for a tendency to overestimate species-level diversity by recognizing allopatric populations as distinct species, especially when populations are small, species are dispersal-limited, and/or only quickly evolving mitochondrial markers are used (de Queiroz 2007; Frankham et al. 2012; Fujita et al. 2012; Giarla et al. 2014). If limited dispersal and allopatry alone were driving the exclusive coalescence of alleles in *F. mitchelli* we might expect three well supported and divergent clades corresponding to the three allopatric populations. We find strong nuclear and mitochondrial support for only two divergent and well supported *F. mitchelli* clades, the Guadalupe group and the Colorado/Brazos group. Individuals from neither the Colorado nor the Brazos were strongly supported as geographically monophyletic clades in any of the six analyses; however the two drainages together (i.e., the Colorado/Brazos group) were supported in an unresolved clade in each analysis except the ML *ITS1* analysis (Fig. 2; Table 3). The reciprocal monophyly of the Guadalupe group and the Colorado/Brazos group supports species delimitation consistent with species model 3 (i.e., *F. mitchelli* in the Guadalupe, *F. iheringi* in the Colorado and Brazos).

However, the soft polytomy of the Colorado and Brazos individuals does not constitute evidence against the possibility of three geographic clades (Fig. 2). Analyses constraining the individuals from Brazos drainage to be monophyletic resolved tree topologies that were either not significantly different (ML) or significantly better (BI) than the optimal topology (Table 2). That is, we cannot reject the possibility that each allopatric population is reciprocally monophyletic and would, under some species concepts (e.g. phylogenetic species concept), be consistent with species model 5. However, the overall genetic similarity of the Colorado and Brazos samples (discussed below) more closely resembles intraspecific geographic variation than speciation.

Mitochondrial and nuclear markers each reveal two distinct haplotype groups corresponding to individuals from the Guadalupe drainage and individuals from the Colorado and Brazos drainages (Fig. 3). Despite the allopatry of the Colorado and Brazos populations they are genetically very similar. Four of the five Colorado and Brazos samples have identical ITS1 haplotypes, suggesting recent or ongoing gene flow between the populations. Of the 23 variable sites among individuals of *F. mitchelli* over half are fixed for the Guadalupe group, while only a single fixed *COI* nt position distinguishes individuals from the Colorado and Brazos drainages (Fig. 4). The Guadalupe group is easily diagnosable from the Colorado/Brazos group at both mitochondrial and nuclear markers suggesting clear genetic isolation between the two groups. The genetic differences among individuals of *F. mitchelli* are not merely products of isolation by distance (*COI*: $p = 0.63$, and *ITS1*: $p = 0.59$). In fact the most geographically distant populations calculated by contemporary river distance (San Saba and Brazos) are genetically more similar than the two geographically closest populations (San Saba and Llano) (Fig. 3).

Bayesian species validation methods also found significant genetic isolation within *F. mitchelli* but each supported two conflicting species models (i.e. species model 3 and 5). BFD resolved significant differences between the various species models but was dependent on the method of marginal likelihood estimation. Harmonic mean estimation and sHME did not discriminate significant differences between any of the five species models (Table 4). The poor discriminatory power of HME and sHME has been expounded elsewhere and was an impetus for developing PS and SS (Lartillot and Philippe 2006; Xie et al. 2011; Grummer et al. 2013). Both PS and SS likelihood estimation determined species model 3 as the best model and was decisively better than every other species model except species model 5. Species model 3 was only marginally better than species model 5 and cannot reject it as a potential hypothesis of species delimitation using Bayes factors.

The species model supported by BPP varied depending on the population demographic scenario implemented (Fig. 5). Four of the six BPP scenarios supported species delineation consistent with species model 3 (Scenarios 1–4). Scenarios 5 and 6 delimited a distinct species in each of the three drainages, consistent with species model 5. Speciation probabilities varied considerably according to the demographic priors implemented; generally they increased with decreasing population size (Fig. 5). This analytical bias towards over-splitting species using BPP has been documented several times in naturally fragmented systems or dispersal-limited species, and rather than delimiting species, may delimit genetically isolated populations (Barley et al. 2013; Carstens and Satler 2013; McKay et al. 2013; Miralles and Vences 2013; Satler et al. 2013; Giarla et al. 2014; Hedin 2015).

Each of our methods found strong support for genetic isolation within *F. mitchelli*, but none give conclusive support for a single species model. Although genetic isolation is a necessary property of speciation, it is not exclusive to such and can also be a product of extrinsic factors such as habitat fragmentation, population bottlenecks, genetic drift, and dispersal limitation. The problem of distinguishing between the genetic architecture of speciation and intraspecific variation of dispersal-limited animals using statistical species delimitation is well known and can be mitigated by relying on multiple non-molecular lines of evidence, such as morphological, behavioral, or ecological traits (Yang and Rannala 2010; Zhang et al. 2011; Fujita et al. 2012; Carstens et al. 2013; Giarla et al. 2014; Hedin 2015). Given the paucity of morphological, behavioral, and ecological data available for *F. mitchelli* and the consistent conflict in the supported species models, we cannot justify regarding the two allopatric clades as distinct species. In the absence of non-molecular synapomorphies and a single supported species model, we error on the side of conservatism and treat *F. mitchelli* as a single species with limited or no gene flow between the drainage-specific populations. However, given that accurate descriptions of morphology, life history, and ecological requirements are major priorities for mussel species conservation (Neves 1997; Haag and Williams 2013), future research may reveal population specific characteristics that could justify the recognition of multiple species (e.g. host use, infection strategy, larval morphology).

Although the number of *F. mitchelli* individuals and loci used in this study was small, our assessment provides a useful and timely foundation for the conservation research community to build on. The implications of accurately delimiting critically endangered species are so great that the coupling of data-rich morphological, ecological, and behavioral data sets with rigorous genome-scale molecular analysis is becoming a fundamental research priority of many taxon-based conservation efforts (Hedin 2015; McCormack and Maley 2015) and given the imperiled status and untested boundaries of many freshwater mussel species such methodology stands to be an important tool in freshwater mussel systematics and conservation.

Systematics and distribution of *F. mitchelli*

Considering that a precise understanding of the historic and current range of species is one of the most important criteria in determining species conservation status, we address and raise some important questions concerning the taxonomy and range of *F. mitchelli.* Various authorities have considered the range of *F. mitchelli* to extend west to the Rio Grande drainage based on the hypothesis that it is synonymous with *Sphenonaias taumilapana* (Howells et al. 1996; Johnson 1999; Howells 2010; Randklev et al. 2013b) (= *F. mitchelli* sensu lato). Although *S. taumilapana* was described 41 years before *F. mitchelli,* the former has incorrectly been regarded as a junior synonym of the latter, presumably because Coney and Taylor (1986) considered *S. taumilapana* as *nomen dubium*. However, the nomenclatural opinion of Coney and Taylor (1986) has no bearing on the taxonomic availability of the specific epithet *taumilapana* (i.e., it can still compete for synonymy) and should have retained priority over *F. mitchelli* if the two were considered to represent the same species (e.g., Frierson 1927; Strecker 1931; Haas 1969). However, based on the fact that *S. taumilapana* appears to be known only from fossil specimens and are generally much larger than *F. mitchelli* from Central Texas (Metcalf 1982; Johnson 1999; Howells 2001, 2003, 2010) we suspect, as have others (Simpson 1914; Metcalf 1982; Graf and Cummings 2007), that *S. taumilapana* is a species distinct from *F. mitchelli*. We follow the most recent global review of species-level freshwater mussel diversity (Graf and Cummings 2007) and recognize *Sphenonaias taumilapana* as valid and consider it endemic to the Rio Grande drainage.

As such, we recognize *F. mitchelli* sensu stricto as endemic to the Guadalupe, Colorado, and Brazos River drainages of Central Texas (Fig. 3). Despite various collecting efforts in the drainages to the east and west of the Brazos and Guadalupe Drainages, the Trinity and Nueces-Frio respectively, *F. mitchelli* s.s. has never been reported (Strecker 1931; Metcalf 1974; Murray 1981; Howells et al. 1996; Randklev et al. 2013b). In the Guadalupe River, *F. mitchelli* s.s. has been primarily reported from the mainstem but is also known from the lower portions of one of its largest tributaries, the San Marcos River (Howells 2010; Randklev et al. 2013b). Despite the San Antonio River watershed comprising over 40 % of the Guadalupe's drainage area, only a single subfossil valve of putative *F. mitchelli* s.s. has been reported (Howells 2002; catalog no. RGH.2001.001). However the weathered condition of the aforementioned valve precludes confident identification to any species and relegates the evidence for the presence of *F. mitchelli* s.s. in the San Antonio River watershed to little or none.

In the Colorado and Brazos River drainages *F. mitchelli* s.s. is known mostly from its larger catchments, particularly the Llano and San Saba, and the Little River watersheds, respectively. *Fusconaia mitchelli* s.s. has been reported from two sites from the Brazos River proper, one of which considerably extends its northern most range (Howells 2010; Randklev et al. 2013b). However this northernmost record (catalog no. OSUM50994: two subfossil valves) represents a misidentified *Truncilla macrodon* and further reduces the known historic range of *F. mitchelli* s.s. While further museum and fieldwork is necessary to more fully determine the historic and current range of *F. mitchelli* s.s., our brief review resolves and raises some important questions concerning its range and should have implications regarding future conservation assessments.

Conclusion

Heretofore, the generic position and species boundary of *Fusconaia mitchelli* were untested and represented a fundamental research need in regards to taxon-based conservation efforts. Understanding the evolutionary history of *F. mitchelli* s.s. can help mitigate the time and data constraints that characterize conservation efforts focused on poorly understood and highly threatened taxa. We hope that this phylogenetic framework can be utilized by the conservation research community to infer aspects of the biology of *F. mitchelli* s.s., apply basic principles of conservation genetics, stimulate future taxonomic and ecological hypotheses, and ultimately help sustain its future.

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