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

Chase H. Smith[1,2,3] | Nathan A. Johnson[4] | Kaitlyn Havlik[1] | Robert D. Doyle[1] | Charles R. Randklev[2]

[1]Biology Department, Baylor University, Waco, TX, USA

[2]Texas A&M Natural Resources Institute, Texas A&M AgriLife Research Center at Dallas, Dallas, TX, USA

[3]Department of Integrative Biology, University of Texas, Austin, TX, USA

[4]U.S. Geological Survey, Wetland and Aquatic Research Center, Gainesville, FL, USA

Correspondence
Chase H. Smith, Department of Integrative Biology, University of Texas, Austin, TX 78712, USA.
Email: chase.smith@austin.utexas.edu

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Abstract

Species are a fundamental unit of biology, and defining accurate species boundaries is integral to effective conservation and management of imperiled taxa. Freshwater mussels (Bivalvia: Unionidae) are among the most imperiled groups of organisms in North America, yet species boundaries remain uncertain for many taxa. The False Spike, *Fusconaia mitchelli* (Simpson in Dall, 1895), is a freshwater mussel considered to be endemic to central Texas (Brazos, Colorado, and Guadalupe drainages). Recent research revealed significant intraspecific genetic variation between geographically separated populations of *F. mitchelli*, which could be indicative of speciation; however, small sample sizes for several of the populations precluded formal taxonomic revision. Here, we increase taxon sampling and use multilocus DNA sequence data and traditional morphometrics to re-evaluate species boundaries in *F. mitchelli*. We sequenced three loci: the protein-coding mitochondrial DNA genes *cytochrome c oxidase subunit 1* and *NADH dehydrogenase 1*, and the nuclear *internal transcribed spacer 1*. Phylogenetic analyses depicted deep genetic divergence between *F. mitchelli* in the Guadalupe and those in the Brazos and Colorado drainages, which was further supported by available biogeographic information. Morphometric analyses and coalescent-based species delimitation models integrating both DNA sequence and morphological data provided strong support for the divergence observed between the two geographically isolated clades of *F. mitchelli*. Based on these results, we revise taxonomy accordingly by elevating the junior synonym *Fusconaia iheringi* (Wright, 1898) to represent the Brazos and Colorado populations and restrict the distribution of *F. mitchelli* to the Guadalupe River drainage. Our findings may impact pending management decisions to protect *F. mitchelli* under the U.S. Endangered Species Act.

KEYWORDS

Balcones Spike, conservation, *Fusconaia iheringi*, integrative taxonomy, phylogenetics

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1 | INTRODUCTION

Species are a fundamental unit of biology, and defining accurate species boundaries is integral to effective conservation and management of imperiled taxa. Freshwater mussels (Bivalvia: Unionida) are a group of aquatic invertebrates comprised of approximately one thousand species worldwide (Graf & Cummings, 2007; Lopes-Lima, Burlakova, et al., 2018), and the greatest diversity of freshwater mussels lies within North America with at least three hundred native species in the family Unionidae alone (Graf & Cummings, 2007; Williams et al., 2017). Mussels contribute significant ecological benefits to freshwater ecosystems by integrating the fluvial food web and providing and stabilizing benthic habitat (Haag & Williams, 2014; Vaughn, 2018; Vaughn, Nichols, & Spooner, 2008). Freshwater mussels are also a compelling system in the study of evolutionary biology. This is due to the unionid life cycle which involves parasitic larvae (glochidia) that must attach to vertebrate hosts (primarily fish) prior to becoming adults (Barnhart, Haag, & Roston, 2008). These coevolutionary relationships have led to a variety of life-history strategies across species, resulting in subsequent biological diversification (Barnhart et al., 2008; Haag, 2012).

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

Molecular genetic studies on freshwater mussels that address phylogenetic placement and species boundaries have been pivotal in inferring important biological characteristics of species (e.g., host use, reproductive traits, habitat preference) and ensuring the taxonomic validity of protected species or those being considered for protection (Johnson et al., 2018; Pfeiffer, Johnson, Randklev, Howells, & Williams, 2016; Smith, Johnson, Pfeiffer, & Gangloff, 2018). Although considerable progress has been made in understanding ecology (Dudding, Hart, Khan, Robertson, Lopez, & Randklev 2019; Hart, Haag, Bringolf, & Stoeckel, 2018; Johnson, McLeod, Holcomb, Rowe, & Williams, 2016; Sietman, Hove, & Davis, 2018) and evolution (Inoue, Harris, Robertson, Johnson, & Randklev, 2020; Lopes-Lima et al., 2017; Pfeiffer, Atkinson, et al., 2019; Pfeiffer, Breinholt, & Page, 2019; Smith, Johnson, Inoue, Doyle, & Randklev, 2019; Smith, Pfeiffer, & Johnson 2020) of freshwater mussels in recent years, many species still remain poorly understood and species validity has not been confirmed using robust molecular genetic analyses (Haag, 2012; Lopes-Lima, Burlakova, et al., 2018). This is certainly the case in the southwestern United States, where diversity, distribution, and ecology of many mussel species are still poorly understood (Haag, 2012; Howells, Neck, & Murray, 1996).

Morphology-driven taxonomic hypotheses in the freshwater mussel tribe Pleurobemini Hannibal, 1912 have been largely invalidated by molecular genetic analyses and resolving accurate phylogeny has been integral toward understanding the evolution of this group (Campbell & Lydeard, 2012b; Inoue et al., 2018). For members in the genus *Fusconaia* Simpson, 1900 in Texas, there have been multiple systematic changes in recent years using DNA sequence data and some sympatric species are even morphologically indistinguishable (Campbell & Lydeard, 2012a; Pfeiffer et al., 2016; Pieri et al., 2018). One member of this genus, *Fusconaia mitchelli* (Simpson in Dall, 1895) or the False Spike, is endemic to the Brazos, Colorado, and Guadalupe drainages of central Texas (Howells et al., 1996). *Fusconaia mitchelli* was presumed extinct until its recent rediscovery in 2011 when several individuals were collected from the lower Guadalupe River (Randklev et al., 2012). At the time of rediscovery, the validity and systematic position of *F. mitchelli* were unknown. The taxon was assigned to the genus *Quadrula* Rafinesque, 1820 based on morphology and phylogenetic positioning of assumed closely related species (Howells et al., 1996; Randklev et al., 2012; Randklev, Tsakiris, Johnson, et al., 2013); however, taxonomic placement remained an enigmatic issue toward understanding the basic biology and ecology of the species. Recent molecular genetic analyses revealed that *F. mitchelli* belonged to the genus *Fusconaia* rather than *Quadrula*, and also reported two distinct clades within *F. mitchelli* corresponding to the Brazos and Colorado drainages, and the Guadalupe drainage (Pfeiffer et al., 2016). Despite high levels of divergence between the two clades, recognizing two distinct species within *F. mitchelli* warranted increased taxon sampling, additional molecular markers, and morphological or life-history data.

Species boundaries in *F. mitchelli* remain a significant knowledge gap for natural resource managers, as conservation efforts based on current taxonomic hypotheses (TPWD, 2010; USFWS, 2009a, 2009b, 2010, 2011, 2016) may lead to unsubstantiated conclusions about its status and bias management and recovery actions. Given the importance of accurate taxonomy in conservation and management of imperiled taxa, the primary objective of this study was to resolve species boundaries within *F. mitchelli* by incorporating both DNA sequence and morphological data. Specifically, we set out to accomplish the following objectives: (a) use multilocus DNA sequence data to resolve a phylogeny of Pleurobemini, with emphasis on *F. mitchelli*; (b) delineate species boundaries within *F. mitchelli* using DNA sequence and morphometric data; and (c) discuss the

implications of our findings toward effective conservation and management practices.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

To evaluate species boundaries within *F. mitchelli*, we generated DNA sequence and morphological data for a total of 49 and 114 individuals of *F. mitchelli*, respectively, from the Brazos, Colorado, and Guadalupe river drainages. We also utilized 20 published sequences for *F. mitchelli* from Pfeiffer et al. (2016) available on GenBank and added additional loci to these individuals. Individuals representing all type species from genera in the Pleurobemini were also included, and *Quadrula quadrula* (Rafinesque, 1820) was selected to function as the root based off of previous molecular genetic assessments of Ambleminae Rafinesque, 1820 (Pfeiffer, Breinholt, et al., 2019).

2.2 | Molecular genetic analyses

Small mantle tissue clips from each specimen were preserved in 100% ethanol and stored at −80°C. For a subset of samples, Isohelix™ DNA swabs (Harrietsham, United Kingdom) were used to non-lethally sample individuals in the field. DNA was isolated from tissue clips and DNA swabs with the Qiagen Puregene DNA Extraction Kit following manufacturer's suggested protocols (Qiagen, Hilden, Germany). We used three loci in our investigation: a portion of the protein-coding mitochondrial genes *cytochrome c oxidase subunit 1* (*COX1*) and *NADH dehydrogenase subunit 1* (*ND1*), and the nuclear *internal transcribed spacer 1* (*ITS1*). The primer sequences used for PCR were as follows: *COX1* 5′-GTTCCACAAATCATAAGGATATTGG-3′ and 5′-TACACCTCAGGGTGACCAAAAAACCA-3′ (Campbell et al., 2005); *ND1* 5′-TGGCAGAAAAGTGCATCAGATTAAAGC-3′ and 5′-CCTGCTTGGAAGGCAAGTGTACT-3′ (Serb, Buhay, & Lydeard, 2003); and *ITS1* 5′-AAAAAGCTTCCGTAGGTGAACCTGCG-3′ and 5′-AGCTTGCTGCGTTCTTCATCG-3′ (King, Eackles, Gjetvaj, & Hoeh, 1999). Amplicon length for each locus was as follows: *COX1* = 709 bp, *ND1* = ~1,000 bp, and *ITS1* = ~640 bp. PCR was conducted using a 12.5 µl mixture of molecular grade water (4.25 µl), MyTaq Red Mix (Bioline; 6.25 µl), 10 µM primers (0.5 µl each), and DNA template (50 ng). PCR product was sent to Molecular Cloning Laboratories (MCLAB, South San Francisco, CA, USA) for bidirectional sequencing on an ABI3730. PCR product for *ITS1* was more difficult to sequence than mtDNA markers considering the possibility of length polymorphisms at *ITS1*. All individuals were sent directly for sequencing, similar to recent studies in freshwater mussels that yielded sequences that were readable without cloning (Johnson et al., 2018; Pfeiffer, Sharpe, Johnson, Emery, & Page, 2018; Pieri et al., 2018; Smith et al., 2018, 2019). Reliable *ITS1* sequences for five individuals of *F. mitchelli* could not be obtained due to substantial heterozygosity, and these individuals were not included in the phylogenetic reconstruction.

Sequences were aligned with MAFFT v 7.311 (Katoh & Standley, 2013) in Mesquite v 3.31 (Maddison & Maddison, 2017) using the L-INS-I method. The protein-coding mtDNA genes were translated into amino acids to ensure absence of stop codons and indels. A total of 70 individuals were included in our 3-locus molecular matrix for phylogenetic inference (Table 1). Of the 210 sequences, 138 were novel sequences generated in this study (Table 1). For the 44 individuals of *F. mitchelli* included in our phylogenetic analysis, 112 were novel sequences generated in this study (Table 1). Phylogenetic inference was performed using MrBayes v 3.2.6 (Ronquist et al., 2012). We utilized PartitionFinder v 2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) to find the best partition schemes and substitution models for the MrBayes analysis. The Bayesian information criterion (BIC) was selected, and branch lengths were linked. MrBayes analyses were conducted using 2 runs of 8 chains for 3×10^7 generations sampling every 1,000 trees. Tracer v 1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018) was used to determine the appropriate burn-in value and ensure convergence of all parameters (ESS > 200). In addition, convergence of the two runs was monitored using the potential scale reduction factor (PSRF) and the average standard deviation of split frequencies (ASDSF). PopART v 1.7 (Leigh & Bryant, 2015) was used to create haplotype networks for mtDNA loci (i.e., *COX1* and *ND1*) and *ITS1* using the TCS Network (Clement, Posada, & Crandall, 2000), and samples were grouped by drainage of origin (i.e., Brazos, Colorado, or Guadalupe). Nucleotide positions with gaps or missing data were not considered during creation of the haplotype networks. To further explore relationships within *F. mitchelli*, we used MEGA-X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) to compute uncorrected p-distances. All codon positions were included, ambiguous sections were handled using partial deletion, and individuals were grouped based on drainage of capture. We used MEGA-X to identify diagnostic sites that distinguish major clades of *F. mitchelli* (i.e., Brazos and Colorado, Guadalupe) at *COX1*, *ND1*, and *ITS1* independently.

To estimate divergence times among well-supported clades, we employed the coalescent-based model *BEAST (Heled & Drummond, 2010) in BEAST v 2.5.1 (Bouckaert et al., 2014). We utilized a coalescent approach considering concatenation methods typically overestimate the divergence times across species trees (Arbogast, Edwards, Wakeley, Beerli, & Slowinski, 2002; Ogilvie, Heled, Xie, & Drummond, 2016). We followed similar methodologies as Pieri et al. (2018) and created a molecular matrix including *Fusconaia* spp. used in BI (Table 1) and included *Pleurobema clava* (Lamarck, 1819) as the out-group. The molecular matrix was re-aligned, and substitution models were evaluated for each locus (i.e., *COX1*, *ND1*, *ITS1*) using PartitionFinder. A strict molecular clock was fit to each locus, and we used the *COX1* substitution rate of $2.56 \times 10^{-9} \pm 0.6 \times 10^{-9}$ substitutions per site per year (Froufe et al., 2016) for the *COX1* partition. Substitution rates were estimated for *ND1* and *ITS1* based on the *COX1* partition. Yule process was used as the species tree prior paired with a piecewise linear and constant root population size

TABLE 1 Material examined in this study for molecular genetic analyses with indication of river drainage where specimens were collected, catalog numbers, and GenBank accession numbers. Museum abbreviations are as follows: JBFMC—Joseph Britton Freshwater Mollusk Collection; NCSM—North Carolina Museum of Natural Sciences; UF—Florida Museum. DNA swab represents individuals sampled using non-lethal methods and NAs represent chimeric individuals from GenBank where collection information was variable. Novel data generated in this study are represented by GenBank accessions MN649033–MN649180

Taxon	Drainage	Source	*COX1*	*ND1*	*ITS1*
Elliptio crassidens (Lamarck, 1819)	Ohio	UF441250	MH633634	MH633586	MH362521
Elliptio crassidens	Ohio	UF441250	KT285622	MN649089	KT285666
Elliptoideus sloatianus (Lea, 1840)	Apalachicola	UF441118	KT285623	MN649081	KT285667
Eurynia dilatata (Rafinesque, 1820)	Tennessee	UF441302	MN649035	MN649084	MN649140
Eurynia dilatata	Tennessee	UF441302	MN649036	MN649085	MN649141
Fusconaia askewi (Marsh, 1896)	Sabine	UF441160	MF961824	MH133663	MH133813
Fusconaia askewi	Sabine	UF441253	KT285625	MH133668	KT285669
Fusconaia burkei (Walker, 1922)	Choctawhatchee	UF441129	KT285628	MH133770	KT285672
Fusconaia burkei	Choctawhatchee	UF441129	MN649034	MN649083	MN649139
Fusconaia chunii (Lea, 1862)	Trinity	UF439075	MF961853	MH133715	MH133855
Fusconaia chunii	Trinity	UF439075	MF961854	MH133716	MH133856
Fusconaia cor (Conrad, 1834)	NA	GenBank	HM230369	KT187953	KT188104
Fusconaia cuneolus (Lea, 1840)	NA	GenBank	AY654998	KT187960	KT188107
Fusconaia escambia Clench and Turner, 1956	Escambia	UF428548	KT285631	MH133772	KT285675
Fusconaia escambia	Escambia	UF428548	MN649040	MN649090	MN649145
Fusconaia flava (Rafinesque, 1820)	Red	UF375436	KT285634	MH133764	KT285678
Fusconaia flava	Red	UF375436	KT285636	MH133765	KT285680
Fusconaia iheringi (Wright, 1898)	Brazos	UF438156	KT285638	MN649099	KT285682
Fusconaia iheringi	Brazos	UF438156	KT285639	MN649100	KT285683
Fusconaia iheringi	Brazos	UF438156	MN649045	MN649101	MN649150
Fusconaia iheringi	Brazos	UF438156	KT285637	MN649102	KT285681
Fusconaia iheringi	Brazos	UF439060	MN649053	MN649110	MN649158
Fusconaia iheringi	Brazos	UF439060	MN649054	MN649111	MN649159
Fusconaia iheringi	Brazos	UF439060	MN649055	MN649112	MN649160
Fusconaia iheringi	Brazos	UF439060	MN649056	MN649113	MN649161
Fusconaia iheringi	Brazos	JBFMC8065.1	MN649078	MN649135	–
Fusconaia iheringi	Brazos	JBFMC8065.2	MN649079	MN649136	–
Fusconaia iheringi	Brazos	JBFMC8102.1	MN649057	MN649114	MN649162
Fusconaia iheringi	Brazos	JBFMC8102.2	MN649058	MN649115	MN649163
Fusconaia iheringi	Colorado	UF441083	MN649076	MN649133	–
Fusconaia iheringi	Colorado	UF438010	KT285650	MN649091	KT285694
Fusconaia iheringi	Colorado	UF438155	KT285640	MN649098	KT285684
Fusconaia iheringi	Colorado	UF438745	MN649052	MN649109	MN649157
Fusconaia iheringi	Colorado	JBFMC8089.1	MN649080	MN649137	–
Fusconaia iheringi	Colorado	JBFMC8502.1	MN649066	MN649123	MN649171
Fusconaia iheringi	Colorado	JBFMC8502.2	MN649067	MN649124	MN649172
Fusconaia iheringi	Colorado	JBFMC8502.3	MN649068	MN649125	MN649173
Fusconaia iheringi	Colorado	JBFMC8502.4	MN649069	MN649126	MN649174
Fusconaia iheringi	Colorado	JBFMC8502.5	MN649070	MN649127	MN649175
Fusconaia iheringi	Colorado	JBFMC8502.6	MN649071	MN649128	MN649176
Fusconaia iheringi	Colorado	JBFMC8502.7	MN649072	MN649129	MN649177
Fusconaia iheringi	Colorado	JBFMC8502.8	MN649073	MN649130	MN649178

(Continues)

TABLE 1 (Continued)

Taxon	Drainage	Source	*COX1*	*ND1*	*ITS1*
Fusconaia iheringi	Colorado	JBFMC8502.9	MN649074	MN649131	MN649179
Fusconaia iheringi	Colorado	JBFMC8502.10	MN649075	MN649132	MN649180
Fusconaia masoni (Conrad, 1834)	Neuse	UF438274	MF961941	MH133773	MH133892
Fusconaia masoni	Pamlico	UF438289	MF961942	MH133774	MH133893
Fusconaia mitchelli (Simpson in Dall, 1895)	Guadalupe	UF441081	KT285651	MH133775	KT285695
Fusconaia mitchelli	Guadalupe	UF441082	KT285652	MH133776	KT285696
Fusconaia mitchelli	Guadalupe	DNA Swab	MN649041	MN649092	MN649146
Fusconaia mitchelli	Guadalupe	DNA Swab	MN649042	MN649093	MN649147
Fusconaia mitchelli	Guadalupe	DNA Swab	MN649043	MN649094	MN649148
Fusconaia mitchelli	Guadalupe	DNA Swab	KT285653	MN649095	KT285697
Fusconaia mitchelli	Guadalupe	DNA Swab	KT285654	MN649096	KT285698
Fusconaia mitchelli	Guadalupe	DNA Swab	MN649044	MN649097	MN649149
Fusconaia mitchelli	Guadalupe	UF438139	MN649046	MN649103	MN649151
Fusconaia mitchelli	Guadalupe	UF438139	MN649047	MN649104	MN649152
Fusconaia mitchelli	Guadalupe	UF438139	MN649048	MN649105	MN649153
Fusconaia mitchelli	Guadalupe	UF438139	MN649049	MN649106	MN649154
Fusconaia mitchelli	Guadalupe	UF438139	MN649077	MN649134	–
Fusconaia mitchelli	Guadalupe	UF438549	MN649050	MN649107	MN649155
Fusconaia mitchelli	Guadalupe	UF438549	MN649051	MN649108	MN649156
Fusconaia mitchelli	Guadalupe	JBFMC8188.1	MN649059	MN649116	MN649164
Fusconaia mitchelli	Guadalupe	JBFMC8188.3	MN649060	MN649117	MN649165
Fusconaia mitchelli	Guadalupe	JBFMC8188.4	MN649061	MN649118	MN649166
Fusconaia mitchelli	Guadalupe	JBFMC8188.6	MN649062	MN649119	MN649167
Fusconaia mitchelli	Guadalupe	JBFMC8188.9	MN649063	MN649120	MN649168
Fusconaia mitchelli	Guadalupe	JBFMC8233.1	MN649064	MN649121	MN649169
Fusconaia mitchelli	Guadalupe	JBFMC8233.2	MN649065	MN649122	MN649170
Fusconaia subrotunda (Lea, 1831)	NA	GenBank	HM230405	KT187998	KT188110
Hemistena lata (Rafinesque, 1820)	Tennessee	UF439083	MN649038	MN649087	MN649143
Parvaspina steinstansana (Johnson and Clarke, 1983)	Pamlico	NCSM43401	MN649033	MN649082	MN649138
Plethobasus cyphyus (Rafinesque, 1820)	Clinch	DNA Swab	MN649039	MN649088	MN649144
Pleurobema clava (Lamarck, 1819)	NA	GenBank	AY655013	AY613802	DQ383449
Pleuronaia barnesiana (Lea, 1838)	Tennessee	UF438232	MN649037	MN649086	MN649142
Quadrula quadrula (Rafinesque, 1820)	Ohio	UF439156	MH633643	MH633595	MH362613

model. The analysis was run for 3×10^7 generations sampling every 5,000 generations and a 10% burn-in. Effective sample size (ESS) was ensured using Tracer, and a maximum clade credibility tree was created using TreeAnnotator v 2.5 (Bouckaert et al., 2014).

2.3 | Morphometric analyses

We collected morphometric data on external shell dimensions for 114 *F. mitchelli* museum specimens from all focal drainages (i.e., Brazos, Colorado, and Guadalupe), including 21 vouchered specimens used in genetic analyses. Three measurements were taken to the nearest 0.01 mm using digital calipers for morphological analyses: maximum length, height, and width. Measurements were $\log_e$-transformed to produce a scale-invariant matrix while preserving information about allometry (Jolicoeur, 1963; Kowalewski et al., 1997; Strauss, 1985) and subsequently converted into three ratios: height/length, width/length, and width/height. Morphological variation was assessed using a principal component analysis (PCA) in the package ggbiplot (Vu, 2011) and a canonical variate analysis (CVA) in the package Morpho (Schlager, 2017) using R v 3.5.3. PCAs allowed for visual inspection of morphological groupings without *a priori* assignment to a specific group. Canonical variate scores were used for cross-validated discriminant analyses (DAs) to assess the ability of morphological

data to assign individuals to (a) drainage of capture (i.e., Brazos, Colorado, Guadalupe) and (b) groupings depicted by DNA sequence data (Brazos + Colorado, Guadalupe). Additionally, we used a permutational multivariate analysis of variance (MANOVA) utilizing 1,000 iterations in the vegan package (Oksanen et al., 2016) to test for significant morphological differences between the Brazos + Colorado and Guadalupe. A significance level of $\alpha = 0.05$ was assumed when assessing the statistical significance.

2.4 | Species delimitation

We implemented the coalescent species delimitation model STACEY v 1.2.4 (Jones, 2017) via BEAST using all available DNA sequence data (*COX1*, *ND1*, and *ITS1*) for *F. mitchelli*. We used PartitionFinder to re-evaluate the best partition and substitution models for the STACEY analyses. We allowed the model to consider all individuals as minimum clusters and assign individuals to appropriate clusters considering STACEY infers species boundaries without *a priori* species designations. A strict molecular clock was set at 1.0 for the 1st position of *COX1* for both analyses, and remaining partitions were estimated by STACEY. Analyses executed 2×10^8 generations and logged every 5000th tree and a 10% burn-in. Tracer v 1.7.1 was used to ensure convergence of all parameters (ESS > 200). The most likely number of species clusters was calculated by SpeciesDelimitationAnalyser (SpeciesDA) v 1.8.0 (Jones, 2017) using a collapse height of 0.0001 and a simcutoff of 1.0.

We integrated DNA sequence and morphological data into a species delimitation framework using the program iBPP v. 2.1.3 (Solís-Lemus, Knowles, & Ané, 2015). This method uses the Bayesian Phylogenetics and Phylogeography (BP&P) model for coalescent species delimitation (Yang & Rannala, 2010) and integrates a Brownian motion model of trait evolution (Solís-Lemus et al., 2015). The data matrix used for the iBPP analysis consisted of all available *COX1*, *ND1*, and *ITS1* sequences for *F. mitchelli*, as well as the PC scores for the 3 PCs created from R to represent morphological differences. For the iBPP analysis, we set the species tree topology to the most likely species cluster scenario as resolved by STACEY (Brazos + Colorado and Guadalupe). We followed the most stringent methodologies presented by Pfeiffer et al. (2016) by using the priors $\theta \sim \Gamma(1, 10)$ and $\tau_0 \sim \Gamma(1, 10)$ for sequence data. A non-informative prior of 0 was used for the control parameters ν and κQ. Algorithm 0 was used as the species delimitation prior with an $\varepsilon = 2$ and default fine-tuning parameters (Yang & Rannala, 2010). We implemented 500,000 reversible-jump Markov chain Monte Carlo (rjMCMC) generations sampling every 10th generation with an initial burn-in of 10%. ESS > 200 for all parameters was ensured for adequate generation time and convergence.

2.5 | Range map

We compiled distributional data from freshwater mussel surveys conducted from 1898 to 2018 in the Brazos, Colorado, and Guadalupe drainages to assess both the contemporary and historical geographic distribution of *F. mitchelli*. Sources of the distribution data were as follows: Baylor University Mayborn Museum, Florida Museum, Fort Worth Museum of Science and History, Houston Museum of Natural Science, Joseph Britton Freshwater Mollusk Collection, Smithsonian National Museum of Natural History, Texas A&M Natural Resources Institute, Texas Department of Transportation, Texas Parks and Wildlife Department, University of Michigan Museum of Zoology, and U.S. Fish and Wildlife Service. We used distribution data for *F. mitchelli* to develop a conservation status assessment map using ArcMap 10.3 (ESRI) following protocols used in previous publications (Johnson et al., 2016; Smith et al., 2019). The spatiotemporal distribution of *F. mitchelli* was illustrated at the Hydrologic Unit Code (HUC) 10-level, and all known survey locations were included to illustrate presence or absence from 1900 to 2018.

3 | RESULTS

3.1 | Molecular genetic analyses

Our molecular matrix included 2,132 bp and a total of 49 *F. mitchelli* (including the five individuals without *ITS1*): Brazos (12), Colorado (15), and Guadalupe (22). Detailed information regarding individuals and alignments used in molecular genetic analyses is available in Table 1, GenBank (novel accessions: MN649033–MN649180), Alignments S1–S3, and ScienceBase (https://doi.org/10.5066/P9Y7K5CD). The best partitioning scheme and substitution models determined by PartitionFinder for the MrBayes analysis were as follows: HKY + G for *COX1* codon 3, SYM + I + G for *COX1* codon 1 and *ND1* codon 1, HKY + I for *COX1* codon 2 and *ND1* codon 2, HKY + G for *ND1* codon 3, and JC + G for *ITS1*. The phylogenetic reconstruction resolved *Fusconaia* as monophyletic and depicted two monophyletic clades within *F. mitchelli*: (a) Brazos + Colorado and (b) Guadalupe (Figure 1). The TCS networks for mtDNA and *ITS1* show clear separation between the Brazos + Colorado and Guadalupe groupings (Figure 2). Intra- and inter-drainage uncorrected p-distances for *F. mitchelli* as well as maximum and minimum values are reported in Table 2. Intra-drainage values for mtDNA markers ranged from 0% to 1.0%, and there was no divergence in *ITS1* for average p-distance (Table 2). For every marker, inter-drainage values for Brazos-Colorado were lower than Brazos-Guadalupe or Colorado–Guadalupe comparisons (Table 2). *Fusconaia mitchelli* from the Brazos + Colorado were diagnosable from the Guadalupe using mtDNA and nDNA sequence data: *COX1* (5), *ND1* (13), and *ITS1* (2 nucleotides and 1 indel).

Our molecular matrix used for *BEAST consisted of 60 individuals and 2,086 bp (*COX1* = 658 bp; *ND1* = 900 bp; *ITS1* = 528 bp). Substitution models for each locus were as follows: *COX1* − HKY + I, *ND1* − HKY + G, and *ITS1* − JC. Convergence of the *BEAST analysis was supported by all parameters having ESS values > 200. The *BEAST topology was generally congruent with BI and resolved two monophyletic clades within *F. mitchelli*

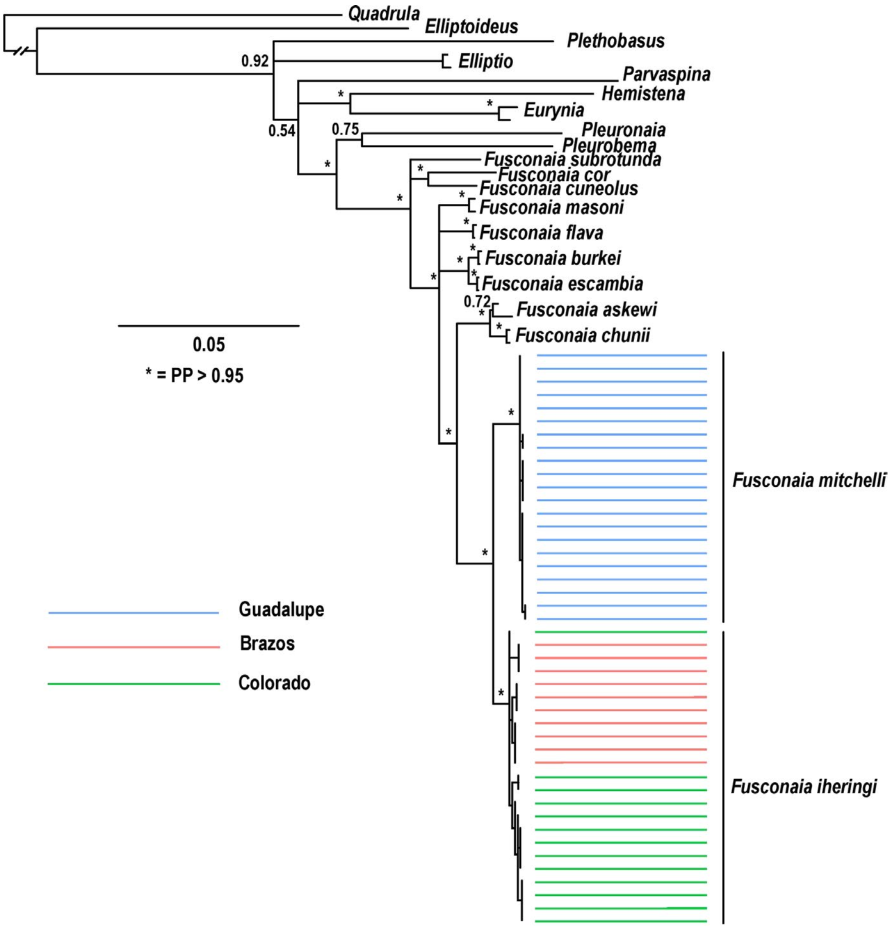

FIGURE 1 Consensus tree generated under Bayesian inference in MrBayes on our molecular matrix. Node labels indicate posterior probability (PP), and values >0.95 are represented by asterisks. Each colored line represents an individual *Fusconaia iheringi* or *Fusconaia mitchelli* sampled, and colors correspond to drainage of capture: red (*Fusconaia iheringi*—Brazos), green (*Fusconaia iheringi*—Colorado), and blue (*Fusconaia mitchelli*—Guadalupe)

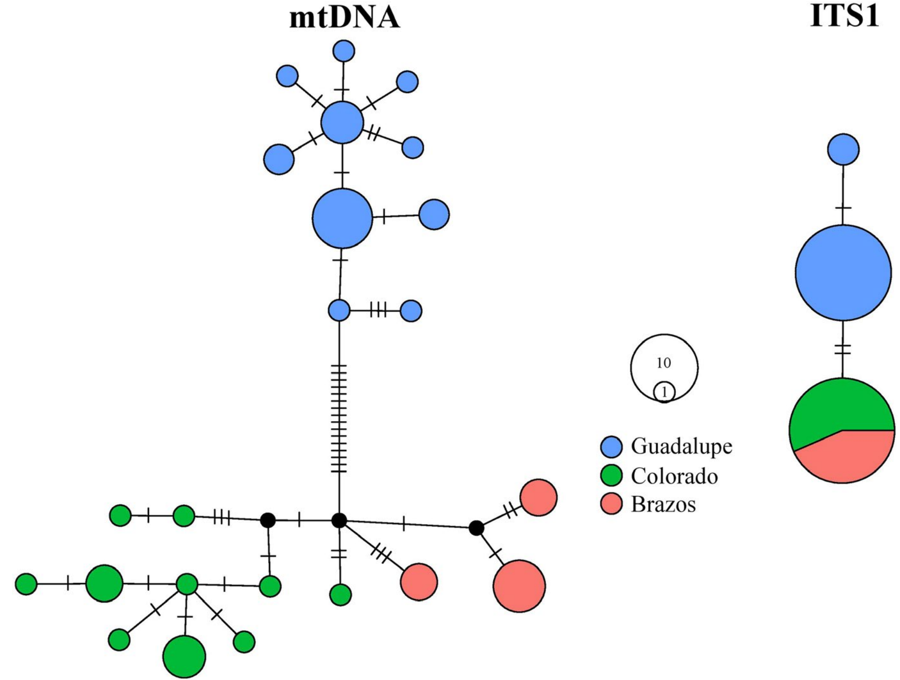

FIGURE 2 *Haplotype network generated from mitochondrial (COX1 and ND1) and nuclear (ITS1) DNA sequence data for Fusconaia iheringi and Fusconaia mitchelli. Dashes represent the number of substitutions between haplotypes, black circles indicate an unsampled haplotype, and colored circles represent a unique haplotype with size relative to the number of individuals with each haplotype. Colors indicate drainage of capture: red (Fusconaia iheringi—Brazos), green (Fusconaia iheringi—Colorado), and blue (Fusconaia mitchelli—Guadalupe)*

(i.e., Brazos + Colorado, and Guadalupe; Figures 1; 3). The split of *F. mitchelli* and east Texas lineages (i.e., *F. askewi* and *F. chunii*) was estimated to have occurred in the late Miocene, ~6.60 Mya (95% CI: 3.78–9.76 Mya; Figure 3). Subsequent diversification between *F. mitchelli* from the Brazos + Colorado and Guadalupe was estimated to have occurred ~3.18 Mya (95% CI: 1.75–4.92 Mya) in the Pliocene/Pleistocene epochs (Figure 3). The split between *F. mitchelli* from the Brazos and Colorado drainages was estimated

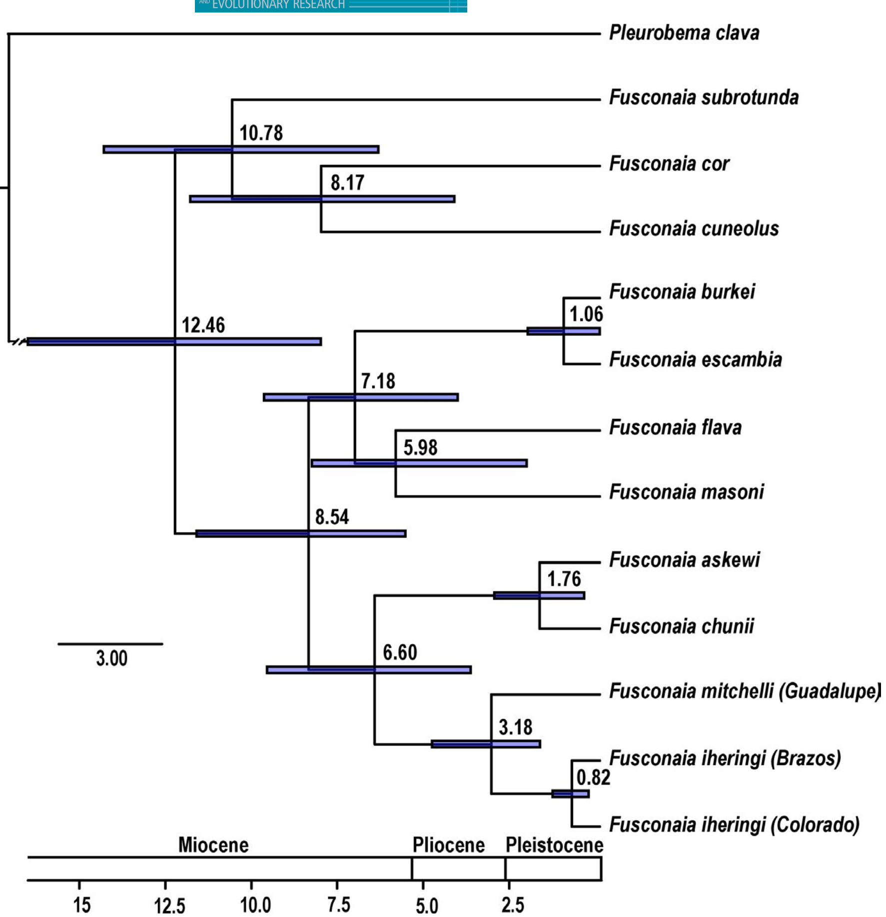

FIGURE 3 Maximum clade credible tree generated from divergence time estimations in *BEAST. Divergence time is scaled to million years before present and node bars represent the 95% CI

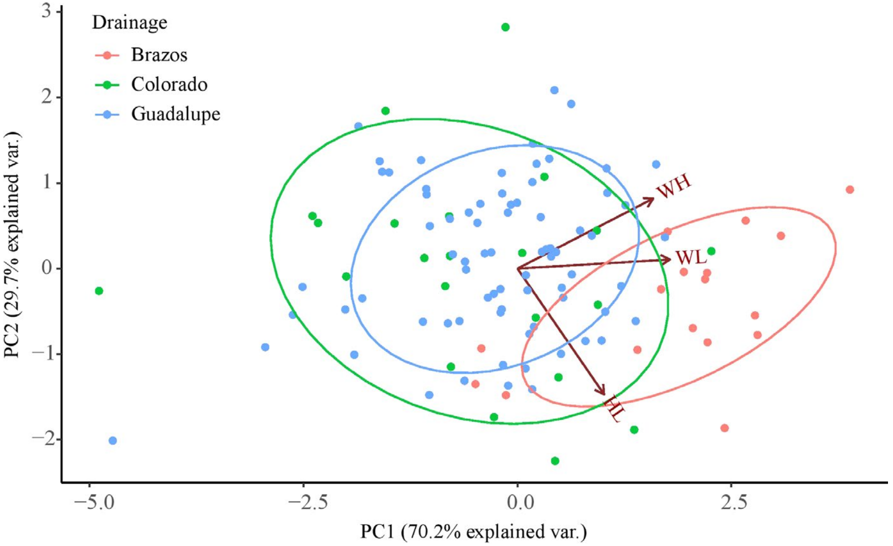

FIGURE 4 PCA biplots from morphometric data with 95% CI ellipses and arrows for biplot variables (HL = height/length, WL = width/length, WH = width/height). Colors indicate the drainage of capture: red (*Fusconaia iheringi*—Brazos), green (*Fusconaia iheringi*—Colorado), and blue (*Fusconaia mitchelli*—Guadalupe)

to be recent, ~0.82 Mya (95% CI: 0.33–1.38 Mya), during the late Pleistocene epoch (Figure 3).

3.2 | Morphometric analyses

We measured 114 individuals for *F. mitchelli* from focal drainages: Brazos (17), Colorado (22), and Guadalupe (75). Detailed information regarding individuals used in the morphological dataset is available in Table S1 and on ScienceBase (https://doi.org/10.5066/P9Y7K5CD). PC1 (70.2%) and PC2 (29.7%) eigenvalues explained 99.9% of the total variability in PCA. The PCA depicted overlap between *F. mitchelli* from the Colorado and Guadalupe drainages, while *F. mitchelli* from the Brazos was shown to be more inflated (Figure 4). Cross-validated DA scores provided an overall classification accuracy of 58.8% by drainage of capture (Brazos = 82.4%,

TABLE 2 Intra- and inter-drainage uncorrected p-distance for *Fusconaia iheringi* and *Fusconaia mitchelli*. Pairwise genetic distances are reported as mean (minimum–maximum)

Drainage	Locus	Intra-drainage	Comparison	Locus	Inter-drainage
Brazos	*COX1*	0.003 (0–0.009)	Brazos ~ Colorado	*COX1*	0.008 (0.005–0.013)
	ND1	0.002 (0–0.004)		*ND1*	0.002 (0.001–0.005)
	ITS1	0		*ITS1*	0
Colorado	*COX1*	0.004 (0–0.010)	Brazos ~ Guadalupe	*COX1*	0.016 (0.014–0.017)
	ND1	0.002 (0–0.003)		*ND1*	0.018 (0.015–0.020)
	ITS1	0		*ITS1*	0.004 (0.004–0.006)
Guadalupe	*COX1*	0.001 (0–0.005)	Colorado ~ Guadalupe	*COX1*	0.019 (0.015–0.021)
	ND1	0.002 (0–0.007)		*ND1*	0.017 (0.014–0.021)
	ITS1	0.001 (0–0.006)		*ITS1*	0.004 (0.004–0.006)

Colorado = 31.8%, Guadalupe = 61.3%) and 71.1% for groupings supported by DNA sequence data (Brazos + Colorado = 43.6%, Guadalupe = 85.3%). The permutational MANOVA between $\log_e$-transformed variables (i.e., H, W, and L) identified significant morphological differentiation between the Brazos + Colorado, and Guadalupe ($\alpha < 0.001$).

3.3 | Species delimitation

The molecular matrix used in STACEY and iBPP was 2,076 bp (*COX1* = 658 bp; *ND1* = 900 bp; *ITS1* = 518 bp). Five partitions and substitution models were selected for STACEY by PartitionFinder: *COX1* and *ND1* 1st position—K80, *COX1* and *ND1* 2nd position—F81, *COX1* 3rd position—HKY, *ND1* 3rd position—HKY, and *ITS1*-JC. Convergence of the STACEY analysis was indicated by all ESS values > 200. STACEY resolved the most likely species model as two species clusters: (i) Brazos + Colorado drainages and (ii) Guadalupe drainage (Figure 5). Convergence of the iBPP analysis was indicated by all ESS values > 200 and iBPP strongly supported (PP = 1.0) the two clusters (i.e., Brazos + Colorado, and Guadalupe) as distinct species (Figure 5).

3.4 | Range map

During our searches of museum records and available field observations, we located collection information for 6,365 freshwater mussel observations conducted from 1898 to 2018 in the Brazos, Colorado, and Guadalupe River drainages. Of these observations, 158 were *F. mitchelli* based on shells (recently dead to subfossil; *n* = 102) and live individuals (fresh dead + live; *n* = 56). Date of collection ranged from 1898 to 2016 for all observations of *F. mitchelli* (Table S2; https://doi.org/10.5066/P9Y7K5CD). *Fusconaia mitchelli* records that could be mapped (*n* = 106) were distributed across 25 HUC units (Brazos 6; Colorado 12; Guadalupe 7; Figure 6; Fig. S1; https://doi.org/10.5066/P9Y7K5CD). The status of the species in each HUC unit was categorized as follows: 20 HUCs with shell only (Brazos 4; Colorado 10; Guadalupe 6); 3 with historical records (fresh dead + live; prior to 1995; Colorado 3); and 9 with current records (fresh dead + live; 2011 to present; Brazos 3; Colorado 3; Guadalupe 3).

3.5 | Taxonomic accounts

Fusconaia mitchelli (Simpson in Dall, 1895).

Common Name: False Spike.

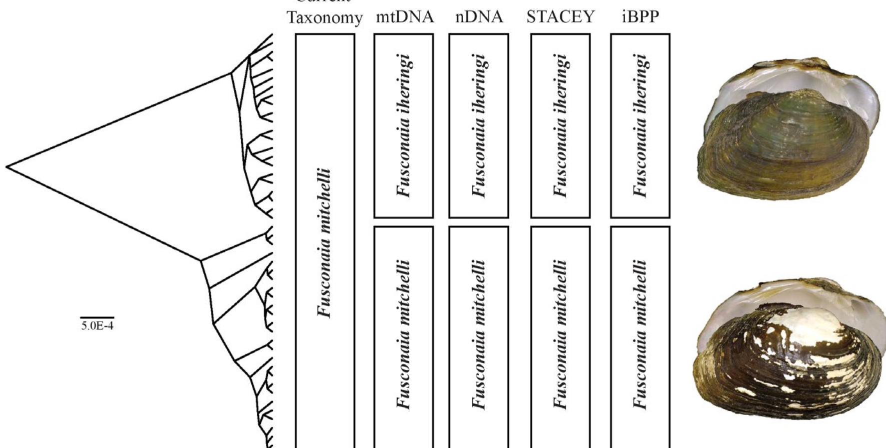

FIGURE 5 Summary of data types collected in this study and the STACEY phylogenetic reconstruction used to guide iBPP analyses. Photographs of shells represent the holotype of *Fusconaia iheringi* (USNM152171) and lectotype of *Fusconaia mitchelli* (USNM128364)

Synonymy: *Unio mitchelli* Simpson in Dall, 1895: 5–6 [Guadalupe River, Victoria County, Texas, Hon. J. D. Mitchell; Rio Salado, near New Leon, Mexico]. Lectotype USNM128364 inadvertently selected by Johnson (1975: 15) as the "figured holotype" and later designated as the lectotype by Johnson (1999: 19).

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

> *Quadrula* (*Quincuncina*) *guadalupensis* Wurtz, 1950: 2, figs. 1–5 [Guadalupe River above Seguin between Routes 123, and 90, Guadalupe County, Texas]. Holotype ANSP185974 fixed by monotypy (ICZN, 1999; Art. 73.1.1).

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

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

Type Material: Lectotype USNM128364. Specimen incorrectly designated as the figured holotype by Johnson (1975: 15) but later correctly designated as the lectotype (Johnson, 1999: 19). The measurements in the original description (Simpson in Dall, 1895) match USNM128364. Paralectotype USNM128364a. Other possible paralectotypes include BV134 and MCZ165695, but the exact date and collection location of specimens cannot be confirmed at this time.

Type Locality: Guadalupe River, Victoria County, Texas. The type locality in the original description of *F. mitchelli* (Simpson in Dall, 1895) was "Guadelupe River, Victoria County, Texas, Hon J.D. Mitchell; Rio Salado, near New Leon, Mexico." However, the type locality for *F. mitchelli* was restricted to the Guadalupe River, Victoria, Texas by Johnson (1975: 15) and we follow this revision.

Distribution: *Fusconaia mitchelli* is endemic to the Guadalupe River drainage in Texas. The distribution of *F. mitchelli* was designated to span from "Southern Texas to New Leon, Mexico" (Simpson, 1900), which included the Brazos, Colorado, Guadalupe, and Rio Grande drainages (Howells et al., 1996; Johnson, 1999). However, Pfeiffer et al. (2016) considered the distribution of *F. mitchelli* restricted to the Brazos, Colorado, and Guadalupe drainages in Texas based on the assumption that specimens identified as *F. mitchelli* in New Leon, Mexico, represent *S. taumilapana* (see above). We agree with the findings of Pfeiffer et al. (2016), and considering the elevation of *F. iheringi* for individuals in the Brazos and Colorado drainages, the distribution of *F. mitchelli* is restricted to the Guadalupe drainage in Texas.

Shell Description: Maximum length at least 68 mm (BV134). Shell moderately thick and moderately inflated. General outline of shell rhomboidal, anterior margin rounded, posterior margin truncate to bluntly pointed. Dorsal margin rounded, ventral margin straight to convex, posterior ridge moderately sharp dorsally to slightly rounded posterioventrally, posterior slope slightly concave. Umbo broad and slightly elevated above the hinge line. Periostracum shiny, light brown to dark brown. Pseudocardinal teeth moderately thick with two in left valve and one in right valve. Lateral teeth short and well-developed, slightly curved, two in left valve and one in right valve. Interdentum short and narrow. Umbo cavity wide moderately deep. Nacre white, usually iridescent.

Comparative Diagnosis: *Fusconaia mitchelli* resembles *F. iheringi* but is not syntopic with the species. *Fusconaia mitchelli* was found to be more compressed than *F. iheringi*; however, there was overlap in this character between *F. mitchelli* and *F. iheringi* from the Colorado (Figure 4). *Fusconaia mitchelli* usually has a rounder posterior ridge and less shiny periostracum when compared to *F. iheringi*. *Fusconaia mitchelli* can be distinguished from *F. iheringi* in our alignments by 5 diagnostic nucleotides at *COX1* (284:C, 295:G, 313:A, 406:T, 479:C), 13 diagnostic nucleotides at *ND1* (33:G, 93:G, 348:C, 403:A, 540:A, 588:T, 636:G, 643:G, 645:T, 720:C, 771:C, 801:T, 868:T), and 3 diagnostic loci at *ITS1* (58:A, 90:G, 325–327:CAA/AAA).

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

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

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

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

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

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

Fusconaia iheringi (Wright, *1898*).

Common Name: Balcones Spike.

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

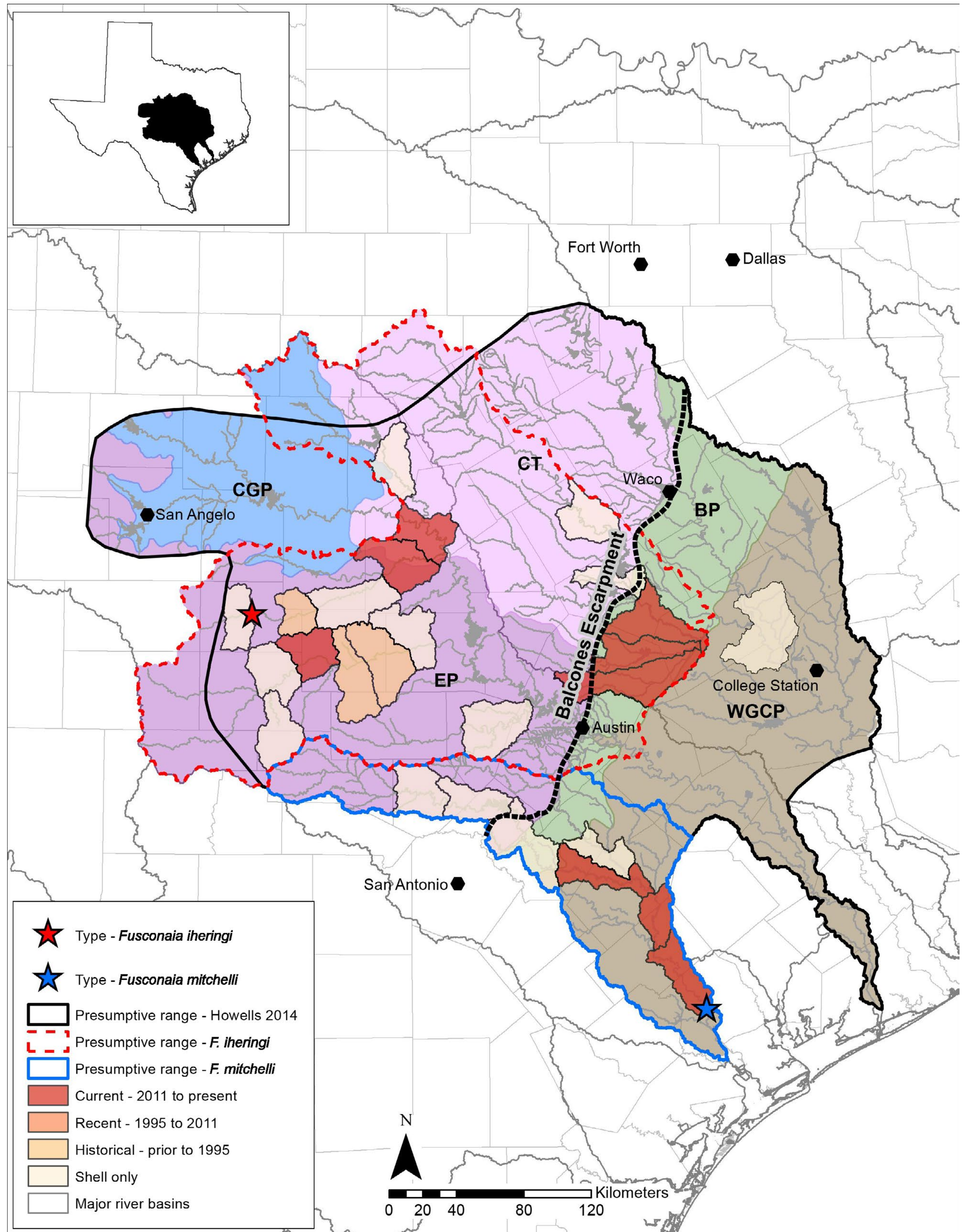

FIGURE 6 Conservation status map for *Fusconaia iheringi* and *Fusconaia mitchelli*. Hydrologic Unit Code (HUC) 10-level is colored to distinguish between live and shell only records. For the former, HUCs are further shaded by when a live specimen of *F. iheringi* or *F. mitchelli* was collected. The presumptive ranges for *F. iheringi* and *F. mitchelli* are denoted by the dashed red line and solid blue line, respectively. Type localities for *F. iheringi* and *F. mitchelli* are represented by red and blue stars, respectively. Ecoregion designations follow Griffith, Bryce, Omernik, and Rogers (2007): Blackland Prairie (BP), Central Great Plains (CGP), Cross Timbers (CT), Edwards Plateau (EP), and Western Gulf Coastal Plain (WGCP)

Type Material: Holotype USNM152171 fixed by monotypy (ICZN, 1999; Art. 73.1.1). Original description based on a single specimen, referred to as "type in National Museum." The same specimen was figured as the type by Simpson, 1900a: 79, pl. 4, fig. 5 and refigured and regarded as the holotype by Johnson, 1967: 7.

Type Locality: San Saba River, Menard County, Texas.

Distribution: *Fusconaia iheringi* is endemic to the Brazos and Colorado River drainages in Texas. *Fusconaia iheringi* appears to be restricted to streams along the Blackland Prairies and Edwards Plateau (Figure 6), including the Llano and San Saba rivers in the Colorado drainage; and Brushy Creek, San Gabriel River, and Little River in the Brazos drainage.

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

Comparative Diagnosis: *Fusconaia iheringi* resembles *F. mitchelli* but is not syntopic with the species. *Fusconaia iheringi* was found to be more inflated than *F. mitchelli*; however, there was overlap in this character between *F. iheringi* from the Colorado and *F. mitchelli* (Figure 4). *Fusconaia iheringi* usually has a sharper posterior ridge and shinier periostracum when compared to *F. mitchelli*. *Fusconaia iheringi* can be distinguished from *F. mitchelli* in our alignments by 5 diagnostic nucleotides at *COX1* (284:T, 295:A, 313:G, 406:T, 479:C), 13 diagnostic nucleotides at *ND1* (33:A, 93:G, 348:T, 403:G, 540:C, 588:C, 636:A, 643:A, 645:C, 720:T, 771:T, 801:A, 868:C), and 3 diagnostic loci at *ITS1* (58:C, 90:T, 325–327:---).

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

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

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

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

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

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

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

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

4 | DISCUSSION

An integrative species concept using multiple independent lines of evidence is a powerful approach to species delimitation (De Queiroz, 2007), and this approach has been utilized with success in resolving taxonomic issues for freshwater mussels (Inoue, McQueen, Harris, & Berg, 2014; Johnson et al., 2018; Keogh & Simons, 2019; Lopes-Lima, Bolotov, et al., 2018; Smith et al., 2018, 2019, 2020). In this study, we utilized multiple data types to re-evaluate species boundaries in *F. mitchelli*. Below, we describe how our holistic approach strongly supports the elevation of the binomial *Fusconaia iheringi* (Wright, 1898) to represent what was formerly referred to as *F. mitchelli* from the Brazos and Colorado drainages.

4.1 | Species delimitation in *Fusconaia iheringi* and *Fusconaia mitchelli*

A previous molecular genetic assessment (Pfeiffer et al., 2016) identified two distinct clades within *F. mitchelli*, and similar to that study, our phylogenetic analyses and distance-based approaches strongly support *F. iheringi* and *F. mitchelli* as distinct species. *Fusconaia iheringi* and *F. mitchelli* were resolved as mutually exclusive based on multilocus sequence data (Figure 1), depicted a clear signal for genetic separation at both mtDNA and nDNA markers using uncorrected p-distances (Table 2), were diagnosable using mtDNA and nDNA sequence data, and did not share haplotypes at mtDNA or nDNA markers (Figure 2). Furthermore, genetic divergence at mtDNA markers between the two species (Table 2) was greater than between congeners *F. burkei* (Walker in Ortmann & Walker, 1922) and *F. escambia* Clench & Turner, 1956 (Pfeiffer et al., 2016), and *F. askewi* (Marsh, 1896) and *F. chunii* (Lea, 1862) (Pieri et al., 2018). Despite nDNA having a slower mutation rate compared with mtDNA (Moore, 1995), *F. iheringi* and *F. mitchelli* did not share haplotypes and were also diagnosable at *ITS1* (Figure 2), while *F. askewi*, *F. chunii*, and *F. flava*; and *F. burkei* and *F. escambia* independently shared *ITS1* haplotypes (Pfeiffer et al., 2016; Pieri et al., 2018).

Biogeography is a critical component to species distribution and genetic divergence in freshwater mussels. Specifically, the host–parasite relationship between mussels and their host fish links their geographic distribution (Haag, 2010; Watters, 1992). Furthermore, dispersal is generally reliant on host fish, which are typically restricted by both terrestrial and marine barriers (Haag, 2012). In the case of *F. iheringi* and *F. mitchelli*, the species are specialized to parasitize freshwater fishes in the family Cyprinidae (Dudding et al., 2019), which are intolerant of marine environments (Matthews & Hill, 1977; Ostrand & Wilde, 2001) making ongoing gene flow between river drainages unlikely. If *F. iheringi* and *F. mitchelli* were conspecifics, populations in the three drainages (i.e., Brazos, Colorado, and Guadalupe) would be expected to be resolved as monophyletic with similar patterns of genetic divergence. However, phylogenetic and phylogeographic analyses using mtDNA and nDNA

resolve two strongly supported groups corresponding to *F. iheringi* (Brazos + Colorado) and *F. mitchelli* (Guadalupe) differing from expected patterns based solely on intraspecific genetic drift. These biogeographic patterns mirror those of other freshwater mussel species endemic to the Edwards Plateau, including two newly described species from the Guadalupe drainage *Cyclonaias necki* Burlakova, Karatayev, Lopes-Lima, & Bogan, 2018 in Burlakova et al. 2018 and *Lampsilis bergmanni* Inoue & Randklev, 2020 in Inoue et al., 2020, further emphasizing the high levels of endemism in the Guadalupe drainage (Inoue et al., 2020; Johnson et al., 2018).

Geological processes have shaped patterns of genetic divergence in many freshwater mussels (Haag, 2010; Inoue et al., 2020; Inoue, Lang, & Berg, 2015; Smith et al., 2018) and account for the observed inconsistencies between geographic and genetic divergence in *F. iheringi* and *F. mitchelli*. Isolation of the western Gulf of Mexico drainages peaked in the late Miocene and early Pliocene (Galloway, Whiteaker, & Ganey-Curry, 2011), and subsequent climatic changes connected drainage fragments to create two "mega-drainages": (a) Mega-Brazos (Brazos, Calcasieu, Sabine, and Trinity rivers) and (b) Mega-Colorado (Colorado and Guadalupe rivers; Blum & Hattier-Womack, 2009). The ancestral Mega-Colorado separated from the Mega-Brazos during the late Miocene, which led to the separation of lineages from central Texas (i.e., *F. iheringi* and *F. mitchelli*) and east Texas lineages (i.e., *F. askewi* and *F. chunii*; Figure 3). Subsequently, the modern fluvial systems of western Gulf of Mexico drainages began to form in the Pliocene–Pleistocene epochs (Galloway et al., 2011), leading to the allopatry of *F. iheringi* and *F. mitchelli* lineages (Figure 3). However, there may have been a more recent stream capture that introduced *F. iheringi* to the Brazos drainage, hence the close genetic relationship and incomplete lineage sorting between the Brazos and Colorado populations (Figure 1). An equally plausible explanation is that during the last glacial lowstand, the Brazos and Colorado drainages were merged (Blum & Hattier-Womack, 2009), which could be the source of introduction or gene flow into the adjacent drainage. However, the lack of fossil records makes the exact pattern of biological invasion uncertain. Available museum records and contemporary distribution support that *F. iheringi* was not distributed throughout the Brazos drainage and only historically occurred in streams flowing along the Blackland Prairie and Edwards Plateau (Figure 6). Recent distributional information supports a stream capture along the Edwards Plateau is likely the source of *F. iheringi* in the Brazos drainage rather than a merger of the two rivers during a lower sea level stand, which would theoretically lead to a wide-ranging distribution in the drainage. This biogeographic pattern is rare in aquatic taxa, but is also found in *Notropis amabilis* (Girard 1856), a small cyprinid with a distribution restricted to the Edwards Plateau in Texas drainages (Colorado, Guadalupe, Nueces, and Rio Grande), and a disjunct population in the San Gabriel River (Brazos drainage) along the Edwards Plateau and Blackland Prairie (Craig, Littrell, & Bonner, 2017; Hubbs, Edwards, & Garrett, 1991).

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

External morphology has long been used by taxonomists to delineate freshwater mussels (Frierson, 1927; Simpson, 1914) and has also been integrated with DNA sequence data to assess species boundaries in previous studies (Inoue et al., 2014, 2020; Johnson et al., 2018; Keogh & Simons, 2019; Pieri et al., 2018; Smith et al., 2018, 2019). However, reliance on conchological characteristics has been particularly problematic within the Pleurobemini, where both generic- and species-level taxonomic hypotheses have been largely invalidated by molecular genetic analyses (Campbell & Lydeard, 2012a, 2012b; Campbell et al., 2005; Inoue et al., 2018; Pfeiffer et al., 2016; Pieri et al., 2018). Furthermore, misidentification in Pleurobemini is problematic due to high levels of interspecific morphological convergence and intraspecific variation (Williams et al., 2017; Williams, Bogan, & Garner, 2008). For example, two sympatric species in east Texas are morphologically indistinguishable (i.e., *F. chunii* and *F. flava* in the Trinity River) further emphasizing the limited morphological divergence present between *Fusconaia* spp. (Pieri et al., 2018). Aligning with these issues, our morphological analyses indicate clear overlap between groups in PCA (Figure 4) and DAs had poor overall accuracy primarily due to the morphological overlap between *F. iheringi* from the Colorado and *F. mitchelli*. Although our ability to distinguish individuals among these drainages using morphometrics was limited, *F. iheringi* from the Brazos was found to be more inflated than both *F. iheringi* from the Colorado and *F. mitchelli*. This morphological divergence likely caused the strong statistical evidence for differences between *F. iheringi* and *F. mitchelli*; however, our data also suggest that morphological variation may be indicative of phenotypic plasticity rather than the presence of diagnostic morphological characters, a common phenomenon in freshwater mussels (Eagar, 1950; Ortmann, 1920). Our morphological results are similar to those in previous studies involving closely related *Fusconaia* spp. (i.e., *F. askewi*, *F. chunii*, and *F. flava*), where there was significant overlap in shell characters yet significant statistical support for differences in shell shape (Pieri et al., 2018). The lack of morphological signal in our dataset may also be due to the scarcity of material available of *F. iheringi*, which limits a robust assessment of morphological diversity in this species.

Although morphological evidence alone was compelling, there were numerous issues with our dataset making reliance on this type of data alone problematic. We addressed these issues by integrating inference from both DNA sequence and morphological data

using the coalescent-based model iBPP (Solís-Lemus et al., 2015). Coalescent approaches are promising in species delimitation studies; however, the reliance on user-defined guide trees can lead to these models over-splitting species (Knowles, Carstens, & Weins, 2007; Leaché & Fujita, 2010; Olave, Solà, & Knowles, 2014; Sukumaran & Knowles, 2017; Yang & Rannala, 2010, 2014). In our analyses, we addressed this issue by employing STACEY before iBPP, which strongly supported two species clusters without *a priori* designation (i.e., *F. iheringi* and *F. mitchelli*) similar to our other molecular genetic approaches (Figure 5). Considering the significant effects of demographic parameters on coalescent-based models (Yang, 2015; Yang & Rannala, 2010, 2014), we also utilized the most conservative priors for species delimitation presented by Pfeiffer et al. (2016). Despite conservative priors, our analyses unified the strong patterns of genetic divergence with significant morphological signal and provided decisive support (i.e., PP = 1.0) for the recognition of *F. iheringi* and *F. mitchelli* as distinct species. Given the results from our holistic approach for delineating species boundaries, we formally elevate the binomial *F. iheringi*.

4.2 | Implications on conservation and management

Species conservation is largely dependent on the ability to distinguish one species from another (e.g., Inoue et al., 2020; Johnson et al., 2018; Keogh & Simons, 2019; Smith et al., 2018, 2019). Results of this study indicate the Brazos + Colorado (*F. iheringi*) and Guadalupe (*F. mitchelli*) groupings correspond to two distinct species, which has important conservation implications. First, the geographic range of *F. mitchelli* is now restricted to the Guadalupe drainage. To date, stronghold subpopulations for this species occur primarily in the lower Guadalupe downstream of Gonzales, Texas (Randklev, Tsakiris, Howells, et al., 2013; Randklev, Tsakiris, Johnson, et al., 2013), and no live records of *F. mitchelli* in the upper Guadalupe have been reported (Figure 6; Fig. S1). Second, historical records indicate *F. iheringi* has always been restricted to streams in the Brazos and Colorado river drainages flowing along the Blackland Prairie and Edwards Plateau, and not those in the coastal plain (Figure 6). Based on this, the historical distribution of the species is much narrower than previously thought (Howells et al., 1996). Extant populations of *F. iheringi* are known from the Llano and San Saba rivers within the Colorado drainage; and Brushy Creek, San Gabriel River, and Little River in the Brazos drainage. One long-dead shell was found on the coastal plain; however, the lone record likely represents shell material transported downstream from waterways along the Blackland Prairie (Figure 6). The distribution and abundance of *F. iheringi* within the Brazos and Colorado drainages is limited, and stronghold subpopulations have not been identified for this species despite a significant amount of survey effort (Randklev et al., 2017, 2018). The exact causes for the rarity of *F. iheringi* are unknown but likely stem from changes in hydrology due to anthropogenic impacts such as groundwater pumping and increased severity of droughts and floods brought about by ongoing climate change (Randklev et al., 2018).

The dependency on host fish exacerbates conservation concerns in all freshwater mussels, as they are threatened by actions directly impacting both mussels and host fish populations (Haag, 2012). *Cyprinella lutrensis* Baird & Girard, 1853 and *C. venusta* Girard, 1856 were identified as putative host fish for *F. mitchelli* (Dudding et al., 2019); however, multiple enigmatic questions remain regarding the early life history for both *F. iheringi* and *F. mitchelli*. Primarily, host use has not been confirmed for *F. iheringi* and is critical toward understanding the basic biology of the species. Additionally, ecological hosts (i.e., natural infections) have not been confirmed for *F. mitchelli* and many sympatric minnow species have not been tested for host suitability (e.g., *Notropis* spp.). Until thorough information is available for *F. iheringi* and *F. mitchelli*, it is uncertain whether the status of host fish populations is contributing to imperilment.

The geographic distribution of mussels is largely shaped by host specificity and the movement of host fish during larval encystment; therefore, barriers preventing the movement of the host fish also disrupt the dispersal of mussels (Barnhart et al., 2008; Haag, 2012; Hoffman, Willoughby, Swanson, Pangle, & Zanatta, 2017; Strayer, 2008; Watters, 1992). This is certainly the case for *F. iheringi* and *F. mitchelli*, as the species are both presumably host specialists with glochidia exclusively transforming on cyprinids (Dudding et al., 2019). Typically, cyprinids have a small home range and limited dispersal capabilities (Chase, Caldwell, Carleton, Gould, & Hobbs, 2015; Johnston, 2000), making ongoing gene flow between suitable habitat patches in anthropogenically affected systems unlikely. These factors make both *F. iheringi* and *F. mitchelli* susceptible to localized extirpation, and it is likely that population recovery will only be possible through reintroduction using captive propagation or other human-mediated recovery efforts. Before these types of recovery actions are performed, comprehensive genetic management plans should be developed to ensure population viability and sustainability (McMurray & Roe, 2017). Our DNA sequence data do not show significant evidence of intra-drainage population structuring (Figure 2); however, more rapidly evolving nuclear markers (i.e., genotype by sequencing, microsatellites, whole-genome resequencing) will facilitate further evaluation of population structure, connectivity, genetic diversity, and viability of extant populations.

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ORCID

Chase H. Smith https://orcid.org/0000-0002-1499-0311
Nathan A. Johnson https://orcid.org/0000-0001-5167-1988
Charles R. Randklev https://orcid.org/0000-0002-6755-1507

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Conservation status map for *Fusconaia iheringi* and *Fusconaia mitchelli*.

Table S1. Morphological material examined in this study with catalog numbers and locality information of where specimens were collected, including river drainage, waterbody, and county.

Table S2. Distribution data used to create the conservation maps of *Fusconaia iheringi* and *Fusconaia mitchelli*.

Alignment S1. Alignment of *COX1* sequences used in molecular analyses

Alignment S2. Alignment of *ND1* sequences used in molecular analyses.

Alignment S3. Alignment of *ITS1* sequences used in molecular analyses.

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