ABSTRACT
Re-evaluating species boundaries in the freshwater mussel *Fusconaia mitchelli*

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Freshwater mussels (Bivalvia: Unionidae) are the most imperiled groups of organisms in North America. Accurately defining species boundaries is vital for ongoing biological conservation and management efforts. The false spike or *Fusconaia mitchelli* (Simpson in Dall, 1896) is a freshwater mussel found in the Brazos, Colorado, and Guadalupe drainages. A previous molecular study depicted significant intraspecific genetic variation which may be indicative of speciation occurring between geographically separated populations. Here, we use multi-locus sequence data to re-evaluate systematic relationships between *F. mitchelli* in the three central Texas drainages. We sequenced both mitochondrial (mtDNA) and nuclear loci: the protein-coding mtDNA genes *cytochrome oxidase subunit 1* and *NADH dehydrogenase 1*, and the nuclear ribosomal *internal transcribed spacer 1*. Phylogenetic and phylogeographic analyses depict molecular divergence between two groups within *F. mitchelli* coinciding to the Guadalupe drainage, and the Brazos and Colorado drainages. The junior synonym *Fusconaia iheringi* is available for the Brazos and Colorado populations and elevating it from synonymy warrants further investigation using an integrative taxonomic approach.

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RE-EVALUATING SPECIES BOUNDARIES IN THE FRESHWATER MUSSEL FUSCONAIA MITCHELLI

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TABLE OF CONTENTS

CHAPTER ONE

Introduction

Freshwater mussels (Bivalvia: Unionida) live in a wide variety of places and can be found in lakes, streams, and rivers on all continents excluding Antarctica. Emanating from the class Bivalvia, freshwater mussels in the order Unionida are a group of aquatic invertebrates comprising around one thousand species worldwide (Haag, 2012). The greatest diversity of freshwater mussels lies within North America with approximately three hundred native species (Williams et al., 2017). Mussels contribute significant ecological benefits to the environments they inhabit. For example, freshwater mussels are an integral part of fluvial food webs, omnivorously filter-feed on organic material and detritus in the water column, and stabilize sediment in streams (Vaughn, Nichols, & Spooner, 2008; Haag and Williams, 2014; Vaughn 2018).

The basic elements of internal and external freshwater mussel anatomy are consistent across species. Adult bivalves consist of a soft body enclosed in a hinged, two-part shell with a line of symmetry running across the hinge. The soft body components include the foot, muscles, internal organs, and the mantle. The foot allows for basic locomotion and facilitates burrowing into substrate, the adductor muscles open and close the shell, and the siphon filters water for food which is digested in the gut (Haag, 2012). By simple observation, the most immediately apparent of all features is the calcified shell which is secreted from the soft mantle (Haag, 2012). Habitat preference differs by species and tends to influence their shell shape (Haag 2012). Species with inflated shells tend to

live in soft sediments and in environments with slow moving water while species with compressed shells thrive in fast currents (Hornbach, Kurth, & Hove, 2010). The inside layer of the shell is typically a pearlescent white but, in some species, color variations include purple, pink, and orange nacres. Nacre or "mother of pearl" is composed of 95% aragonite and 5% organic materials such as proteins and polysaccharides (Sun & Bhushan, 2012). The process which forms the nacre can also create pearls if an irritating particle such as a grain of sand infiltrates the shell (Gosling 2008). The external surface of the shell is often brown but it can be green or yellow in some species (Haag, 2012). It tends to be smooth but several species have intricate surface texturing such as *Cyclonaias pustulosa* and *Amblema plicata* which have pustule-like growths and well-defined ridges, respectively. Annual growth rings on the exterior can be used to estimate the age of individuals (Haag & Commens-Carson, 2008). These rings contain clay deposits and ions gathered from the water which researchers can use to reconstruct the history of the organism's environment, including water quality and air temperatures (Schone, 2004). They also can be used to estimate the age of individual mussels (Haag & Commens-Carson, 2008). Certain mussel species are among the longest living invertebrates in the world with individuals in some populations reaching ages in excess of one hundred years (Anthony, Kesler, Downing, & Downing, 2001).

Freshwater mussels provide a unique system in the study of evolutionary biology due to the affect their life cycle on biological diversification. The unionid life cycle involves parasitic larvae (glochidia) that must attach to vertebrate hosts (primarily fish) prior to becoming sessile adults; therefore, they rely on larval parasitism to propagate and disperse offspring (Vaughn, Nichols, & Spooner, 2008; Barnhart et al., 2008). This life

history has likely significantly contributed to the radiation of this group considering adaptations to parasitism has led to differing life histories across species. Some species are generalists, targeting numerous species of fish, while other species have developed specialized adaptations to invest a single host. Most generalists use a strategy called broadcasting which is simply dispersing larva into the water (Schwalb, Morris, & Cottenie 2015), while other species have evolved a specialized lure to attract specific host fish. When a fish strikes the lure, the mussel releases its larval offspring, called glochidia, into the water column. The glochidia are taken in by the fish's respiration and latch onto its gills (Cunjak & McGladdery 1991); however, some species have adapted to latch onto the fins (Dudgeon & Morton, 1984). Other mussels use free floating packages of glochidia called conglutinates instead of attached lures (Barnhart et al., 2008), which similarly after a fish attack, the glochidia attach to the gills of the fish. Once glochidia attach, fish carries the larva until they metamorphose into juveniles and drop off in suitable habitat (Strayer, 2008). Considering this, the geographic distribution of mussels is largely influenced by the movement of the fish to which the glochidia attach. Upstream dispersal is only possible while glochidia are attached to their host since the movement capabilities of adult mussels becomes much more limited (Kat, 1984). Juvenile and adult mussels are restricted to burrowing, crawling, and moving with the current downstream to a more suitable habitat location. The aquatic nature of mussels makes the dry land surrounding rivers serve as a natural barrier to inhibit ongoing gene flow between drainages. Changes in drainage geography also affects the distribution of mussel species as the dispersion of larva is heavily contingent on fish behavior and movement patterns

(Kat, 1984). While the drainages do connect at the ocean, if host fish are intolerant of salinity, marine waters also serves as a geographic barrier for many species of mussels.

Freshwater mussels are the most imperiled group of animals in North America due to many contributing factors (Anthony, Kesler, Downing, & Downing, 2001). Anthropogenic alterations to freshwater ecosystems have largely hindered the geographic distribution and density of mussel populations (Watters, 1996). The reproduction and dispersion of mussels is contingent on the health of the host fish population, and thus the species richness of fish in a given area and mussels in that same habitat are positively related (Vaughn, & Taylor, 2000). Considering the reliance on host fish the completion of their life cycle, declines in fish populations reduces the viability mussel populations (Ronaldo, Vanessa, Allan, & Martina, 2015). Climate change can alter the average temperature and weather patterns of regions. These changes can cause faunal shifts in mussels which may be more or less viable depending on the features of adjacent habitats (Inoue & Berg, 2016). The introduction of non-native species to regions of North America often has negative effects on the health of native mussel populations since native mussels can be outcompeted in food gathering and habitat. In locations with high densities of invasive species, populations of native mussels decline in number (Ricciardi, Neves, & Rasmussen, 1998). Industrial runoff can also affect the health of mussel populations as the filter-feeding nature of freshwater mussels makes them sensitive to common pollutants such as copper and ammonia (Wang, et al., 2007). Considering all these factors that affect survival, freshwater mussels are the most imperiled group of organisms in North America with 70% of species considered endangered, threatened, or special concern (Williams et al., 1993). Many species are already on or being considered

for the endangered species list with 60% of all North American freshwater mussel species considered endangered or threatened and 12% presumed extinct (Anthony, Kesler, Downing, & Downing, 2001; Haag & Williams, 2014). Worldwide, 40% of all mussel species are near-threatened, threatened, or extinct, and global cooperation is necessary to conserve the remaining species (Lopes-Lima et al., 2018).

Conservation concerns have brought significant attention to freshwater mussels in recent years, especially using molecular techniques to validate current taxonomic hypotheses. Traditional methods of classifications were based primarily on the morphology of shell characteristics, but recent efforts using molecular data have largely invalidated the traditional taxonomy (Strayer, 2008). Therefore, implementing molecular data to understand species boundaries and resolve evolutionary relationships is essential for accurately defining diversity and the establishment of effective conservation and management plans. The subject of this paper, *Fusconaia mitchelli* (Simpson in Dall, 1896), is a member of the subfamily Ambleminae and tribe Pleurobemini. Commonly called the false spike, *F. mitchelli* is a Texas native mussel found in the Brazos, Colorado, and Guadalupe drainages. While not much is known about the host fish and specific life history of *F. mitchelli*, other species in the same tribe (Pleurobemini) have more documentation. Pleurobemini contains the genera, *Elliptio, Elliptoideus, Fusconaia, Plethobasus,* and *Pleurobema*. While some species of mussel use lures to attract fish, species in Pleurobemini release packaged, free-floating bundles of glochidia and unfertilized eggs called pelagic conglutinates which attract fish (Barnhart et al., 2008). Mussels which use this type of reproductive strategy are commonly specialists and use minnows which feed on small particles in the water column (Patterson et al., 2018).

Fusconaia mitchelli was presumed extinct until a recent rediscovery in 2011 when a small group of individuals were collected from the Guadalupe River (Randklev, et al., 2012). The species is currently under review for protection under the Endangered Species Act. Its rediscovery has prompted research into the evolutionary history and species boundaries of *F. mitchelli* and review for inclusion as an endangered species has made that research a priority. It was moved from the genus *Quadrula* to *Fusconaia* after a recent study compared the molecular data to that of other species in the *Fusconaia* genus (Pfeiffer et al. 2016). In addition to the name update, two distinct clades were resolved within the species which became the basis for further analysis of additional molecular markers (Pfeiffer, Johnson, Randklev, Howells, & Williams, 2016). The main purpose of this experiment is to re-evaluate species boundaries within populations of *F. mitchelli*. Here, we add additional molecular markers and increased sample size to determine whether the two distinct clades of *F. mitchelli* should be split based on genetic and geographical disparities.

CHAPTER TWO

Materials and Methods

Sampling and molecular data acquisition

To test species boundaries within *F. mitchelli,* we sampled individuals from the Brazos, Colorado, and Guadalupe drainages. Small clippings of tissue were taken from the mantle of each specimen were preserved in 100% ethanol and stored at -80°C. DNA was isolated with the Qiagen PureGene DNA extraction kit following manufacturer's suggested protocols (Gentra Systems, Inc., Minneapolis, MN, USA). We used three loci in this experiment: a portion of the mitochondrial genes *cytochrome c oxidase subunit 1* (CO1) and *NADH dehydrogenase subunit 1* (ND1), and the nuclear spacer gene *ribosomal internal transcribed spacer 1* (ITS1). The primer sequences used for PCR were: CO1 5'-GTTCCACAAATCATAAGGATATTGG-3' and 5'-TACACCTCAGGGTGACCAAAAAACCA-3' (Campbell et al., 2005) ND1 5'-TGGCAGAAAAGTGCATCAGATTAAAGC-3' and 5'-CCTGCTTGGAAGGCAAGTGTACT-3' (Serb, Buhay, & Lydeard, 2003) ITSI 5'-AAAAAGCTTCCGTAGGTGAACCTGCG-3' and 5'-AGCTTGCTGCGTTCTTCATCG-3' (King, Eackles, Gjetvaj, & Hoeh, 1999). These three markers are commonly used for reconstructing phylogeny, DNA barcoding, and species delimitation in many studies of freshwater mussels (Campbell & Lydeard, 2012b, 2012a; Inoue, McQueen, Harris, & Berg, 2014; Johnson et al., 2018; Perkins, Johnson, & Gangloff, 2017; Pfeiffer, Johnson, Randklev, Howells, & Williams, 2016;

Pfeiffer, Sharpe, Johnson, Emery, & Page, 2018; Smith, Johnson, Pfeiffer, & Gangloff, 2018).

PCR allows for the amplification of a specific fragment of DNA through the use of a primer which serves as a template. During PCR, the samples are first heated to denature the DNA, then, primers previously added to the samples bind to the denatured DNA as the temperature is lowered in a process called annealing and finally, the temperature is raised again for the final process of polymerization or extension (Garibyan & Avashia, 2013). Temperatures can be adjusted and customized to specific primers (Weier & Gray, 1988). A series of these cycles produces billions of copies of a specific DNA product (Garibyan & Avashia, 2013). The whole process is automated by a device which controls the temperatures and timing of the reactions.

PCR processes used in this experiment include a mixture of molecular grade water (4.25 µl), MyTaq Red Mix (6.25 µl), (Bioline) (6.25 µl), specific primers (0.5 µl each), and a DNA template (50 ng) totaling 12.5µl. The product of the PCR reaction was sent to Molecular Cloning Laboratories (MCLAB, South San Francisco, CA, USA) for bidirectional sequencing on an ABI3730. PCR product for ITSI was more difficult to sequence than mtDNA markers considering the possibility of multiple copies at ITSI. All individuals were sent directly for sequencing, similar to previous studies in freshwater mussels that yielded sequences that were readable without cloning (e.g., Grobler et al., 2005; Jones et al., 2006; Campbell et al., 2008; Pfeiffer et al., 2016). Reliable ITSI sequences could not be obtained for some heterozygous individuals; and these individuals were, therefore, excluded from phylogenetic analyses.

Molecular analyses

The portions of the data received from MCLAB can be viewed in the form of a chromatogram. Chromatograms are graphical depictions of a sequence of nitrogenous bases. Chromatograms were examined and edited using Geneious version 10.2.3 (Kearse et al., 2012). Sequences were aligned using MAFFT v 7.311 (Katoh & Standley, 2013) in Mesquite v 3.31 (Maddison & Maddison, 2018) using the L-INS-I method. The protein-coding mtDNA genes were translated into amino acids to ensure open reading frame and no indels.

Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian Inference in IQ-TREE version 1.6.8. (Trifinopoulos, Nguyen, von Haeseler, & Minh, 2016) and MrBayes v 3.2.6 (Ronquist et al. 2012), respectively. Before phylogenetic analyses, sequences must be run through a program to find the best fit scheme for partitioning the dataset. The user inputs the first, second, and third codon positions into the program for coding genes such as CO1 and ND1. Modeling programs which treat different codons as separate sites produce more accurate results than homologous models when researching evolution (Bofkin & Goldman, 2006). For ML analyses in IQ-TREE, ModelFinder (Kalyaanamoorthy et al, 2017) was used to find the best partitioning scheme and nucleotide substitution models. After partitioning schemes were determined, IQ-TREE analyses conducted 10 independent runs of an initial tree and 1000 ultrafast bootstrap replicates (Hoang, Chernomor, von Haeseler, Minh, & Vinh, 2017) for nodal support. The ultrafast bootstrap method efficiently reduces computing time while maintaining accuracy when compared to the traditional bootstrap replicate (Hoang, Chernomor, von Haeseler, Minh, & Vinh, 2017).

Not all substitution models available for IQ-TREE can be used in MrBayes; therefore, we utilized Partionfinder v 2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) to find the best partition schemes and substitution models for BI analyses. The analysis used PhyML which estimated the maximum likelihood phylogenies by interchanging nearest neighbors to improve tree accuracy (Guindon et al., 2010). The Bayesian information criterion (BIC) was selected and branch lengths were linked. The BIC is a system for developing models based on their posterior probability (Schwarz, 1978). As its name suggests, MrBayes uses the Bayesian method and Bayes's theorem which, given a likelihood, calculates a posterior distribution of data forming a statistical distribution. Bayesian inference makes probability statements about the validity of a model (Nascimento, dos Reis, & Yang, 2017). MrBayes uses Markov chain Monte Carlo integration (MCMC), a method for solving integration problems commonly used in population genetic inference (Drummond, Nicholls, Rodrigo, & Solomon, 2002). The proportion of convergence is given with a value called the average standard deviation of split frequencies (ASDSF). The ASDSF should approach 0.0 as the independent runs begin to show matching results, giving a quantitative number which monitors convergence (Ronquist et al., 2012). An additional method for monitoring convergence is with the effective sample size (ESS) value.

The program PopART is useful for creating diagrams to visualize the relationships between datasets of intraspecific individual sequences. It was used to visualize genetic structure with respect to geographic distribution. PopART version 1.7 was used to create haplotype networks using the TCS Network (Clement et al., 2002). Samples were grouped by drainage of origin. PopART does not include samples with

unknown bases or gaps so these were removed. The TCS network is an algorithm which evaluates statistical parsimony before estimating and constructing cladograms (Templeton, Crandall, & Sing, 1992). To further explore relationships within *F. mitchelli*, we used MEGA7 (Kumar, Stecher, & Tamura 2016) to compute p-distance and average p-distances within and between drainages. All codon positions were included and ambiguous sections were removed.

Species Delimitation Analyses

We implemented the coalescent species delimitation model STACEY v 1.2.4 (Jones, 2017) via BEAST v 2.5.1 (Bouckaert et al., 2014) on a concatenated alignment of CO1, ND1, and ITS1 for all individuals of *F. mitchelli*. We utilized Partitionfinder (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) to re-evaluate the best partitions and substitutions 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 CO1 for both analyses and remaining partitions were estimated by STACEY. Analyses executed 2x10^{8} generations and logged every 5000th tree and a 10% burn-in. Tracer v 1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018) 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.

CHAPTER THREE

Results

Taxon sampling

Our dataset included a total of 44 samples of *F. mitchelli* with 10 from the Brazos, 13 from the Colorado, and 21 from the Guadalupe. In addition to the sequences from the *F. mitchelli* specimens, representatives from other species in *Fusconaia* were included: *F. askewi, F. burkei, F. chunii, F. cor, F. cuneolus, F. escambia, F. flava, F. masoni,* and *F. subrotunda*. We also included species from other genera to serve as outgroups: *Elliptio crassidens, Elliptoideus sloatinanus, Eurynia dilatata, Hemistena lata, Parvaspina steinstansana, Pleurobema clava, Pleuronaia barnesiana,* and *Quadrula quadrula. Quadrula quadrula* functioned as a root as it belongs to the tribe Quadrulini while the others all belong to Pleurobemini.

Phylogenetic tree reconstruction

The best partitioning scheme and substitution models determined by ModelFinder for IQ-TREE analyses were: HKY+F+G4 for CO1 codon 3, TIM3e+I+G4 for CO1 codon 1 and ND1 codon 1, TN+F+I for CO1 codon 2 and ND1 codon 2, TN+F for ND1 codon 3, and JC+I for ITS1. The best partitioning scheme and substitution models determined by PartitionFinder for MrBayes analyses were: HKY+G for CO1 codon 3, SYM+I+G for CO1 codon 1 and ND1 codon 1, HKY+I for CO1 codon 2 and ND1 codon 2, HKY+G for

ND1 codon 3, and JC+G for ITS1. Both ML and BI phylogenetic reconstructions depicts two monophyletic clades within *F. mitchelli* coinciding to the Guadalupe drainage; and the Brazos and Colorado drainages (Figures 1 & 2). These results are very similar to the IQ-TREE model with a split indicated between the Guadalupe drainage away from the Brazos and Colorado.

Each haplotype network indicated separation between the samples taken from the Guadalupe drainage and those from the Brazos and Colorado. The TCS network for CO1 depicts 6 points of difference between the Guadalupe grouping and the Brazos/Colorado grouping (Figure 3). The TCS network for ND1 indicates 12 mutation differences between the Brazos/Colorado grouping and the Guadalupe grouping (Figure 4). TCS Haplotype network for ITS1 shows 2 points of difference between groupings and it also shows no genetic differences between samples from the Colorado and Brazos drainages (Figure 3). We calculated average p-distances within each drainage group as well as maximum and minimum values (Table 1). The average intra-drainage p-distances were all 0.002 for ND1 in each drainage and 0.000 for ITS1 in each drainage. CO1 varied in value with Brazos: 0.004 (0.000-0.009), Colorado 0.007 (0.004-0.010) and Guadalupe 0.002 (0.000-0.005). We also compared the p-distances between drainages and calculated the average, maximum, and minimum values (Table 2). The average p-distances between Brazos and Colorado is 0.008 (0.007-0.013) for CO1, 0.002 (0.001-0.003) for ND1 and 0.000 for ITS1 (Table 2).

Species Delimitation

The molecular matrix used in the STACEY analyses was aligned to 2076 bp and included all individuals of *F. mitchelli*. Five partitions and substitution models were selected for STACEY by PartitionFinder: CO1 and ND1 1st position- K80, CO1 and ND1 2nd position- F81, CO1 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: *F. mitchelli* from the Guadalupe drainage, and *F. mitchelli* from the Brazos and Colorado drainages (Figure 4.).

CHAPTER FOUR

Discussion

Species concepts and speciation mechanisms

When discussing speciation it may be beneficial to examine the various concepts that evolutionary biologists use to distinguish between species. Perhaps the most fundamental of all species concepts, the biological species concept states that any interbreeding or potentially interbreeding groups of organisms which produce fertile, viable offspring are the same species (De Queiroz, 2005). Diagnosability is important when dealing with species delimitation. For instance, testing the biological species concept with endangered mussels would not be very feasible. In addition, the populations are separated allopatrically in separate drainages so they would not breed with one another naturally.

Another way to determine species boundaries, traditionally used in with museum taxonomy and paleontology, is with morphology (De Queiroz, 2007). Shell morphology was the common way to distinguish and classify species of mussels before genetic testing was developed. Using shell morphology to distinguish between different species of mussels can be problematic as habitat can heavily influence shell characteristics and add additional weathering to the shells (Haag, 2012). Species misidentification is not uncommon even among clean, archetypical shells (Shea, Peterson, Wisniewski, & Johnson, 2011). Because of this, genetic information must also be taken into consideration if possible when using morphology to describing species.

Other species concepts involve the evolutionary relationships between organisms such as the phylogenetic species concept which consists of monophyletic groups which contain an ancestor and all of its descendants (De Queiroz, 2007). The above concepts are helpful when examining speciation but a unified species concept may be more helpful when it comes to species delimitation (De Queiroz, 2007). A unified species concept would use multiple lines of evidence to diagnose whether a species is a separately evolving lineage (De Queiroz, 2007). To determine whether groups of mussels form separately evolving lineages, we can examine their life history and geographic distribution.

The life cycle and habitat of freshwater mussels shape their biodiversity in interesting ways as mussels have opportunities for multiple types of genetic divergence. Both sympatric and allopatric speciation are possibilities when considering mussels' life cycles. Sympatric speciation occurs after populations which share the same geographic range diverge genetically (Dieckmann, U., & Doebeli, M. 1999). In mussels, this is often because of adaptations regarding the host fish mussels use to reproduce. As a novel host fish it acquired, mussels have new opportunities for dispersal and reproduction (Graf, 1997). There is also an inherent arms race in the parasitic life cycle of freshwater mussels. As mussels are selected to better parasitize host fish, the fish also adapt to counteract this parasitism. Host fish can produce antibodies to counteract glochidia infection (Dodd, B. J. et al., 2005).

As mentioned previously, there are many barriers which prevent gene flow between populations of freshwater mussels. Any barrier which hinders or prevents the movement of the host fish also disrupts the dispersal of mussels (Kat, 1984). The land

between waterways serves as a physical barrier which prevents host fish and mussels from crossing into other drainages. In addition, marine water may serve as a barrier to species of host fish which are intolerant of high salinity. Populations separated allopatrically can diverge through selection to new environments or through genetic drift. In regards to *F. mitchelli* our analysis indicates that geographic distribution is the primary means of speciation in this instance as these species are allopatrically separated in different drainages as the land between drainages serves as a geographic barrier that isolates the populations.

Loci selection

Mitochondrial DNA (mtDNA) loci are consistently used to reconstruct phylogeny in non-model taxa due to their rapidly evolving nature (Brown, George, & Wilson, 1979). Maternal inheritance and absence of recombination make mtDNA valuable for the construction of phylogenies (Ladoukakis, & Zouros, 2017). Variation in mtDNA and elevation in mutation rate compared to nuclear DNA result in areas of the genome which are more conserved than others which makes mtDNA useful for both comparisons within the same population and comparisons across distant species (Ladoukakis, & Zouros, 2017). One mitochondrial gene used in this study, ND1, is a coding sequence which contains the information necessary for producing the *NADH dehydrogenase 1* protein. The other mitochondrial gene, CO1, is often used in DNA barcoding as it has been shown to be species specific in eukaryotes (Marshall, 2005). Its use as a barcode has been proposed as a global bio-identification system for animals (Herbert, Cywinska, Ball, & deWaard, 2003). Over 95% of animals have a diagnostic sequence of CO1 which can be

used for species level identification (Ratnasingham & Hebert, 2013). Within a single species, deviation between individual CO1 sequences rarely exceeds a 2% difference (Hebert et al., 2003). The distance between intraspecific p distance and interspecific p distance is the barcode gap which can be useful in identifying species and in determining species divergence (Ratnasingham et al., 2013).

Unlike the previous genes, ITS1 is spacer DNA between two ribosomal genes, 5.8S and 18S, and is therefore non-coding (King, Eackles, Gjetvaj, & Hoeh, 1999). The non-coding nature of this sequence makes it a rapidly and neutrally evolving marker (Edger et al., 2014) as it experiences lower selective pressure compared to coding sequences (Hřibová et al., 2011). Its short length also gives it significant advantages in the PCR process compared with similar barcoding genes (Wang et al., 2014). Like the previous genes, ITS1 is a popular and frequently-used genetic marker for producing phylogenies in many eukaryotic groups (Edger et al., 2014); however, it is nuclear (nDNA) resulting in a slower mutation rate and larger effective population size when compared to mitochondrial DNA loci (Moore, 1995; Funk & Omland, 2003; Ballard and Whitlock, 2004; McCracken and Sorenson, 2005).

Statistical analysis

Here we use molecular data and examine the data received from statistical analysis programs. There is separation evident between the Guadalupe and Brazos/Colorado populations in all three haplotype networks (Figure 4). For each marker, the Guadalupe are more separated from the Brazos and Colorado than the Brazos and Colorado are from each other. In addition, we found no separation between the Colorado and Brazos group

in the haplotype network for ITS1 loci. These separations indicate genetic distance between populations which could be congruent with speciation.

Genetic distance can also be measured by calculating p distances between populations. As suggested by the haplotype networks, the ITS1 showed 0.000 average p-distance between the Brazos and Colorado drainages (Table 2). For every marker, the Brazos-Colorado average intra-p-distance was lower than either the Brazos-Guadalupe or Colorado-Guadalupe. This further indicates that there is more separation and genetic distance between the Guadalupe population and the other two drainages. Colorado had the most intra-drainage variation especially in the CO1 region. This agrees with the CO1 haplotype network (Figure 4). ND1 was the most consistent among drainages with 0.002 intra-drainage variation for each drainage. Previous molecular analysis has found similarly greater distance between the Guadalupe and Brazos/Colorado groups (Pfeiffer et al., 2016). The mean percent sequence divergence between Brazos/Colorado and Guadalupe for CO1 was found to be 1.79 (1.52-2.22) which is comparable to the distance between *Fusconaia escambia* and *Fusconaia burkei* which is mean: 0.86 (0.77-1.11) (Pfeiffer et al., 2016). This indicates greater distance between drainage populations than between two separate species. In addition, both BI and ML phylogenies resolve a single monophyletic clade for the Guadalupe samples and another for the Brazos and Colorado samples.

The possible species concepts are depicted in Figure 3; the first being that all populations of *F. mitchelli* are all of the same species and the second being that the Guadalupe is a distinct species from the Brazos and the Colorado. STACEY analysis has resolved a species model with two separate species with >99.9%. This species model

agrees with both the BI and ML analysis as well as the haplotype networks and p-distance. Each system of analysis consistently resolved more separation between the Guadalupe samples and the samples from the other two drainages.

Naming and future studies

The name *Fusconaia iheringi* is available for the Brazos and Colorado populations as it is associated with a holotype specimen from the San Saba River (USNM 152171). If species delimitation models indicate the presence of more than one species, this name can be applied to the Brazos and Colorado species. Currently, there appears to be strong statistical support for the new species model which separates Guadalupe from the other drainages. Additional data may increase support for species delimitation and improvements to the methods can be taken for improvements. A larger sample size is always helpful for improving the precision and reliability of statistical results. Additional markers may also be necessary to increase the power of the analysis. In detecting bottlenecks, testing twice the number of markers was equally or more influential for power than multiplying the number of individuals by three (Hoban, Gaggiotti, & Bertorelle, 2013) and this may also be the case for species delimitation. Further investigation into other forms of evidence such as morphological characteristics and combining those with molecular data may increase support.

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Figures

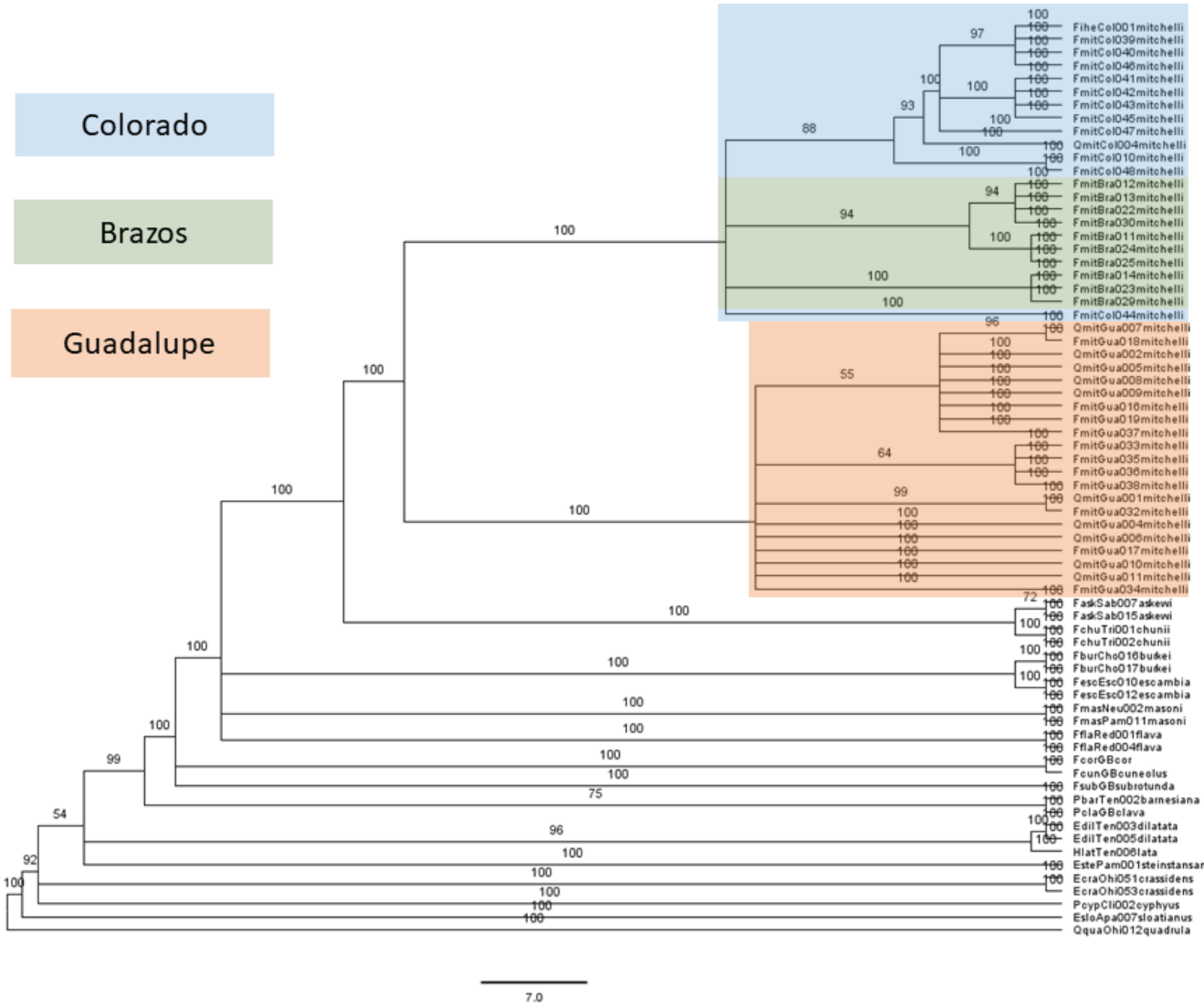

Figure 1. Phylogenetic reconstruction generated by MrBayes. Node labels indicate posterior probability (PP). Significant support represented by PP>95.

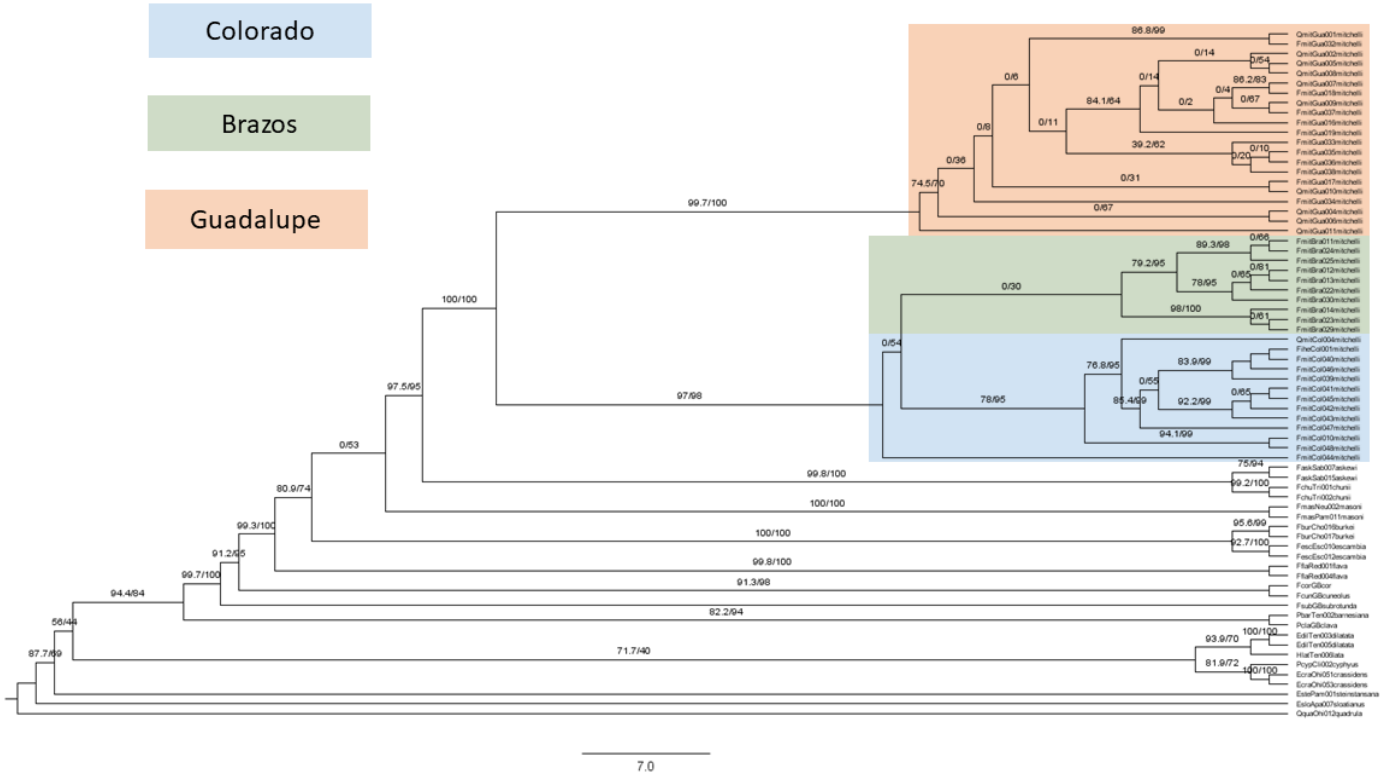

Figure 2. Maximum likelihood (ML) phylogenetic reconstruction.

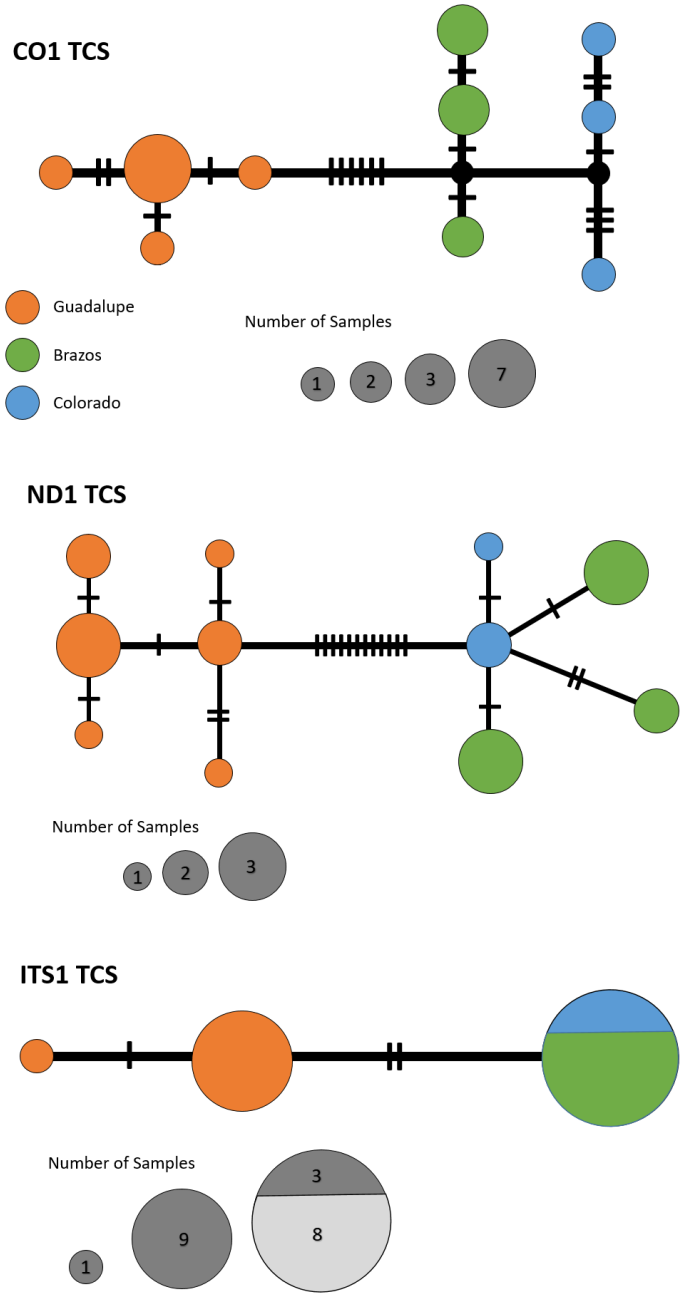

Figure 3. Haplotype network depicting CO1, ND1, and ITS1 loci constructed in PopArt utilizing a TCS network construction

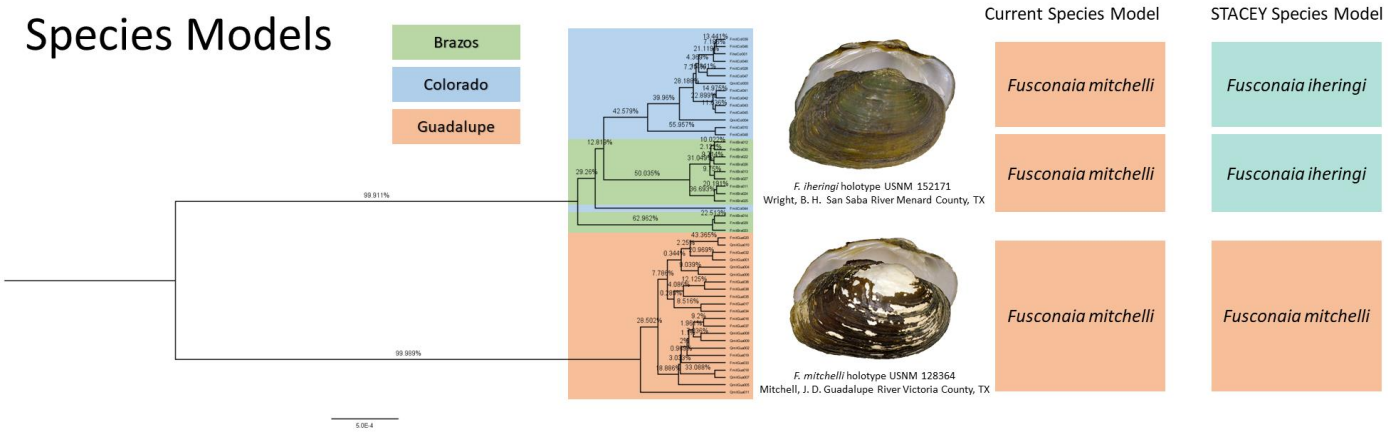

Figure 4. Comparison of the current species model and the STACEY species model

		Average p distance within drainage
Brazos	CO1	0.004 (0.000-0.009)
	ND1	0.002 (0.000-0.004)
	ITS1	0.000
Colorado	CO1	0.007 (0.004-0.010)
	ND1	0.002 (0.000-0.003)
	ITS1	0.000
Guadalupe	CO1	0.002 (0.000-0.005)
	ND1	0.002 (0.000-0.006)
	ITS1	0.000

Table 1. MEGA7 p distances within drainages

		Average p distance between drainage
Brazos-Colorado	CO1	0.008 (0.007-0.013)
	ND1	0.002 (0.001-0.003)
	ITS1	0.000
Brazos-Guadalupe	CO1	0.016 (0.014-0.018)
	ND1	0.018 (0.016-0.021)
	ITS1	0.004 (0.004-0.006)
Colorado-Guadalupe	CO1	0.019 (0.017-0.022)
	ND1	0.017 (0.014-0.020)
	ITS1	0.004 (0.004-0.006)

Table 2. MEGA7 p distances between drainages