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Genetic And Morphological Assessment Of Pigtoe Mussels (unionidae: Pleurobemini: Fusconaia/pleurobema Spp.) in Ozark Drainages

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BY

LOGAN TYLER PHELPS

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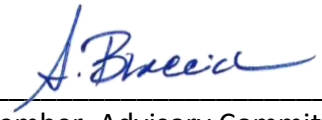

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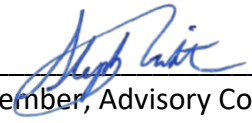

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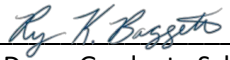

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LOGAN TYLER PHELPS

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ABSTRACT

North America is home to nearly 300 species of native freshwater mussels. Many species within this group are in need of conservation efforts and for these efforts to be effective, species delimitations must be as accurate as possible. Intraspecific variation and interspecific convergence are sources of confusion within morphology-based taxonomy, particularly within the Pleurobemini. Molecular phylogenetic work has revealed multiple problems within currently accepted Pleurobemini classifications. Specifically, *Fusconaia* has been shown to harbor cryptic diversity within drainages of the Ozarks. Further, *Pleurobema rubrum* and *P. sintoxia* have been shown to be possible conspecifics despite have differing shell morphologies. This study sought to use the COI mitochondrial gene to investigate the taxonomic identity of Pleurobemini specimens from within the Ozarks and to use geometric morphometrics to investigate inter- and intraspecific morphological variation. Genetic data revealed that an unnamed *Fusconaia* lineage, previously identified in other studies, occupies all major river systems draining the Ozark Highlands except the White River system. *F. flava* and *F. ozarkensis*, however, were only found in the White River system. *P. rubrum* and *P. sintoxia* were not differentiated at the COI gene. This was consistent in all drainages sampled. Geometric morphometric data revealed varying degrees of morphological overlap between all taxa in regards to both shell outline and shell inflation shapes, although discriminant analysis reclassification was largely successful in identifying data points.

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I. Introduction

Freshwater mussels (Bivalvia: Unionida) are a nearly globally distributed group of animals. The highest diversity in the group can be found in North America, where it is largely concentrated in the family Unionidae (Graf & Cummings 2007). The most current revision of freshwater mussel taxonomy in the United States and Canada considered 298 species valid within the two families Margaritiferidae (1 genus comprised of 5 spp.) and Unionidae (54 genera comprised of 293 spp.) (Williams et al. 2017). Since this revision, researchers have proposed new genera and species (e.g., Watters 2018) and have also found evidence of taxonomic uncertainties in accepted species (e.g., Inoue et al. 2018). This incredible diversity is largely at risk. The freshwater mussels are often considered some of the most imperiled organisms on earth. In regard to North American species, current research estimates that approximately 10% of species are already extinct and more than 50% of extant species should be considered endangered, threatened, or vulnerable and possibly in need of conservation efforts (Williams et al. 1993; Haag & Williams 2014). For conservation efforts to be effective, species delimitations must be as accurate as possible. Efforts towards this goal using molecular methods have found that many taxonomic changes are still warranted in this group.

Many contemporary names of North American unionid species date back to the 1800's as they were formally described largely by naturalists such as Isaac Lea, Constantine Samuel Rafinesque, and Thomas Say (Haag 2012). These researchers described species based largely on one of the best pieces of evidence of freshwater mussel taxonomy that was available at the time: shell morphology. While the collection

of live mussels allows the soft anatomy (i.e., what is inside the shell) to be examined, the hard shells of these animals can exist in the environment for some time after the animal has died and can be easily transported dry. Because of that, shell material has historically served as an important source of taxonomic information. It is now well documented that shells within a single species can vary significantly based on environmental factors. For example, shell inflation varies based on individuals' longitudinal position within a watershed. Individuals that are located in smaller streams tend to be more compressed, while individuals collected from larger rivers tend to be more inflated and convex (known as Ortmann's Law of Stream Position) (Ortmann 1920). Other studies have related variation in shell thickness and shape to substrate type and water chemistry (Bailey & Green 1988; Hinch et al. 1989). This means that the use of these morphological characteristics can often fail to adequately delimit species. This variation has, in many cases, led to variants of single species being described as different (e.g., Inoue et al. 2013). In other cases, divergent evolutionary lineages have been described as the same species due to convergent evolution. These cases of cryptic diversity have largely been revealed through molecular phylogenetics (e.g., Jones et al. 2006; Smith et al. 2019; Smith et al 2020). Because of this intraspecific variation and interspecific convergence, species delimitation based on morphology alone can become blurred.

The use of shell morphology to describe and identify species requires looking at physical characteristics such as shell sculpturing (e.g., presence of pustules, nodules, sulcus, etc.), distinct periostracum color, and ray presence. For many species, these

characteristics are readily observable if present. For other species, researchers have turned to morphometrics and shape analysis. Traditional morphometric analysis relies on measurements of the length, height, and width of shells. These types of measurements contain little information about shape and the use of ratios (e.g., length/width, height/length, etc.) in statistical analysis has been considered problematic by some researchers (reviewed in Zelditch et al. 2004). Geometric morphometry relies on the use of landmarks that can be better used to assess distances and angles between landmarks at the same time. Some organisms, such as cypriniform fishes, have a standardized set of distinct homologous landmarks that are used in this type of analysis (Armbruster 2012). Mussels, however, do not have many such structures on the external shell that can be used in this way. In situations where true landmarks are absent, semi-landmarks can be plotted instead (Bookstein 1997). Researchers using these techniques on mussels have analyzed interior shell landmarks as well as semi-landmarks placed around shell outlines. Geometric morphometry, using both types of landmarks, has been used to describe variation in both bivalve (e.g., Morais et al. 2014) and gastropod shells (e.g., Minton & Wang 2011). In a study of marine bivalves, both interior landmarks and outline semi-landmarks were able to distinguish phenotypic differences, with semi-landmarks providing better resolution (Leyva-Valencia et al. 2012).

The Pleurobemini (Bivalvia: Unionidae) is a tribe of freshwater mussels that has particularly caused taxonomic confusion. The Pleurobemini is within the Ambleminae subfamily and contains multiple genera that have been non-monophyletic in past

classifications (Campbell et al. 2005). There are currently 75 species in 9 genera recognized within this tribe, with nearly half of those belonging to the genera *Fusconaia* and *Pleurobema* (Williams et al. 2017). Recent molecular phylogenetic studies, however, have revealed issues in species delimitations in these genera. For example, researchers have used genetic evidence to document that one lineage in a widespread species, *Fusconaia flava*, potentially represents an undescribed species from the Illinois, Osage, and Neosho River systems (Burdick & White 2007; Campbell & Lydeard 2012; Inoue et al. 2018). These researchers suggested that this undescribed lineage may be attributable to *F. hebetata* (Conrad, 1854) or *F. flava sampsoniana* (Frierson, 1927). The same research has also documented that the widespread *F. flava* and the more restricted (only occurring in Gulf Coast drainages east of the Mississippi River) *F. cerina* seem to be genetically indistinguishable and are likely conspecifics. Additionally, the relationships between other *Fusconaia* species such as *F. askewi* and *F. lananensis* needs resolution, as these species may be conspecifics as well (Pieri et al. 2018). Within *Pleurobema*, recent genetic studies have provided evidence that the relationship between *Pleurobema rubrum*, a species that is petitioned for Federal Protection in the United States under the Endangered Species Act (ESA), and a more common species *P. sintoxia* is not totally resolved and that these species may be conspecifics (Jones et al. 2015; Inoue et al. 2018). For conservation efforts of *P. rubrum* to be effective, this relationship requires further work. Further, Inoue et al. (2018) highlighted many other cases of likely conspecifics within this genus as well as a potential undescribed lineage within *P. ridellii*. All these cases reveal that more genetic taxonomic work is still needed within this

group, especially in order to address cryptic diversity that is confined to small geographic areas or single river drainages.

The Ozark Highlands is a region of the Central Highlands in North America. This upland area makes up the southern portion of Missouri as well as the northern portion of Arkansas. It has a biology and geology that are historically linked to both the Ouachita Highlands and the Eastern Highlands. Mayden (1985) suggested that the Ozarks, Ouachitas, and Eastern Highlands represented, at one time, a more contiguous upland area with a broad ranging aquatic fauna. Fish distributions and patterns of endemism in these areas support the idea that Pleistocene glaciation and associated shifts in eastern North America's major waterways forced geographical separation into the three distinct regions we see today (Mayden 1988). The Ozark and Ouachita highlands together have been recognized as a distinct biogeographical unit for both fishes (Mayden 1988) and mussels (Haag 2010). A similar pattern was also documented with regards to regional North American stonefly faunas (Nelson 2008). The Ozarks, as well has the Ouachita and Eastern highlands, has become a hotspot for aquatic biodiversity and endemism. Besides the genetically distinct *F. "flava"*, there is evidence that the region also harbors a genetically distinct lineage of *Lasmigona costata* (Hewitt et al. 2016).

It is already known that at least two drainages, the Osage and Neosho rivers, in this region contain a genetically distinct *Fusconaia* lineage (hereafter as *F.* cf. *hebetata/sampsoniana*). It is possible that this lineage is more widespread through the region. The use of molecular data to identify specimens in the region may provide more information on this lineage's extent. It has been suggested that the names *F. hebetata*

or *F. sampsoniana* may be attributable to this lineage. Unfortunately, the type locality information associated with these names is extremely vague. The use of morphological information may be able to provide evidence that one of these names may be more appropriate than the other. Further, both *P. sintoxia* and *P. rubrum* are known from this geographic region, and the relationship between those two species has not yet been thoroughly investigated using specimens from all major Ozark drainages. It remains to be established whether specimens from this region are genetically distinguishable or not. The goals of this study are to:

1. Phylogenetically assess *Fusconaia* and *Pleurobema* specimens from various drainages in the Ozarks region so that molecular identities can be established and relationships among species can be assessed.
2. Investigate the geographic extent of the undescribed *Fusconaia* based on molecular identities.
3. Use geometric morphometric techniques to investigate shell shape of genetically identified specimens so that morphological distinctness can be assessed.
4. Use geometric morphometric techniques to investigate whether the names *Fusconaia hebetata* or *Fusconaia sampsoniana* might be attributable to the distinct Ozark *Fusconaia* lineage.

II. Methods

Mussel specimens were collected from 2012 to 2014 from 10 different locations largely by the efforts of the Arkansas State University Museum of Zoology (ASUMZ) and the Missouri Department of Conservation (MDC). The streams included in this effort were the Gasconade and Sac rivers of the Missouri River system; Bryant Creek and the Black, James, and North Fork White rivers of the White River system; the Spring River of the Arkansas River system; the St. Francis River, a direct tributary to the Mississippi River; and the Bourbeuse and Meramec rivers of the Meramec River system. From these streams, a total of 164 individuals were collected and deposited in the ASUMZ collection (Table 1, Figure 1).

Tissue samples were collected as clips from the mantle of specimens and preserved in 95% EtOH. DNA was extracted from tissue samples using a CTAB extraction protocol (Saghai-Maroof et al. 1984). Yields were quantified using a Qubit fluorometer broad-range assay (Invitrogen) and concentrations were standardized to 10 µg/mL and amplified by polymerase chain reaction (PCR). The Cytochrome c oxidase subunit I (COI) mitochondrial gene was chosen for analysis because it has been previously relied on by other researchers to investigate relationships within the Unionidae (e.g. Campbell & Lydeard 2012; Lopes-Lima et al. 2017). PCR amplification of the entire COI region was unsuccessful, presumably because DNA had continued to degrade despite preservation in EtOH. Because of this, the gene was partially amplified as two smaller fragments at the 5’ and 3’ end of the region. For most specimens, PCR primers and PCR conditions follow those described by Burdick and White (2007) for use on specimens that had been

preserved for longer than 4 years. For the 5’ fragment, the upstream primer was COI-L, 5’-GTC AAC AAA TCA TAA AGA TAT TGG-3’ (Folmer et al. 1994) and the downstream primer was COI-H3, 5’-AAC ACC CCT CTC CAC TAA-3’. For the 3’ fragment, the upstream primer was COI-L5, 5’-TTA GTG GAG AGG GGT GTT GG-3’ and the downstream primer was COI-H, 5’-AAA CTT CAGGGT GAC CAAAAA ATC A-3’ (Folmer et al. 1994). PCR was carried under the following conditions: initial denaturing at 95°C for 3 min; 30 cycles of 95°C denaturing for 45 s, 49°C annealing for 30 s, 72°C extension for 90 s; and a final extension at 72°C for 5 min. For *F. ozarkensis* specimens, primers were used that are more specific to the species. The COI-L primer was paired with COI-H3oz, 5’-AAC ACC CCT CTC TAC CAA-3’ and the COI-H primer was paired with COI-L5oz, 5’-TTG GTA GAG AGG GGT GTT GG-3’. Reaction success was based on product visualization using agarose gel electrophoresis. Crude PCR product was plated and sent to Eurofins Genomics (Louisville, KY) for Sanger sequencing.

Sequences returned from Eurofins were trimmed manually using the program DNADynamo (BlueTractor Software Ltd.). They were aligned to the pleurobemini and outgroup sequences used to construct the phylogeny published in Inoue et al (2018) using MUSCLE within the program AliView (Edgar 2004; Larsson 2014). Additional *F. mitchelli* and *F. iherngi* sequences from Smith et al. (2020) were downloaded from GenBank and included in the alignment. Duplicate sequences that were not generated in this study were removed in AliView. Model selection was performed with the ModelFinder tool within IQ-TREE (Kalyaanamoorthy et al. 2017; Nguyen et al. 2015). A

maximum likelihood phylogenetic tree was produced in IQ-TREE with 1000 ultrafast bootstrap replicates (Hoang et al., 2017; Tamura & Nei 1993).

All photographs for morphometric analysis were taken with a Canon EOS 5D Mark IV equipped with the Canon EF 17-40 f/4L USM Lens and photographs were taken using the Ortery Photosimile 200 photobox and the Ortery Capture software V1.0.8.45. To capture the shells outline, photographs were taken of the right valve from a top-down angle. Exterior photographs of 6 specimens contained in Ohio State University Museum of Biological Diversity (OSU) lots 13648 and 39652 were provided by John Harris at ASUMZ. These specimens were included as representatives of *Fusconaia* cf. *hebetata/sampsoniana* based on Burdick & White (2007). The program MakeFan8 (Sheets 2014) was used to overlay a fan of 30 equally spaced rays. The fan was aligned to two homologous structures on each shell: the posterior end of the hinge ligament and the vertex of the angle formed between then umbo and anterior side of the shell. The intersection of each ray with the edge of the shell represented a semilandmark (Appendix B, Figure 2). The program tpsDig2 (Rohlf 2005) was used to digitize the two homologous points, fourteen semilandmarks between them along the ventral side of the shell, and four semilandmarks along the umbo. This gave a total of twenty landmarked locations on each shell. Landmarks were exported as a TPS file for analysis.

To capture the inflation profile of each shell, a General Tools & Instruments 6-inch stainless steel contour-gauge was used. The right valve of each specimen was placed on the image of a circle with 5° increments marked. The vertex of the umbo and anterior side of the shell was aligned to the center of the circle and the posterior end of

the hinge ligament was aligned to the 180° line. The contour-gauge was aligned with the 210° line and pressed straight down onto the shell. This created an outline of the shell that was photographed with the posterior side on the left and the anterior side on the right. MakeFan8 was used to overlay a fan of 30 equally spaced rays. The fan was aligned to two homologous points on each contour: the posterior and anterior ends. Each place that a ray intersected the contour tool represented a semilandmark. The two homologous points and 14 points between them were digitized in tpsDig2. This gave a total of 16 landmarked locations on each shell (Appendix B, Figure 3). Landmarks were exported as a TPS file for analysis.

Prior to analysis, five specimens were removed from the analysis due to excessively worn umbos. Analysis of landmark data was performed using the program PAST v4.03 (Hammer et al. 2001). In order to remove variation related to size and rotation of specimens, the raw landmarks were aligned using Procrustes superimposition. Shape variation was analyzed using two ordination methods. Principal component analysis (PCA) was used to investigate the variation among individuals and Linear Discriminant Analysis (LDA) was used to investigate the variation between groups using *a priori* designations and to reclassify data to evaluate confusion between groups. LDA ordinations were conducted using genetic identification as the grouping factor for comparing all specimens. All ordinations were visualized using the first and second axes.

III. Results

DNA amplification for both fragments was successful in 79 specimens, representing the entire geographic range of collection excluding the North Fork White River. Model selection indicated that the TIM2+F+G4 model was the best descriptor of substitution patterns. The Phylogenetic tree was constructed from 3210 sequences, with a total of 949 positions in the final dataset. The resulting tree had a log-likelihood of -6732.4609 (s.e. 373.5042).

New sequences clustered in 5 different clades. Fifty-seven specimens grouped with *P. rubrum* and *P. sintoxia* sequences. This clade was represented by specimens from every major drainage in the northern Ozarks. The two species were undifferentiated within the phylogeny. Six specimens grouped with *F. flava* sequences. This clade was represented by specimens only from the Black River of the White River system. Four specimens grouped with *F. ozarkensis* sequences. This clade was represented by specimens from multiple localities within the White River system. Twelve specimens grouped with the *F.* cf. *hebetata/sampsoniana* clade that includes the F8 & F9 haplotypes reported in Burdick & White (2007). This clade was represented by specimens from every major drainage in the northern Ozarks except for the White River system & the St. Francis River system (Appendix A). A single specimen from the Gasconade River clustered with *Regenaia ebena* sequences but was not included in further analyses.

The PCA of shell outline shapes revealed very little distinction between any groups. The plot suggested that specimens within the *P. sintoxa/rubrum* and *F.*

hebetata/sampsoniana clades were the most variable, while the *F. flava* and *F. ozarkensis* specimens produced more compact groupings. *F. ozarkensis* was the only species displaying little overlap with any other species (Figure 4). PC axis 1 accounted for 52.93% of variation and PC axis 2 accounted for 28.63% of variation. All other PC axes accounted for less than 10% of variation individually. The CVA of shell outline shapes classified by genetic species identity yielded 3 distinct canonical variates explaining 58.81%, 30.54%, and 10.65% of variance, respectively. Reclassifications based on this CVA produced a 100.00% accuracy in assigning individuals to the correct species (Table 2). Although all reclassifications were correctly assigned, the visualized LDA plot did reveal overlap between *F. ozarkensis* and both *F.* cf. *hebetata/sampsoniana* and *P. rubrum/sintoxia* (Figure 5).

The PCA of shell inflation shapes revealed some separation of *F. ozarkensis* from all other groups. *F. hebetata/sampsoniana* and *P. rubrum/sintoxia* were, again, the most variable of the 4 groups and heavily overlapped. *F. flava* mostly overlapped with *P. rubrum/sintoxia* (Figure 6). PC axis 1 accounted for 82.12% of the variation and PC axis 2 accounted for 9.98% of the variation. All other PC axes accounted for less than 4% of variation individually. The CVA of shell inflation shapes classified by genetic species identity yielded 3 distinct canonical variates explaining 57.18%, 37.37%, and 5.45% of variance, respectively. Reclassifications based on this CVA produced an 88.46% accuracy in assigning individuals to the correct species (Table 3). This analysis suggests confusion of *F.* cf. *hebetata/sampsoniana* for *P. rubrum/sintoxia* (n=1, 7.69%) and confusion of *P. rubrum/sintoxia* for *F.* cf. *hebetata/sampsoniana* (n=8, 14.55%). The visualized CVA plot

did reflect this overlap between *F.* cf. *hebetata/sampsoniana* and *P. rubrum/sintoxia*, while *F. flava* and *F. ozarkensis* were both isolated (Figure 7).

IV. Discussion

The geographical extent of *F. flava* in the Ozarks was restricted to the Black River. The presence of *F. flava* in the Black River is consistent with Burdick & White (2007), which reported it not only in the Black River, but in a tributary of the Black (Strawberry River) as well as the broader White River system of northern Arkansas and southern Missouri. The geographic extent of *F. ozarkensis* was also restricted to the White River system. The presence of this species in the White River system has been confirmed by other genetic studies (Campbell & Lydeard 2012; Inoue et al. 2018). These results indicate that the distributions of *F. flava* and *F. ozarkensis* in the Ozarks may be much more limited than historically recognized. Past records of this species from the Arkansas, Meramec, & Missouri River systems may be representatives of *F.* cf. *hebetata/sampsoniana* that were misidentified. Past studies have identified this distinct genetic lineage from the Illinois, Neosho, and Osage rivers (Burdick & White 2007; Inoue et al. 2018). With this in mind, it may be appropriate to reevaluate historically collected Ozarks data on *Fusconaia* life history and ecology. For example, Barnhart and Riusech (1997) described the conglutinates and glochidia of *F. ozarkensis* specimens from the Spring River and reported 3 successful hosts of glochidia: *Luxilus zonatus, L. cardinalis,* and *Chrosomus erythrogaster*. It is possible that theses descriptions and results represent *F.* cf. *hebetata/sampsoniana* instead. The current study expands the known range of this lineage to the Bourbeuse, Meramec, and Sac rivers. This lineage seems to be centered around the Ozark Highlands and consists of at least three potentially isolated distributional units: that in the Arkansas River System, that in the Meramec River System, and that in the Missouri River system. A lack of geographical structure

within the *F.* cf. *hebetata/sampsoniana* clade was potentially due to large sequence gaps associated with the amplification methodology. While conclusions on the fine-scale geography of this species cannot be drawn from these data, the observed northern-western Ozark distribution of *F.* cf. *hebetata/sampsoniana* is consistent with patterns seen in other aquatic species (e.g., Echelle et al. 2015).

The close relationship between *F.* cf. *hebetata/sampsoniana* and Gulf drainage species such as *F. askewi* and *F. mitchelli* (Figure 8) might suggest a historical connection between contemporary Gulf Coast drainages in Texas and Ozark drainages. A potential explanation for this relationship is historical connection through the hypothesized Ancestral Plains Stream (APS) (Metcalf 1966; Cross et al. 1986). This Pleistocene stream is thought to have drained much of the great plains in a north-south fashion. The presence of this stream would explain this relationship within *Fusconaia* by providing a direct link between tributaries of the upper Arkansas River to Gulf drainages in Texas. Pieri et al. (2018) estimated that the *F. askewi/chunii* clade split from the rest of *Fusconaia* sometime between 2.49 and 2.03 million years ago, during the early to middle Pleistocene. That time frame is consistent with possibility that the APS could have temporarily linked the already-established *F. askewi/chunii* lineage with northern drainages. This would have allowed expansion northward, followed by vicariance events that isolated the two lineages. This drainage has been used by ichthyologists as an explanation for distributions and genetic structuring of Great Plains fishes (e.g., Kreiser et al. 2001; Busso et al. 2013); however, other studies have refuted the existence of the ancient drainage (Hoagstrom & Berry 2006). Elfrink (2007) suggested a similar Pre-

Illinoisan drainage in the form of the Teays River flowing westward, north of the Ozarks, and draining to the Sabine River.

The results that *P. rubrum* and *P. sintoxia* were undifferentiated at the CO1 region is consistent with recently published data (Jones et al. 2015; Inoue et al. 2018). Researchers reporting this relationship between the two currently accepted species have already suggested the possibility that the two may be conspecifics. Inoue et al. (2018) included samples of *P. rubrum* and *P. sintoxia* over much of their geographic range and this study fills in a previously unsampled region where both species are considered sympatric.

Determining the nature of the relationship of these species will be crucial for effective conservation efforts. *Pleurobema rubrum* is currently under review by the U.S. Fish & Wildlife Service (USFWS) as a candidate for federal protections following a 2010 petition for listing. If these two species are truly morphological variants of a single genetic lineage, that information will impact the way that USFWS, as well as state conservation agencies, can act to protect these animals. As noted in Inoue et al. (2018), further genetic and ecological information should be considered to properly assess this relationship. For example, multiple cyprinid fishes & the bluegill (Centrarchidae: *Lepomis macrochirus*) have been reported as glochidia hosts for these two species (Hove 1995; Watters et al. 2005; Culp et al. 2009). However, the only host reported for both species is the spotfin shiner (*Cyprinella spiloptera*). Further testing may reveal more overlap in fishes capable of hosting either species but may also reveal that both species have a distinct set of hosts.

The PCA of shell outlines revealed significant overlap between most groups. The exception to this was *F. flava* and *F. ozarkensis*, which were distinctly separated from each other. Both *P. rubrum*/sintoxia and *F. hebetata/sampsoniana* exhibited a greater amount of variation than *F. flava* and *F. ozarkensis*. The general shape in shell shape from negative to positive was most obvious along the posterior and anterior margins of the shell. Negative PC1 scores tended to be associated with shells displaying elongated and narrowed posterior margins as well as a broadly rounded anterior margins with less concavity between the posterior end of the shell and the umbo. Positive PC1 scores tended to be associated with a more bluntly squared shape on both posterior and anterior margins. Thin-plate spline transformation grids also suggested a shift along the ventral margin of the shell, but this was likely also associated with elongation of the posterior end as concavity along the ventral margin did not appear to change significantly. Negative PC2 scores tended to be associated with an overall more triangular shell shape that has a very convex ventral margin and a higher umbo. Positive PC2 scores tended to be associated with shells that displayed a much more ovoid shape. With even rounder anterior, posterior, and ventral margins. Although reclassifications based on the CVA were 100% correct, the plot appeared to show a high amount of overlap between *F.* ozarkensis and *F. hebetata*/*sampsoniana*. There was also a small amount of overlap between *F. ozarkensis* and *P. rubrum*/*sintoxia.*

Both PCA analyses produced similar results and suggested that *P. rubrum/sintoxia* and *F. hebetata/sampsoniana* may display a higher degree of morphological variation than both *F. ozarkensis* and *F. flava*. These results exemplify the

fact that *P. rubrum* and *P. sintoxia* are currently recognized as morphologically distinct species even though it appears that they are the same genetic lineage. *F. flava* and *F. ozarkensis* are also considered, to be morphologically distinct species. The higher degree of morphological variability within the *F. hebetata/sampsoniana* lineage suggests an explanation for why specimens of this lineage have been misclassified in the past as both *F. flava* and *F. ozarkensis*.

The results and interpretations of these geometric morphometric analyses should be taken with some caution. Sample sizes for each species were uneven & limited to very few or only a single sample locality. More sampling of *F. flava* and *F. ozarkensis* should be performed to ensure accurate morphological assessment. It is possible that with the addition of more specimens of these species, the PCA plots may reflect that they tend to be as variable as the *P. rubrum/sintoxia* and *F. hebetata/sampsoniana* lineages. Another possibility is that with additional data on all lineages, the morphological associations between *F. hebetata/sampsoniana* and *F. ozarkensis* suggested by the LDA plots may become even less distinct.

Biological diversity worldwide is becoming increasingly threatened by climate change as well as direct anthropogenic habitat alteration & destruction. Freshwater mussels are no exception to this, with 40% of global species being at least near threatened (Lopes-Lima et al. 2018). These declines highlight the ever-growing need for the training and funding of experts in taxonomic and systematic research. Scientific knowledge of the diversity of species within a group of animals like freshwater mussels is far from complete, with new research every year pointing to flaws in currently

accepted species. Within the Unionidae alone there are known cases of cryptic diversity that have yet to be formally described & named, for example the case presented here involving *F. flava* & *F.* cf. *hebetata/sampsoniana*. In other cases, diversity has been inflated due to the description of genetically indistinguishable morphotypes being described as separate species, potentially what has happened in the case presented here involving *P. rubrum* & *P. sintoxia*. The scientific process of describing and naming species alongside investigating both inter- and intraspecific relationships is at the base of contemporary conservation and management goals. If the currently accepted concept of a particular species is flawed, then conservation efforts applied to that species will be less effective.

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Tables

Table 1. Museum, field ID, and locality information for specimens included in the study. "Genetic" column indiciates how many specimens from the lot were genetically analyzed. "GM" column indicates how many specimens from the lot were morphometrically analyzed.

Lot	Field ID	Locality	Genetic	GM
ASUMZ 2761	*P. sintoxia*	Illinois River, AR	n=1[a]	n=1
ASUMZ 2911	*F. flava sampsoniana*	Illinois River, AR	n=1[a]	n=1
ASUMZ 3684	*F. ozarkensis*	Jacks Fork Current River, MO	n=1[a]	n=1
ASUMZ 4900	*F.* sp.	Illinois River, AR	n=1[a]	n=1
ASUMZ 4904	*F.* sp.	Illinois River, AR	n=1[a]	n=1
ASUMZ 1103	*F. flava*	Bourbeuse River, Franklin Co., MO	n=3	n=2
ASUMZ 1104	*P. sintoxia*	Bourbeuse River, Franklin Co., MO	n=3	n=3
ASUMZ 1105	*F.* sp.	Bourbeuse River, Franklin Co., MO	n=5	n=5
ASUMZ 1108	*F.* sp.	Gasconade River, Gasconade Co., MO	n=1	n=1
ASUMZ 1109	*P. sintoxia*	Gasconade River, Gasconade Co., MO	n=8	n=8
ASUMZ 1110	*P.* sp.	Gasconade River, Gasconade Co., MO	n=3	n=3
ASUMZ 1111	*P.* sp.	Gasconade River, Gasconade Co., MO	n=1	n=1
ASUMZ 1112	*P. sintoxia*	James River, Green Co., MO	n=2	n=2
ASUMZ 1113	*F. ozarkensis*	James River, Green Co., MO	n=2	n=2
ASUMZ 1114	*F. flava*	Black River, Butler Co., MO	n=6	n=6
ASUMZ 1115	*P.* sp. *cf cordatum*	Black River, Butler Co., MO	n=2	n=2
ASUMZ 1116	*P.* sp. *cf sintoxia*	Black River, Butler Co., MO	n=2	n=2
ASUMZ 1117	*P.* sp. *cf rubrum*	Black River, Butler Co., MO	n=2	n=2
ASUMZ 1118	*P.* sp. *cf sintoxia*	Black River, Butler Co., MO	n=5	n=5
ASUMZ 1119	*P.* sp. *cf cordatum*	Black River, Butler Co., MO	n=1	n=1
ASUMZ 1120	*F. ozarkensis*	North Fork White River, Douglas Co., MO	n=1	n=1
ASUMZ 1121	*F. ozarkensis*	Bryant Creek, Ozark Co., MO	n=1	n=1
ASUMZ 1122	*P. sintoxia*	Bryant Creek, Ozark Co., MO	n=1	n=1
ASUMZ 1149	*F. flava*	Meramec River, St. Louis Co., MO	n=2	n=2
ASUMZ 1150	*P. sintoxia*	Meramec River, St. Louis Co., MO	n=5	n=5
ASUMZ 1151	*P. rubrum*	Meramec River, St. Louis Co., MO	n=2	n=2
ASUMZ 1152	*P. sintoxia*	Sac River, St. Claire Co., MO	n=8	n=8
ASUMZ 1153	*F. flava*	Sac River, St. Claire Co., MO	n=7	n=7
ASUMZ 1154	*P. sintoxia*	St. Francis River, Wayne Co., MO	n=5	n=1
ASUMZ 1179	*P. sintoxia*	Spring River, Jasper Co., MO	n=1	n=1
OSUM 13648	*F. flava*	Osage River, Miller Co., MO	n=1[b]	n=2[c]
OSUM 39652	*F. flava*	Neosho River, Lyon Co., KA	n=2[b]	n=4[c]

Notes: [a] Genetic data analyzed by Inoue et al (2018). [b] Genetic data analyzed by Burdick and White (2007). [c] specimens included in analysis of outline semilandmarks but not analysis of inflation semilandmarks.

Table 2. Confusion matrix from shell outline LDA reclassification. Rows are the given groups and columns are the predicted groups.

	F. cf. *hebetata/sampsoniana*	*P. rubrum/sintoxia*	*F. ozarkensis*	*F. flava*	Total
F. cf. *hebetata/sampsoniana*	19	0	0	0	19
P. rubrum/sintoxia	0	55	0	0	55
F. ozarkensis	0	0	4	0	4
F. flava	0	0	0	6	6
Total	19	55	4	6	84

Table 3. Confusion matrix from shell inflation LDA reclassification. Rows are the given groups and columns are the predicted groups.

	F. cf. *hebetata/sampsoniana*	*P. rubrum/sintoxia*	*F. ozarkensis*	*F. flava*	Total
F. cf. *hebetata/sampsoniana*	12	1	0	0	13
P. rubrum/sintoxia	8	47	0	0	55
F. ozarkensis	0	0	4	0	4
F. flava	0	0	0	6	6
Total	20	48	4	6	78

Figures

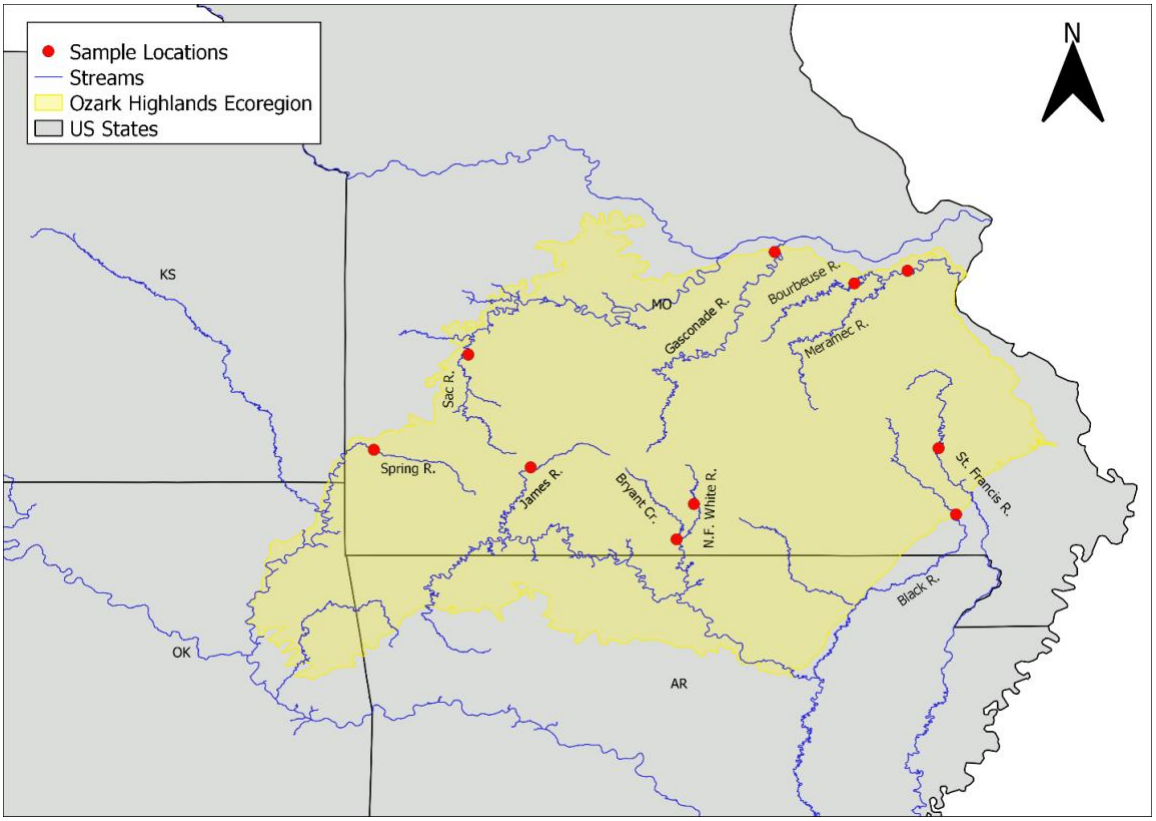


Figure 1. Map displaying sampling locations and major river basins. Sample locations for specimens analyzed in previous studies omitted.

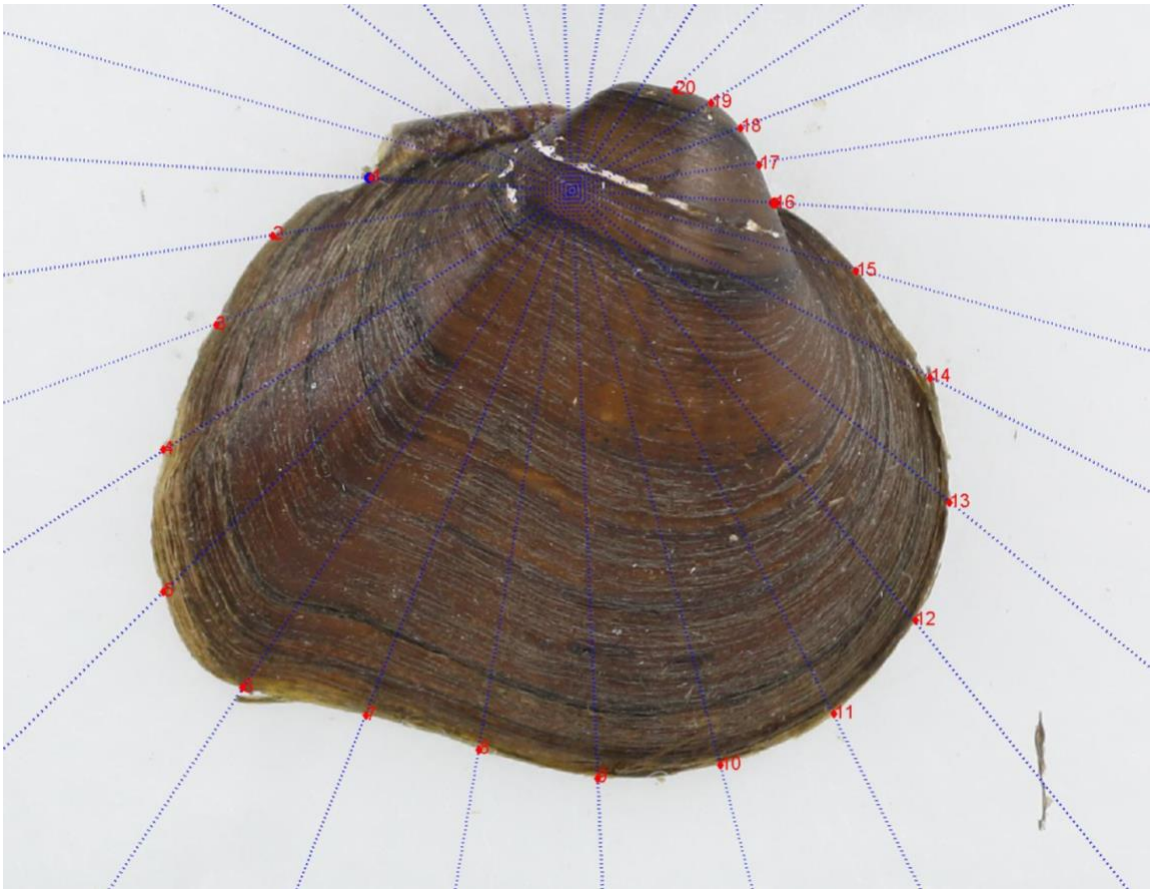


Figure 2. Example placement of radial fan and semilandmarks on shell outline photographs used in geometric morphometric analysis.

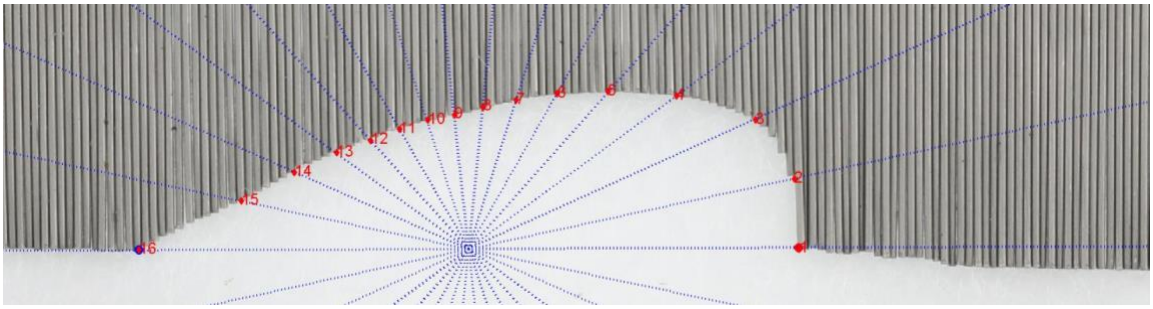


Figure 3. Example placement of radial fan and semilandmarks on shell inflation photographs used in geometric morphometric analysis.

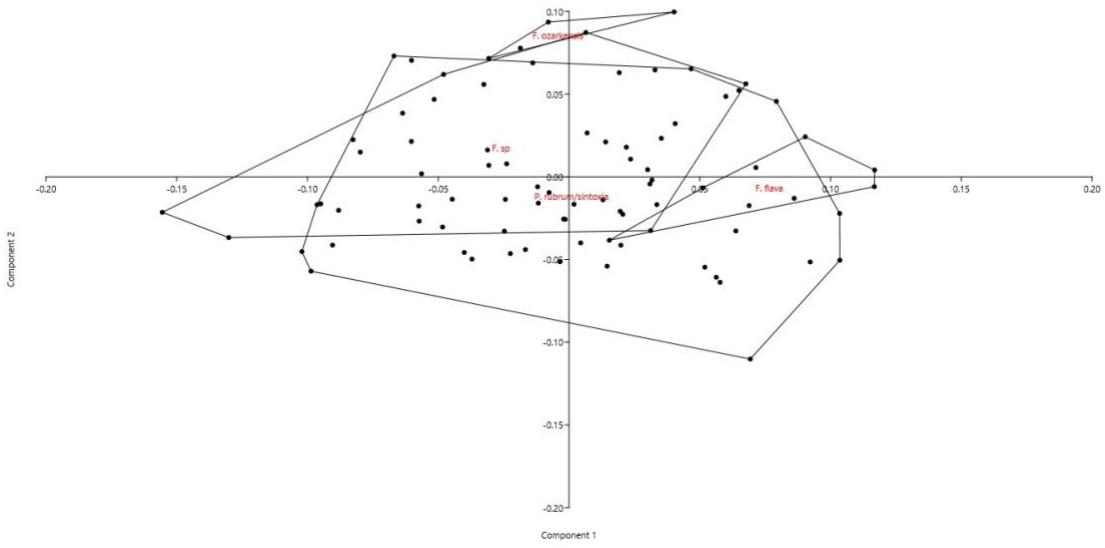


Figure 4. PCA ordination plot for shell outline semilandmarks visualized by first and second axes.

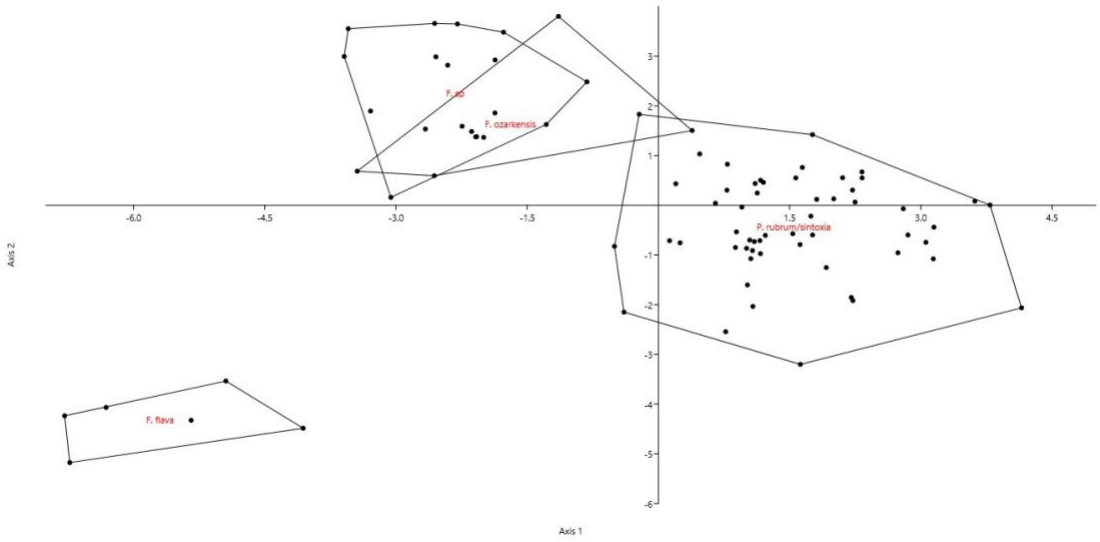


Figure 5. LDA Ordination plot for shell outline semilandmarks visualized by first and second axes.

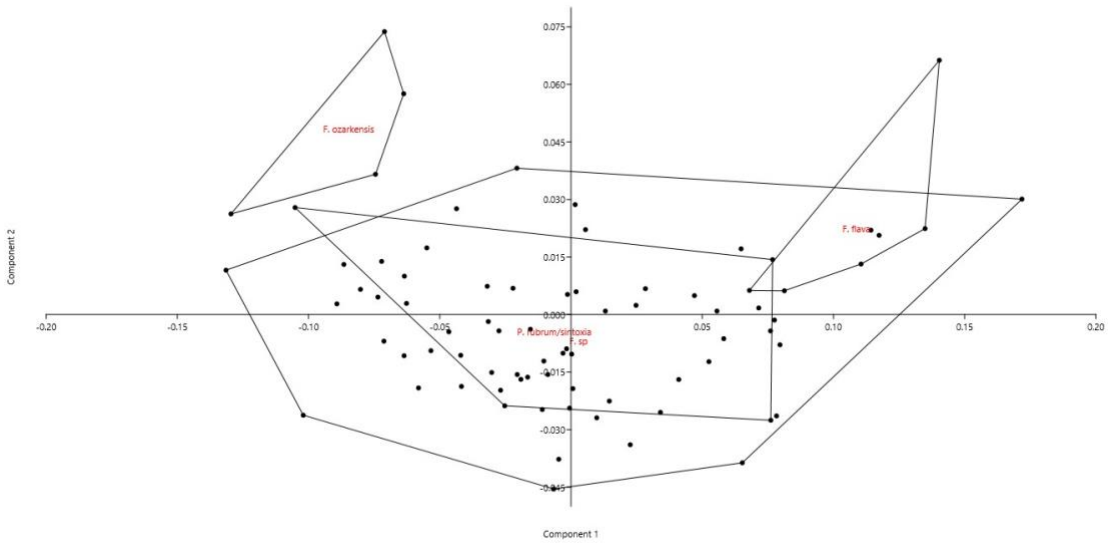


Figure 6. PCA ordination plot for shell inflation semilandmarks visualized by first and second axes.

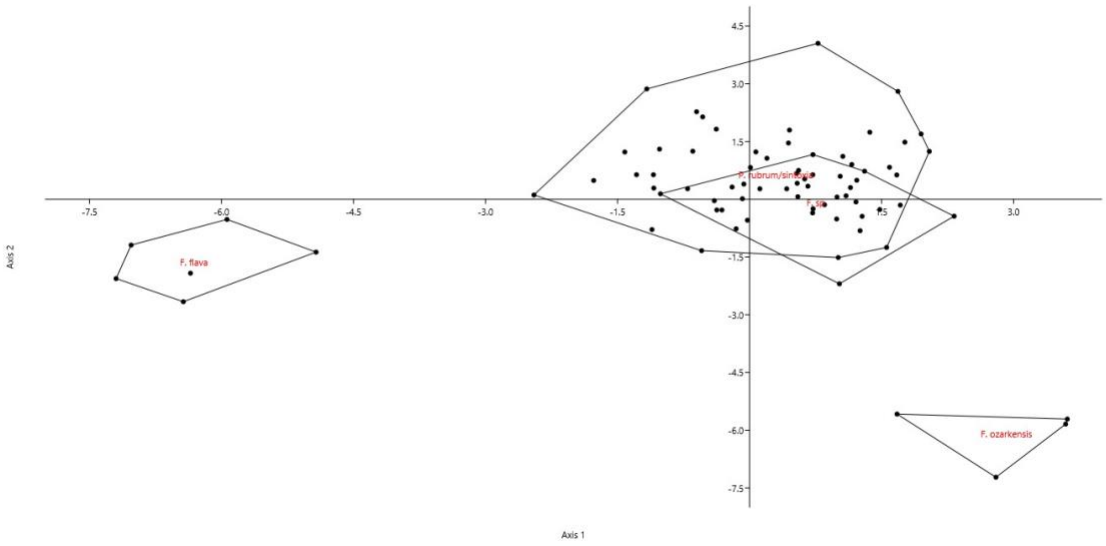


Figure 7. LDA ordination plot for shell inflation semilandmarks visualized by first and second axes.

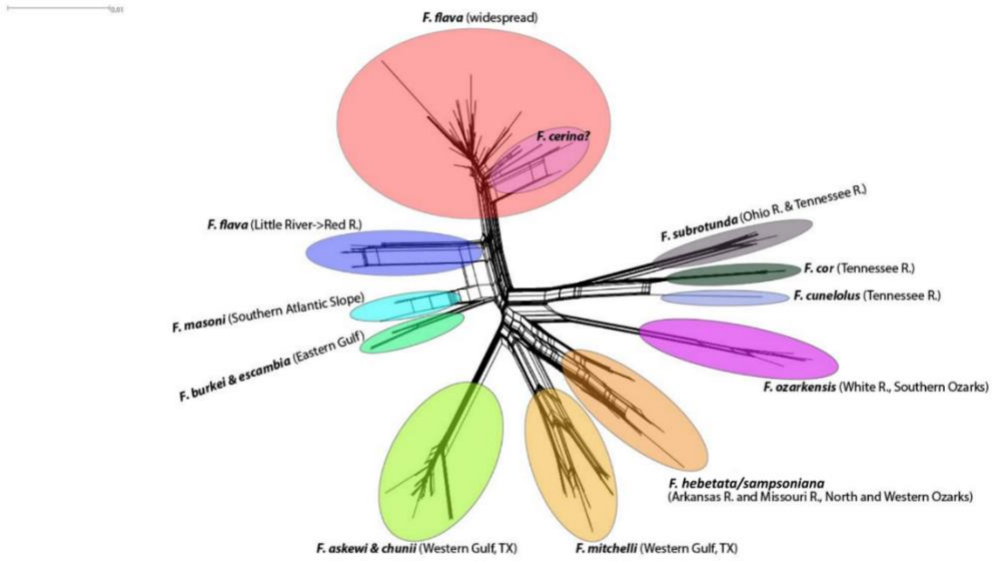
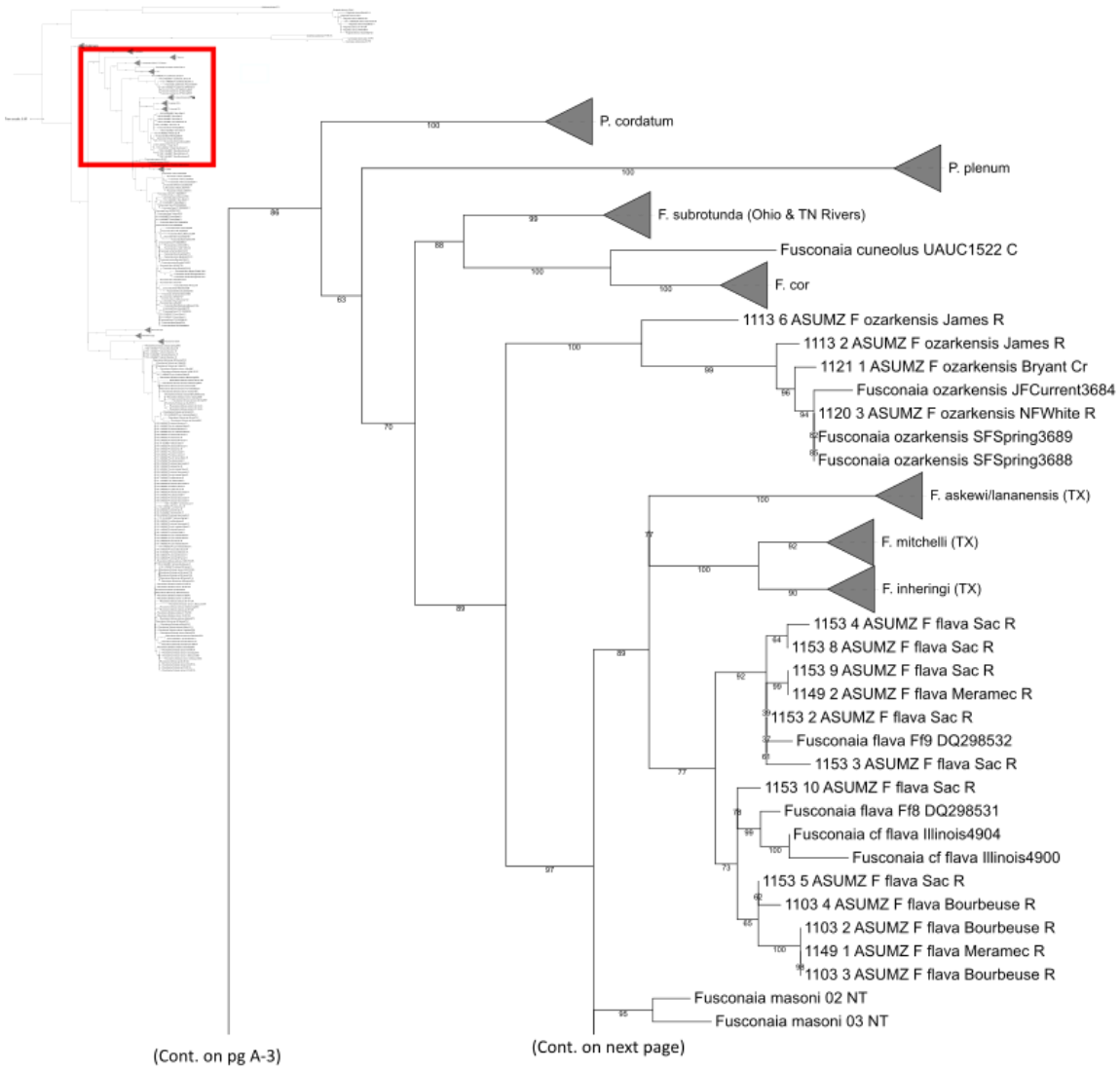


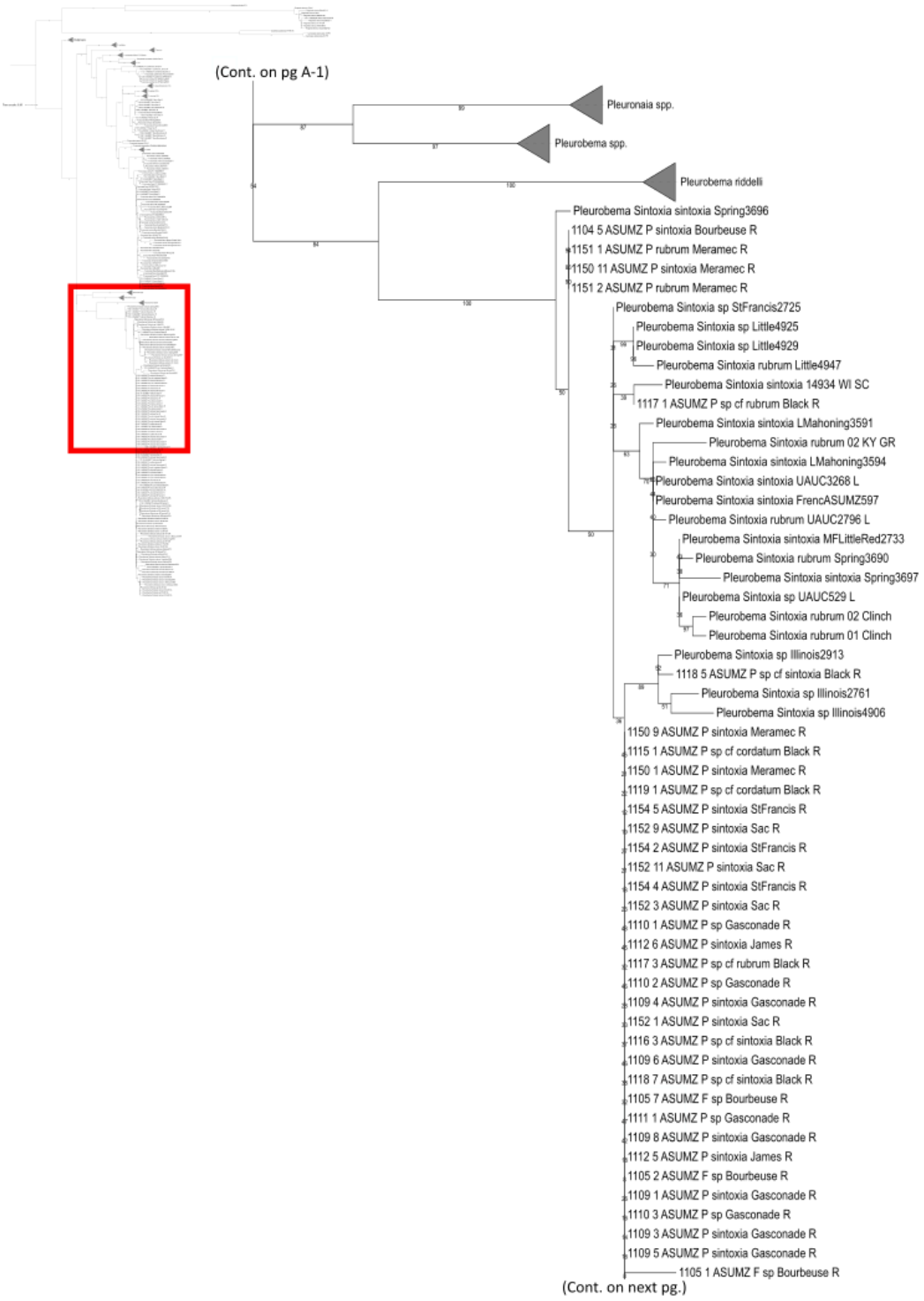
Figure 8. Unrooted phylogenetic network detailing interspecific relationships within Fusconaia.

APPENDICES

Appendix A:
Phylogeny of Pleurobemini with noninformative clades collapsed, outgroups not shown in detail


P. cordatum
P. plenum
F. subrotunda (Ohio & TN Rivers)
Fusconaia cuneolus UAUC1522 C
F. cor
1113 6 ASUMZ F ozarkensis James R
1113 2 ASUMZ F ozarkensis James R
1121 1 ASUMZ F ozarkensis Bryant Cr
Fusconaia ozarkensis JFCurrent3684
1120 3 ASUMZ F ozarkensis NFWhite R
Fusconaia ozarkensis SFSpring3689
Fusconaia ozarkensis SFSpring3688
F. askewi/lananensis (TX)
F. mitchelli (TX)
F. inheringi (TX)
1153 4 ASUMZ F flava Sac R
1153 8 ASUMZ F flava Sac R
1153 9 ASUMZ F flava Sac R
1149 2 ASUMZ F flava Meramec R
1153 2 ASUMZ F flava Sac R
Fusconaia flava Ff9 DQ298532
1153 3 ASUMZ F flava Sac R
1153 10 ASUMZ F flava Sac R
Fusconaia flava Ff8 DQ298531
Fusconaia cf flava Illinois4904
Fusconaia cf flava Illinois4900
1153 5 ASUMZ F flava Sac R
1103 4 ASUMZ F flava Bourbeuse R
1103 2 ASUMZ F flava Bourbeuse R
1149 1 ASUMZ F flava Meramec R
1103 3 ASUMZ F flava Bourbeuse R
Fusconaia masoni 02 NT
Fusconaia masoni 03 NT
(Cont. on pg A-3)
(Cont. on next page)


Fusconaia escambia UF428548 MN649040
F. burkei
Fusconaia cf flava Little4628
Fusconaia cf flava Little4942
Fusconaia cf flava Little4923
Fusconaia cf flava Cossatot2811
Fusconaia cf flava Cossatot2816
Fusconaia cf flava Little4910
Fusconaia cf flava Little4931
Fusconaia cerina Fc1 DQ298537
Fusconaia flava LMissouri2900
1114 7 ASUMZ F flava Black R
1114 2 ASUMZ F flava Black R
Fusconaia flava Ff5 DQ298528
Fusconaia flava Ff7 DQ298530 1
Fusconaia flava WFPRC2751
Fusconaia flava Cadron5210
1114 9 ASUMZ F flava Black R
1114 4 ASUMZ F flava Black R
Fusconaia flava Ff13 DQ298536
Fusconaia flava Ff1 DQ298524
Fusconaia flava Ouachita2804
Fusconaia flava Ff4 DQ298527
Fusconaia flava LMissouri2884
Fusconaia flava Ouachita2780
Fusconaia flava Saline3784
Fusconaia flava Ff3 DQ298526
Fusconaia flava UAUC3391 L
Fusconaia flava 14451 MN CW
Fusconaia cerina BundickCr5410
Fusconaia flava Ouachita2776
Fusconaia cerina BundickCr5413
Fusconaia cerina BundickCr5412
Fusconaia cerina BundickCr5409
Fusconaia flava White2738
Fusconaia cerina BundickCr5411
Fusconaia flava BogueChittol6 NAJ
Fusconaia cerina Pascagoula8 NAJ
Fusconaia cerina Pascagoula7 NAJ
Fusconaia flava PetitJean2744
Fusconaia flava Ouachita
Fusconaia flava White2736
Fusconaia flava NFSaline3698
Fusconaia flava Ouachita2766
Fusconaia cerina BundickCr5408
Fusconaia flava White2737
Fusconaia flava StFrancis2732
Fusconaia flava Little4907
Fusconaia flava BigCypressBayou9 NAJ
Fusconaia flava Ouachita2779
Fusconaia flava Ff11 DQ298534
1114 5 ASUMZ F flava Black R
1114 6 ASUMZ F flava Black R
Fusconaia flava Ff12 DQ298535
Fusconaia flava Black2709
Fusconaia flava Ff6 DW298529


(Cont. on pg A-1)
Pleuronaia spp.
Pleurobema spp.
Pleurobema riddelli
Pleurobema Sintoxia sintoxia Spring3696
1104 5 ASUMZ P sintoxia Bourbeuse R
1151 1 ASUMZ P rubrum Meramec R
1150 11 ASUMZ P sintoxia Meramec R
1151 2 ASUMZ P rubrum Meramec R
Pleurobema Sintoxia sp StFrancis2725
Pleurobema Sintoxia sp Little4925
Pleurobema Sintoxia sp Little4929
Pleurobema Sintoxia rubrum Little4947
Pleurobema Sintoxia sintoxia 14934 WI SC
1117 1 ASUMZ P sp cf rubrum Black R
Pleurobema Sintoxia sintoxia LMahoning3591
Pleurobema Sintoxia rubrum 02 KY GR
Pleurobema Sintoxia sintoxia LMahoning3594
Pleurobema Sintoxia sintoxia UAUC3268 L
Pleurobema Sintoxia sintoxia FrencASUMZ597
Pleurobema Sintoxia rubrum UAUC2796 L
Pleurobema Sintoxia sintoxia MFLittleRed2733
Pleurobema Sintoxia rubrum Spring3690
Pleurobema Sintoxia sintoxia Spring3697
Pleurobema Sintoxia sp UAUC529 L
Pleurobema Sintoxia rubrum 02 Clinch
Pleurobema Sintoxia rubrum 01 Clinch
Pleurobema Sintoxia sp Illinois2913
1118 5 ASUMZ P sp cf sintoxia Black R
Pleurobema Sintoxia sp Illinois2761
Pleurobema Sintoxia sp Illinois4906
1150 9 ASUMZ P sintoxia Meramec R
1115 1 ASUMZ P sp cf cordatum Black R
1150 1 ASUMZ P sintoxia Meramec R
1119 1 ASUMZ P sp cf cordatum Black R
1154 5 ASUMZ P sintoxia StFrancis R
1152 9 ASUMZ P sintoxia Sac R
1154 2 ASUMZ P sintoxia StFrancis R
1152 11 ASUMZ P sintoxia Sac R
1154 4 ASUMZ P sintoxia StFrancis R
1152 3 ASUMZ P sintoxia Sac R
1110 1 ASUMZ P sp Gasconade R
1112 6 ASUMZ P sintoxia James R
1117 3 ASUMZ P sp cf rubrum Black R
1110 2 ASUMZ P sp Gasconade R
1109 4 ASUMZ P sintoxia Gasconade R
1152 1 ASUMZ P sintoxia Sac R
1116 3 ASUMZ P sp cf sintoxia Black R
1109 6 ASUMZ P sintoxia Gasconade R
1118 7 ASUMZ P sp cf sintoxia Black R
1105 7 ASUMZ F sp Bourbeuse R
1111 1 ASUMZ P sp Gasconade R
1109 8 ASUMZ P sintoxia Gasconade R
1112 5 ASUMZ P sintoxia James R
1105 2 ASUMZ F sp Bourbeuse R
1109 1 ASUMZ P sintoxia Gasconade R
1110 3 ASUMZ P sp Gasconade R
1109 3 ASUMZ P sintoxia Gasconade R
1109 5 ASUMZ P sintoxia Gasconade R
1105 1 ASUMZ F sp Bourbeuse R
(Cont. on next pg.)

(Cont. on previous page)

1152 7 ASUMZ P sintoxia Sac R
1152 4 ASUMZ P sintoxia Sac R
1152 10 ASUMZ P sintoxia Sac R
1109 2 ASUMZ P sintoxia Gasconade R
1179 14 ASUMZ P sintoxia Spring R
1105 4 ASUMZ F sp Bourbeuse R
1109 7 ASUMZ P sintoxia Gasconade R
1115 2 ASUMZ P sp cf cordatum Black R
1122 1 ASUMZ P sintoxia Bryant Cr
1108 1 ASUMZ F sp Gasconade R
1118 3 ASUMZ P sp cf sintoxia Black R
1118 2 ASUMZ P sp cf sintoxia Black R
1152 2 ASUMZ P sintoxia Sac R
1118 1 ASUMZ P sp cf sintoxia Black R
1116 8 ASUMZ P sp cf sintoxia Black R
1104 3 ASUMZ P sintoxa Bourbeuse R
1150 10 ASUMZ P sintoxia Meramec R
1150 2 ASUMZ P sintoxia Meramec R
1154 3 ASUMZ P sintoxia StFrancis R
Pleurobema Sintoxia sintoxia 14964 WI MS
1104 2 ASUMZ P sintoxia Bourbeuse R
1154 1 ASUMZ P sintoxia StFrancis R
Pleurobema Sintoxia rubrum UAUC3230 L
Pleurobema Sintoxia sp StFrancis2719
Pleurobema Sintoxia sp StFrancis2723
Pleurobema Sintoxia sp StFrancis2718
Pleurobema Sintoxia sp StFrancis2721
Pleurobema Sintoxia sintoxia 02 AR SA
Pleurobema Sintoxia rubrum 06 AR SA
Pleurobema Sintoxia sp Black3768
Pleurobema Sintoxia sp StFrancis2722
Pleurobema Sintoxia sintoxia 03 BSFC
Pleurobema Sintoxia rubrum 12 AR SA
Pleurobema Sintoxia sintoxia B1 KY GR
Pleurobema Sintoxia rubrum LMissouri2879
Pleurobema Sintoxia sintoxia LMahoning3592
Pleurobema Sintoxia sintoxia A2 KY GR
Pleurobema Sintoxia sintoxia 01 PA WB
Pleurobema Sintoxia rubrum 03 AR SA
Pleurobema Sintoxia sintoxia Saline3273
Pleurobema Sintoxia sp SFSpring2741
Pleurobema Sintoxia sp Black3762
Pleurobema Sintoxia sintoxia Saline3715
Pleurobema Sintoxia rubrum Ouachita2789
Pleurobema Sintoxia rubrum Saline3279
Pleurobema Sintoxia sintoxia PetitJean2753
1105 3 ASUMZ F sp Bourbeuse R
Pleurobema Sintoxia sintoxia Saline3716
Pleurobema Sintoxia sintoxia 4247 MN ST
Pleurobema Sintoxia sintoxia Ouachita2803
Pleurobema Sintoxia rubrum 08 AR SA
Pleurobema Sintoxia rubrum Ouachita2795
Pleurobema Sintoxia rubrum LMissouri2865
Pleurobema Sintoxia rubrum LMissouri2856
Pleurobema Sintoxia sp 04 AR SA
Pleurobema Sintoxia rubrum 09 AR SA
Pleurobema Sintoxia sp 05 AR SA
Pleurobema Sintoxia rubrum 05 AR SA