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The Pleurobemini (Bivalvia : Unionida) revisited: molecular species delineation using a mitochondrial DNA gene reveals multiple conspecifics and undescribed species

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Abstract. The Pleurobemini (Bivalvia: Unionida) represent approximately one-third of freshwater mussel diversity in North America. Species identification within this group is challenging due to morphological convergence and phenotypic plasticity. Accurate species identification, including characterisation of currently unrecognised taxa, is required to develop effective conservation strategies because many species in the group are imperiled. We examined 575 *cox1* sequences from 110 currently recognised species (including 13 *Fusconaia* and 21 *Pleurobema* species) to understand phylogenetic relationships among pleurobemine species (mainly *Fusconaia* and *Pleurobema*) and to delineate species boundaries. The results of phylogenetic analyses showed no geographic structure within widespread species and illustrated a close relationship between *Elliptio lanceolata* and *Parvaspina collina*. Constraint tests supported monophyly of the genera *Fusconaia* and *Pleurobema*, including the subgenus *P.* (*Sintoxia*). Furthermore, results revealed multiple conspecifics, including *P. hanleyianum* and *P. troschelianum*, *P. chattanoogaense* and *P. decisum*, *P. clava* and *P. oviforme*, *P. rubrum* and *P. sintoxia*, *F. askewi* and *F. lananensis*, and *F. cerina* and *F. flava*. Species delimitation analyses identified three currently unrecognised taxa (two in *Fusconaia* and one in *Pleurobema*). Further investigation using additional genetic markers and other lines of evidence (e.g. morphology, life history, ecology) are necessary before any taxonomic changes are formalised.

Additional keywords: DNA barcode, freshwater mussels, generalised mixed Yule-coalescent, molecular systematics, phylogenetics, species delimitation.

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Introduction

External morphologies, such as molluscan shells and arthropod exoskeletons, are the primary characteristics used by taxonomists to describe and identify species. However, external morphology can be influenced by the environment, biological interactions, and genetics (i.e. phenotypic plasticity) (Via *et al.* 1995). Thus, a single nominal species may exhibit multiple distinct morphologies. In contrast, cryptic diversity can arise under those same conditions leading to cases where two or more distinct species share similar morphological characteristics, making them externally indistinguishable (i.e. cryptic species) (Bickford *et al*. 2006). In relatively immobile organisms, there is often high morphological variation due to interaction between genetic factors and ambient environmental conditions. For example, in freshwater mussels (Bivalvia: Unionida; hereafter, freshwater mussels), it is known that shell shape and sculpture can vary with stream position and hydrodynamic conditions (Ortmann 1920; Watters 1994). At the same time, evolutionary convergence in shell morphologies is relatively common among freshwater mussel species (see Haag 2012 for a summary). Thus, phenotypic plasticity combined with the existence of cryptic species makes morphology-based identification of certain

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taxonomic groups of freshwater mussels a challenge and likely leads to under- and overestimates of the total species diversity within given taxa. The advent of species delimitation via molecular genetic techniques can address both issues by determining putative species boundaries within and among species and allow hidden biodiversity to be uncovered.

Freshwater mussels reach their greatest species diversity in North America, with ~300 currently recognised species (Haag and Williams 2014), although diversity may be higher than is currently recognised due to imperfect understanding of mussel taxonomy, which is based primarily on shell morphology (Williams *et al*. 1993; Turgeon *et al*. 1998; Graf and Cummings 2007). For example, recent molecular phylogenetics and species delimitation studies have uncovered cryptic diversity among freshwater mussels (Serb *et al*. 2003; Gangloff *et al*. 2006; Jones *et al*. 2006; Inoue *et al*. 2013; Pfeiffer *et al*. 2016; Perkins *et al*. 2017), providing empirical support that mussel diversity within North America continues to be underestimated. The current classification within the family Unionidae recognises seven subfamilies, of which three occur in North America: Ambleminae, Anodontinae, and Gonideinae (Lopes-Lima *et al.* 2017). Ambleminae contains four tribes: Amblemini, Lampsilini, Pleurobemini, and Quadrulini (Lopes-Lima *et al.* 2017). Many species within these groups have not been examined using molecular phylogenetics, resulting in their exact taxonomic placement being uncertain. Resolving these taxonomic and systematic uncertainties is important for the development of sound conservation actions, such as listing under the US *Endangered Species Act* and design of associated recovery plans.

The Pleurobemini was recognised as a distinct subfamily by Hannibal (1912). Campbell and Lydeard (2012*a*) provided the first modern review of the systematic and taxonomic history of this group using external morphology, anatomy, and molecular phylogenetics, which resulted in several nomenclatural revisions. For example, the genera *Quincuncina* and *Lexingtonia* were synonomised within *Fusconaia*, and *Pleuronaia* was elevated from synonomy within *Fusconaia*. Several genera within Pleurobemini were removed from the tribe, which included *Cyclonaias* Ortmann & Walker, 1922, *Uniomerus* Conrad, 1853, and the new genus *Reginaia* Campbell & Lydeard, 2012. Furthermore, due to phylogenetic distinctiveness, Campbell and Lydeard (2012*a*) suggested elevation of the subgenera *Eurynia* and *Sintoxia* from synonomy within *Elliptio* and *Pleurobema*, respectively, and potential placement of *Pleurobema collina* in a new genus. Subsequently, Perkins *et al*. (2017) analysed the molecular systematics of *P. collina* and described the new genus *Parvaspina*, which included the species previously recognised as *P. collina* and *Elliptio steinstansana* Johnson & Clarke, 1983. As a result of previous systematic and taxonomic studies (Campbell and Lydeard 2012*a*, 2012*b*; Lopes-Lima *et al.* 2017; Perkins *et al*. 2017), Pleurobemini is now thought to comprise 10 genera/subgenera: *Elliptio*, *Elliptoideus*, *Eurynia*, *Fusconaia*, *Hemistena*, *Parvaspina*, *Plethobasus*, *Pleurobema*, *Pleurobema* (*Sintoxia*), and *Pleuronaia*. Despite many taxonomic and systematic studies of Pleurobemini, questions remain regarding the species affinities and the higher-level relationships within the tribe, especially in *Fusconaia* and *Pleurobema*.

We used molecular phylogenetic and species delimitation approaches to revisit phylogenetic differentiation among species within Pleurobemini using additional species/populations collected for this study and data from previous studies (Tables S1 and S2). Our objectives were to: (1) delimit putative species within Pleurobemini, focusing on the genera *Fusconaia* and *Pleurobema*, and (2) examine cryptic diversity within Pleurobemini in the context of phylogenetic patterns among other mussel species.

Methods

Taxon sampling, sample preparation, and sequencing

We included 575 specimens (110 species, 35 genera/subgenera) (Tables S1 and S2), of which 223 specimens (105 species, 35 genera/subgenera) (Table S2) were obtained from GenBank, and we assimilated previously unpublished data for 352 specimens from 42 species representing 11 genera for the current study. A list of taxa, waterbody of collection, unique identifiers, and GenBank accession numbers are provided in Table S1. We extracted genomic DNA from mantle tissue via a CTAB/chloroform extraction method (Saghai-Maroof *et al*. 1984) or using the PureGene DNA extraction kit (Gentra Systems, Minneapolis, MN) following manufacturer's guidelines. After quantification using fluorescence (Labarca and Paigen 1980), extracted DNA was diluted to 10 ng μL^{-1} and used as a template in polymerase chain reaction (PCR) for the mtDNA cytochrome *c* oxidase subunit I (*cox1*). We used *cox1* primers and the thermal conditions for PCR described in Campbell *et al*. (2005). Products from PCR were visualised using 1% agarose gel electrophoresis and purified with QIAquick Gel Extraction Kit (Qiagen) or ExoSAP-IT (Affymetrix). We performed cycle sequencing reactions with BigDye 3.1 and sequenced bidirectionally using an ABI Genetic Analyzer (Applied Biosystems). Sequences were visually aligned in DNADynamo (Blue Tractor Software, Ltd) and an open-reading frame was verified. We used Mafft 7 (Katoh and Standley 2013) to perform multiple sequence alignment.

Phylogeny estimation

The primary aim of this study was to examine phylogenetic relationships within two genera (*Fusconaia* and *Pleurobema*) in Pleurobemini; therefore, we included 62 species from 10 pleurobemine genera/subgenera (*Elliptio*, *Elliptoideus*, *Eurynia*, *Fusconaia*, *Hemistena*, *Parvaspina*, *Plethobasus*, *Pleurobema*, *Pleurobema* (*Sintoxia*), and *Pleuronaia*) and 47 species representing three subfamilies in Unionidae from North America (Ambleminae, Anodontinae, and Gonideinae), and used *Margaritifera monodonta* (Margaritiferidae) as the outgroup; these sequences are listed in Tables S1 and S2. Throughout the study, we used nomenclature synthesised from previous studies (Graf and Cummings 2007; Williams *et al*. 2008; Campbell and Lydeard 2012*a*; Pfeiffer *et al*. 2016; Lopes-Lima *et al*. 2017; Perkins *et al*. 2017). Prior to the analyses, we used the package 'haplotypes' in R 3.3.1 (R Core Team 2015) to identify unique haplotypes and MetaPIGA 3.1 (Helaers and Milinkovitch 2010) to evaluate substitution saturation in the dataset. We used only unique haplotypes for the subsequent analyses.

Initially, we examined network relationships for *cox1* in SplitsTree4 4.14.4 (Huson and Bryant 2006), and used the uncorrected *P*-distance and the NeighbourNet algorithms to visualise the network. Phylogenetic trees were reconstructed using Bayesian inference for *cox1*. The Bayesian inference tree was inferred using MrBayes 3.2.6 (Ronquist *et al*. 2012). We used Kakusan4 (Tanabe 2011) to estimate best-fit models of nucleotide substitution for each codon partition of *cox1*. Based on the Bayesian information criterion, the best substitution model for the *cox1* dataset was GTR+Γ for the first and third codons and HKY+Γ for the second codon. Two simultaneous Markov Chain Monte Carlo runs (MCMC; each chain containing three heated chains and one cold chain) were executed for 8 000 000 generations, with trees sampled every 1000 generations, for a total of 8001 trees for each run in the initial samples. We assessed the convergence of MCMC by plotting the log-likelihood scores for each sampling point using Tracer 1.5 (Rambaut and Drummond 2009). When the likelihood values reached a plateau with sufficient effective sample sizes (ESS >200), we considered the Markov chains stationary. Therefore, we discarded the first 25% of trees as burn-in for each run, and the remaining trees were retained and evaluated using the 50% majority rule for a consensus tree.

Topology testing

To test support strength for the intrageneric relationships of the three genera/subgenera (*Fusconaia*, *Pleurobema*, *P.* (*Sintoxia*)), we constrained various taxon sets as monophyletic in the MrBayes analyses. The analyses were performed including only members of Pleurobemini (292 haplotypes from 62 species and 10 genera/subgenera). We ran MrBayes with a stepping-stone analysis to obtain a marginal likelihood estimate (MLE) for each constraint model. We performed 10 different constraints; each consisted of a positive constraint (i.e. a set of taxa was forced to be a monophyletic clade) and a negative constraint (i.e. a set of taxa was forced to be *not* a monophyletic clade) (Table 1). We developed the constraint models based on taxonomic revisions proposed by Campbell and Lydeard (2012*a*) and results from the present study (Figs 1, 2) (see Results). In particular, we focussed on placement of the subgenus *Sintoxia* relative to the phylogenetic position of *Fusconaia* and *Pleurobema* (Table 1). All stepping-stone analyses were run for 50 path steps with 100 000 generations each for a total of 5 000 000 generations. In addition, we ran an unconstrained model (i.e. no taxa were constrained to monophyly) to obtain a baseline MLE. On the basis of the MLE values, we calculated Bayes factors by taking twice the difference of the MLE from the highest MLE model for each genus (2 L*n*BF: Kass and Raftery 1995). We used the following criteria to determine the best model: 2 L*n*BF <2, low support and not worth mentioning; 2 L*n*BF 2–6, positive support; 2 L*n*BF 6–10, strong support; 2 L*n*BF >10, very strong support (Nylander *et al*. 2004).

Species delimitation

We used genetic distance and Yule-coalescent methods to delimit putative species for *Fusconaia* (116 haplotypes from 13 species) and *Pleurobema* (131 haplotypes from 21 species) only. We used Automated Barcode Gap Discovery (ABGD) (Puillandre *et al*. 2012) to identify DNA barcode gaps and thereby delimit species boundaries. Instead of using arbitrary divergence thresholds, ABGD statistically detects the barcode gap as the first significant gap beyond the range of prior intraspecific divergences and uses it to partition the data (Puillandre *et al*. 2009). This process is applied recursively to previously obtained groups to get finer partitions until there is no further partitioning. We set prior parameters for the minimum intraspecific genetic distance (p_{min}) as 0.01 and maximum intraspecific genetic distance (p_{max}) as 0.1 and used the Kimura 2-parameter (K2P) distance model (Kimura 1980).

We employed two generalised mixed Yule-coalescent (GMYC) models: single-threshold GMYC model (ST-GMYC) (Pons *et al*. 2006) and Bayesian GMYC model (bGMYC) (Reid and Carstens 2012). Briefly, the GMYC model identifies boundaries between branching events that are either divergent between species (Yule process) or diversification within

Table 1. Statistical comparison of 10 constraint models for *Fusconaia*, *Pleurobema*, and *Pleurobema* (*Sintoxia*)

Each constraint model consisted of a positive constraint (i.e. a set of taxa was forced to be a monophyletic clade) and a negative constraint (i.e. a set of taxa was forced to be *not* a monophyletic clade). Marginal likelihood estimate (MLE) for each constraint model was estimated from stepping-stone analysis in MrBayes and Bayes factors for the optimal model (2 L*n*BF; two times the difference of the MLE from the highest MLE model) was calculated for each genus/subgenus. The MLE of the unconstrained model (i.e. none of taxa was constrained to a monophyly) was –8942.56. The optimal model for each genus/subgenus is shown in bold

Model	Constraints	Positive		Negative	
		MLE	2 L*n*BF	MLE	2 L*n*BF
Fusconaia	*Fusconaia* spp.	–8765.31	17.5	–8930.61	348.14
***Fusconaia* s.l.**	*Fusconaia* spp. & *Pleurobema* (*Sintoxia*) *cordatum*	**–8756.54**	–	–8918.68	324.28
Pleurobema	*Pleurobema* spp. including *Pleurobema* (*Sintoxia*) spp.	**–8747.32**	–	–8930.26	365.88
Pleurobema s.s. 1	*Pleurobema* spp. except *Pleurobema* (*Sintoxia*) spp.	–8819.69	144.7	–8945.02	395.40
Pleurobema s.s. 2	*Pleurobema* s.s. 1 & *P.* (*Sintoxia*) *cordatum*	–8800.82	107.0	–8943.68	392.72
Pleurobema s.s. 3	*Pleurobema* s.s. 1 & *P.* (*Sintoxia*) *plenum*	–8831.55	168.5	–8923.01	351.38
***Pleurobema* (*Sintoxia*)**	*Pleurobema* (*Sintoxia*) spp.	**–8788.18**	–	–8933.91	291.46
Pleurobema (*Sintoxia*) s.s. 1	*Sintoxia* spp. except *P.* (*Sintoxia*) *cordatum* & *P.* (*Sintoxia*) *plenum*	–8813.95	51.5	–8929.95	283.54
Pleurobema (*Sintoxia*) s.s. 2	*Sintoxia* spp. except *P.* (*Sintoxia*) *cordatum*	–8810.49	44.6	–8946.62	316.88
Pleurobema (*Sintoxia*) s.s. 3	*Sintoxia* spp. except *P.* (*Sintoxia*) *plenum*	–8812.15	47.9	–8911.58	246.80

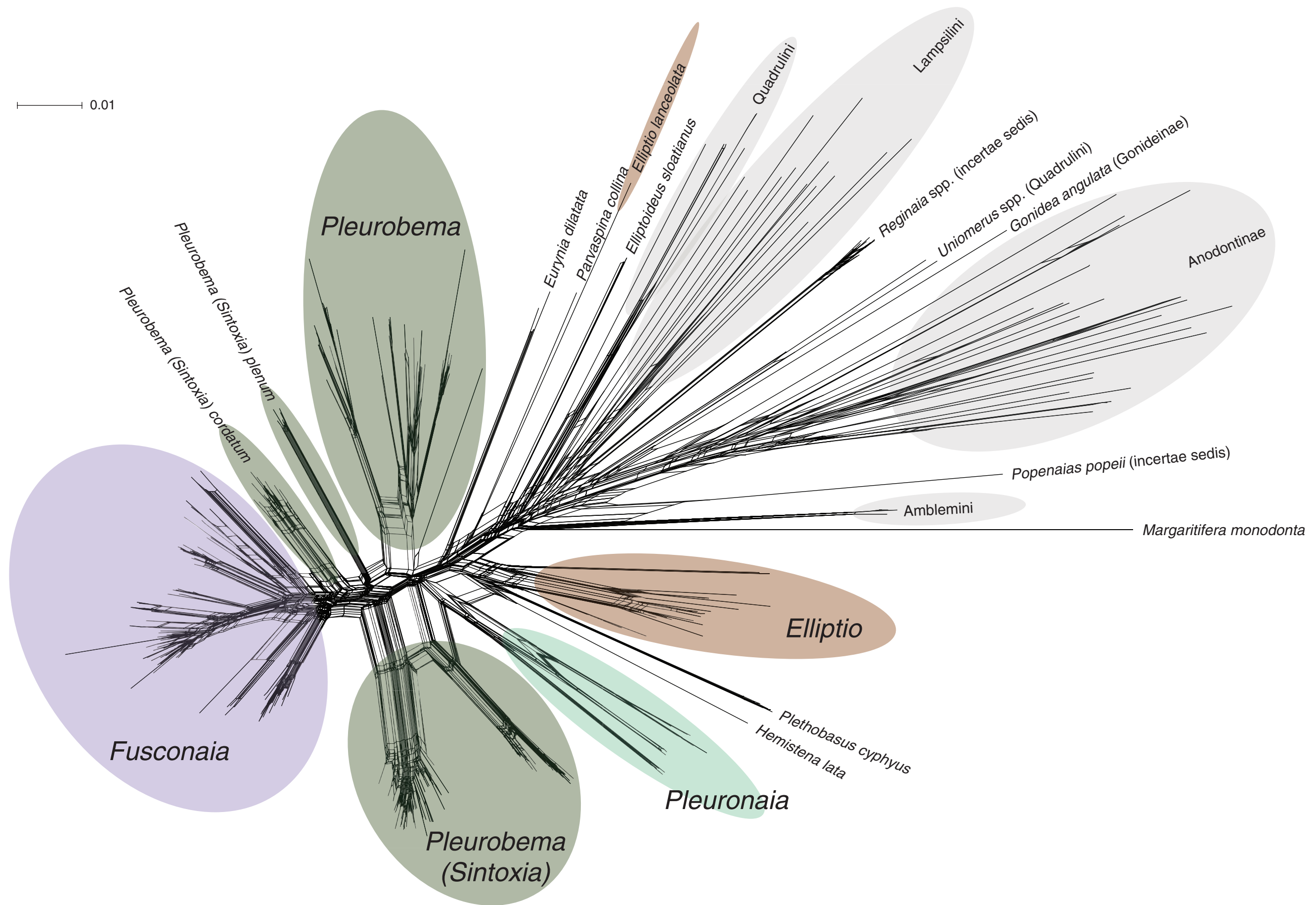

Fig. 1. Phylogenetic network developed using SPLITSTREE4 based on uncorrected *P*-distance and the NeighbourNet algorithm. Shaded ellipses emphasise clusters of various subfamilies, tribes, genera and subgenera.

species (coalescence) under the assumption that speciation processes occur at a lower rate than population processes (Fujita *et al*. 2012). Because GMYC models require ultrametric trees to estimate species boundaries, we first used BEAST 2.4.4 (Bouckaert *et al*. 2014) to generate ultrametric trees. We used HKY+Γ as a substitution model, a log-normal relaxed clock model, and a Yule species model, and ran the MCMC chain for 100 000 000 generations, storing the state every 1 000 000 generations and logging trees every 100 000 generations. The first 25% of the trees were discarded as burn-in based on MCMC convergence determined in TRACER, as previously described. With 750 trees after burn-in, we created the consensus tree using the maximum clade credibility method and setting the posterior probability limit to 0. We used the 'splits' package (Ezard *et al*. 2013) in R to conduct ST-GMYC analysis on the consensus tree using the single-threshold method and default intervals. For the bGMYC analysis, we randomly selected 100 ultrametric trees from the 750 trees after burn-in from BEAST. With the 'bGMYC' package (Reid and Carstens 2012) in R, we ran 50 000 MCMC generations, sampled the state every 100 generations, and discarded 40 000 trees as burn-in. We set the threshold parameter priors, t1 and t2, to 2 and 60, respectively, and the starting parameter value was set at 30. We used a single point estimate approach to delimit species boundaries; thus, we used bgmyc.point function to set the delimitation threshold relative to the posterior mean of the analysis at 0.5. The MRBAYES and BEAST analyses were performed on the CIPRES Science Gateway 3.3 (Miller *et al*. 2010).

Results

We examined 575 *cox1* sequences from 110 currently recognised species (13 *Fusconaia* and 21 *Pleurobema* species) and recovered 351 unique haplotypes used in the analyses (Tables S1 and S2); 352 new sequences collected for this study were submitted to GenBank (accession numbers: MF961804–MF962155). We did not find any indication of substitution saturation in the *cox1* dataset.

The phylogenetic network developed using SPLITSTREE4 revealed most genera/subgenera in Pleurobemini as monophyletic clusters, although *Elliptio* and *Pleurobema* were paraphyletic (Fig. 1). *Elliptio lanceolata* formed a distinct cluster sister to *Parvaspina collina*. The subgenus *Sintoxia* split into three clusters, while the rest of the *Pleurobema* species formed a single cluster. Within *Sintoxia*, two species – *P*. (*Sintoxia*) *cordatum* and *P*. (*Sintoxia*) *plenum* – each formed

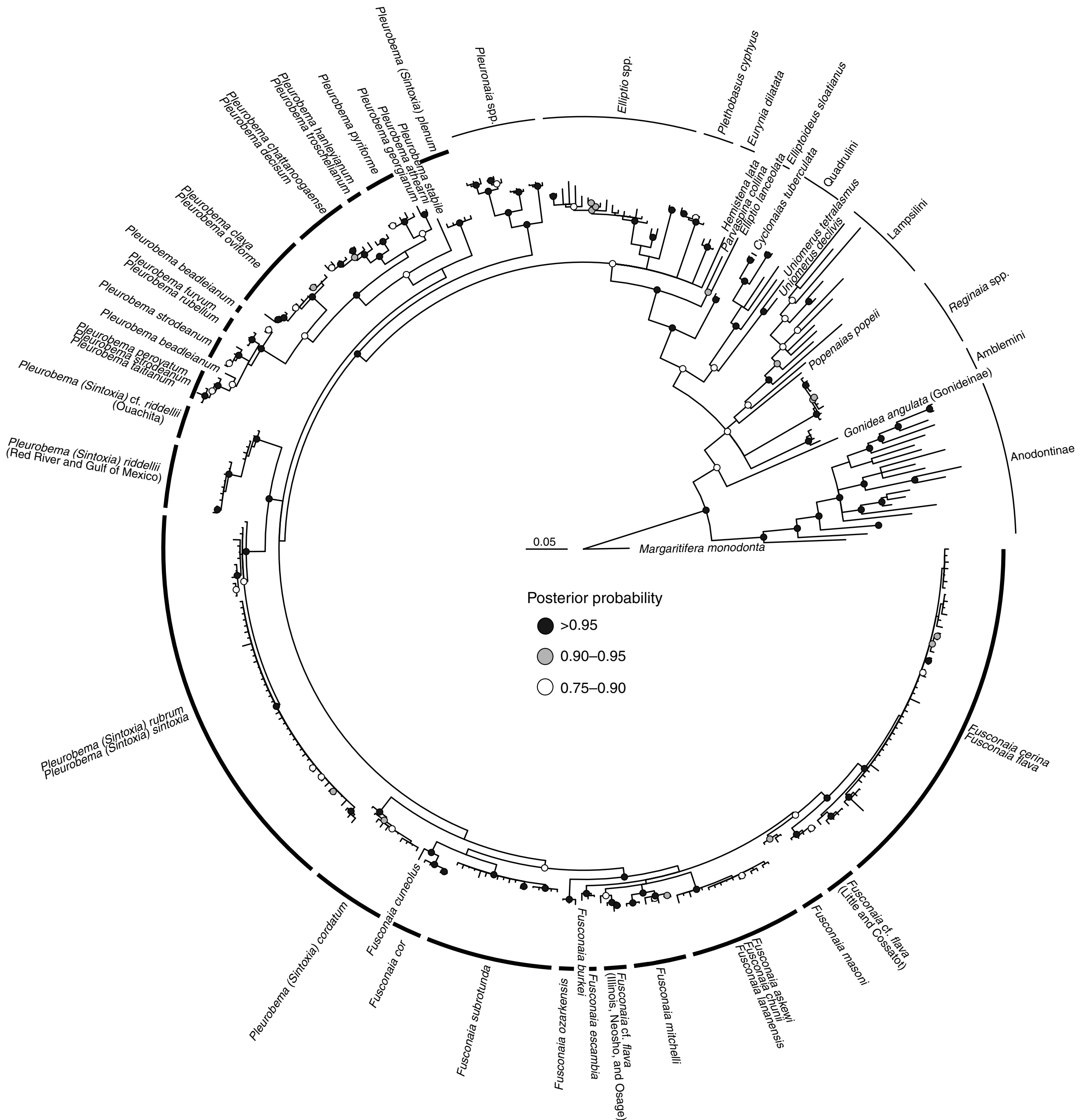

Fig. 2. Bayesian inference of phylogeny for Unionidae. Posterior probability values from Bayesian analysis are shown in shaded circles along the nodes (white = 0.75–0.90, grey = 0.90–0.95, black >0.95). The tree was rooted with *Margaritifera monodonta* (Margaritiferidae). Major clades are annotated. Bold bars along clades correspond to the focal genera.

separate clusters, while three species – *P.* (*Sintoxia*) *riddellii*, *P.* (*Sintoxia*) *rubrum*, and *P.* (*Sintoxia*) *sintoxia* – formed a single cluster.

The Bayesian inference phylogeny showed a monophyletic clade for the tribe Pleurobemini (*Elliptio*, *Elliptoideus*, *Eurynia*, *Fusconaia*, *Hemistena*, *Parvaspina*, *Plethobasus*, *Pleurobema*, and *Pleuronaia*) with high posterior probability support, with the Quadrulini the next most closely related (Fig. 2). Within the Pleurobemini, phylogenetic relationships among genera were generally congruent with the phylogenetic network. *Elliptoideus sloatianus* comprised the basal clade of Pleurobemini, followed by a clade comprising *E. lanceolata* and *P. collina*. Each genus

generally formed a monophyletic clade, except for *E. lanceolata* and *Pleurobema* species. The genus *Pleurobema* formed paraphyletic clades, where *P.* (*Sintoxia*) *riddellii*, *P.* (*Sintoxia*) *rubrum*, and *P.* (*Sintoxia*) *sintoxia* formed a single distinct clade; *P.* (*Sintoxia*) *cordatum* formed a sister clade with *Fusconaia* species; and the remaining *Pleurobema* species formed a monophyletic clade with *P.* (*Sintoxia*) *plenum*. However, these paraphyletic clades were polytomies with low posterior probability support values for individual clades (Fig. 2).

Within *Pleurobema*, there were several monophyletic clades comprising multiple currently recognised species. We found well supported clades shared between *P. hanleyianum* and *P. troschelianum*, *P. chattanoogaense* and *P. decisum*, *P. clava* and *P. oviforme*, *P. furvum* and *P. rubellum*, and *P.* (*Sintoxia*) *rubrum* and *P.* (*Sintoxia*) *sintoxia*; and among *P. perovatum*, *P. strodeanum*, and *P. taitianum*; however, none of these species shared the same *cox1* haplotypes with other species, except between *P.* (*Sintoxia*) *rubrum* and *P.* (*Sintoxia*) *sintoxia*.

Pleurobema beadleianum and *P. strodeanum* each split into two paraphyletic clades. *Pleurobema* (*Sintoxia*) *riddellii* formed two distinct, well supported monophyletic clades: one clade comprised individuals from the Ouachita River drainage in Arkansas, and the other clade from the Red River and the Gulf of Mexico drainages in Arkansas, Louisiana and Texas.

The genus *Fusconaia* formed a monophyletic clade. Within *Fusconaia*, species generally formed monophyletic clusters, although a few species shared clades. These included clades comprising *F. cerina* and *F. flava* and *F. askewi*, *F. chunii*, and *F. lananensis. Fusconaia cerina* and *F. flava* shared the same haplotypes and individuals identified as *F.* cf. *flava* split into two distinct clades: one comprising individuals from the Little and Cossatot rivers in Arkansas, and the other from the Illinois, Neosho, and Osage rivers in Arkansas, Kansas, and Missouri. Several widespread species (i.e. *F. flava*, *F. subrotunda*, *P.* (*Sintoxia*) *cordatum*, *P.* (*Sintoxia*) *rubrum*, and *P.* (*Sintoxia*) *sintoxia*) did not show geographic structure in *cox1*. Furthermore, *F. askewi* and *F. lananensis* shared the same haplotypes, but *F. chunii* possessed a unique haplotype.

Tests for the intrageneric relationships of the three genera/subgenera (*Fusconaia*, *Pleurobema*, and *P.* (*Sintoxia*)) showed that all positive constraint models received higher MLE values than the unconstrained model or the negative constraint models (Table 1). On the basis of 2 L*n*BF, the best model for *Fusconaia* was a model constraining the nominal *Fusconaia* species and *P.* (*Sintoxia*) *cordatum* to be monophyletic (*Fusconaia* s.l. model: Table 1). For subgenus *Sintoxia*, a model constraining the monophyly of the five nominal *P.* (*Sintoxia*) species was the best model (*Pleurobema* (*Sintoxia*) model: Table 1). The overall best model determined by the highest MLE values was the *Pleurobema* model, where *Pleurobema*, including the subgenus *P.* (*Sintoxia*), was monophyletic (Table 1).

Species delimitation analysis based on genetic distance and Yule-coalescent methods within the three genera/subgenera revealed several undescribed putative taxa and multiple conspecifics among nominal taxa (Fig. 3). The three methods yielded similar patterns of species delimitations, which generally agreed with the 34 currently recognised species. We identified 25 putative species from ABGD, 28 putative species from bGMYC, and 30 putative species from ST-GMYC within the three genera/subgenera. These findings suggest the presence of cryptic diversity and need for taxonomic revision regarding species-level diversity in Pleurobemini.

In *Fusconaia*, all analyses showed the following taxa may be conspecific: *F. cerina* and *F. flava*; *F. askewi*, *F. chunii*, and *F. lananensis*; and *F. burkei* and *F. escambia* (Fig. 3A). Both GMYC models identified undescribed putative *Fusconaia* cf. *flava* from the Little and Cossatot rivers in Arkansas and *F.* cf. *flava* from the Illinois, Neosho, and Osage rivers in Arkansas, Kansas, and Missouri. Only the ST-GMYC model recognised two putative species within *F. mitchelli*.

In *P.* (*Sintoxia*), both ABGD and ST-GMYC approaches identified *P.* (*Sintoxia*) *rubrum* and *P.* (*Sintoxia*) *sintoxia* as conspecific, while bGMYC recognised the two as separate species (Fig. 3B). Furthermore, all models identified two putative species within *P.* (*Sintoxia*) *riddellii*: one from the Red River and the Gulf of Mexico drainages and the other from the Ouachita River drainage.

Within *Pleurobema*, we found these groupings conspecific: *P. perovatum*, *P. strodeanum*, and *P. taitianum*; *P. furvum* and *P. rubellum*; *P. clava* and *P. oviforme*; *P. chattanoogaense* and *P. decisum*; *P. hanleyianum* and *P. troschelianum*; and *P. athearni* and *P. georgianum* (Fig. 3C). All models identified two putative species within *P. beadleianum*: one collected from Twelvemile Creek (a tributary of the Tickfaw River) and the other collected from the Pearl River. Similarly, individuals identified as *P. strodeanum* showed two putative species: one collected from the Escambia River (conspecific with *P. perovatum* and *P. taitianum*), and the other collected from the Choctawhatchee River. Furthermore, ST-GMYC recognised a distinct putative species in *P. chattanoogaense*.

Discussion

In this study, we re-examined the taxonomy and phylogenetic relationships among members of the tribe Pleurobemini and found (1) robust support for most currently recognised species and (2) indications that the taxonomic status of a few nominal species should be refined. In general, the results of our phylogenetic analyses were congruent with those of previous studies (e.g. Campbell and Lydeard 2012*a*); however, additional taxon sampling resolved certain features of tree topology and supported the discovery of cryptic diversity. Our phylogenetic analyses and topology testing with constraint models supported eight nominal genera as monophyletic clades in the Pleurobemini (*Elliptoideus*, *Eurynia*, *Fusconaia*, *Hemistena*, *Parvaspina*, *Plethobasus*, *Pleurobema*, and *Pleuronaia*), whereas *Elliptio* was paraphyletic and warrants additional investigation (Figs 1, 2). *Pleurobema* showed polyphyly in the phylogenetic network and Bayesian inference tree in which *P.* (*Sintoxia*) species were split into three clades. Campbell and Lydeard (2012*a*) suggested recognising *Sintoxia* as a subgenus of *Pleurobema* due to uncertainties regarding phylogenetic placement of *P.* (*Sintoxia*) *cordatum* and *P.* (*Sintoxia*) *plenum* within *Pleurobema*. However, constraint models supported the monophyly of *P.* (*Sintoxia*) + *Pleurobema* species (Table 1); therefore, we recognise the five nominal species (*P. cordatum*,

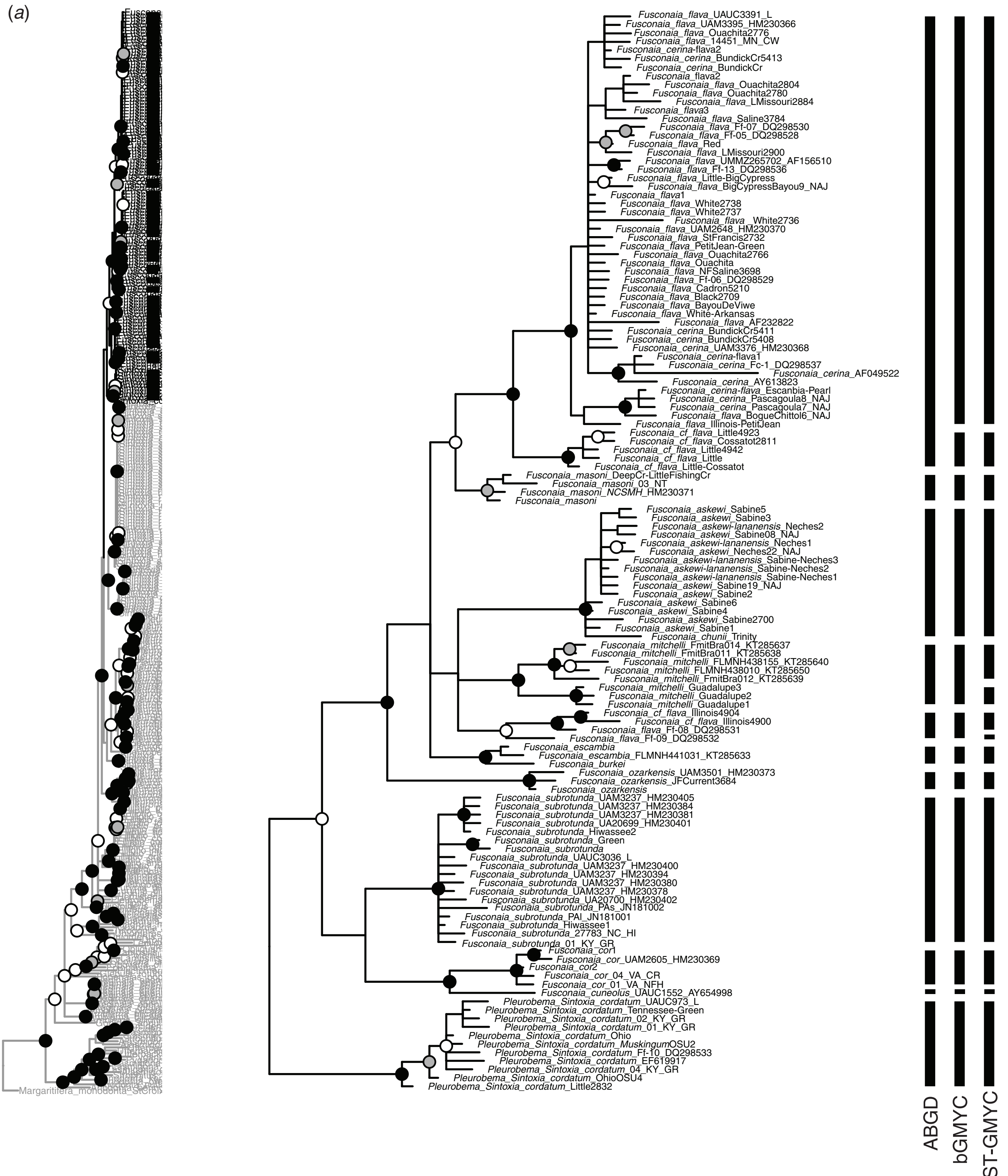

Fig. 3. Bayesian inference of phylogeny with close-up view of (*A*) *Fusconaia*, (*B*) *Pleurobema* (*Sintoxia*), and (*C*) *Pleurobema* and results of three delimitation models: ABGD, bGMYC and ST-GMYC. Each vertical bar corresponds to a clade in the Bayesian inference phylogeny on the left and represents a putative species from the delimitation methods: ABGD, bGMYC and ST-GMYC, respectively. Posterior probability values are shown in shaded circles along the nodes (white = 0.75–0.90, grey = 0.90–0.95, black >0.95).

Fig. 3. (*continued*)

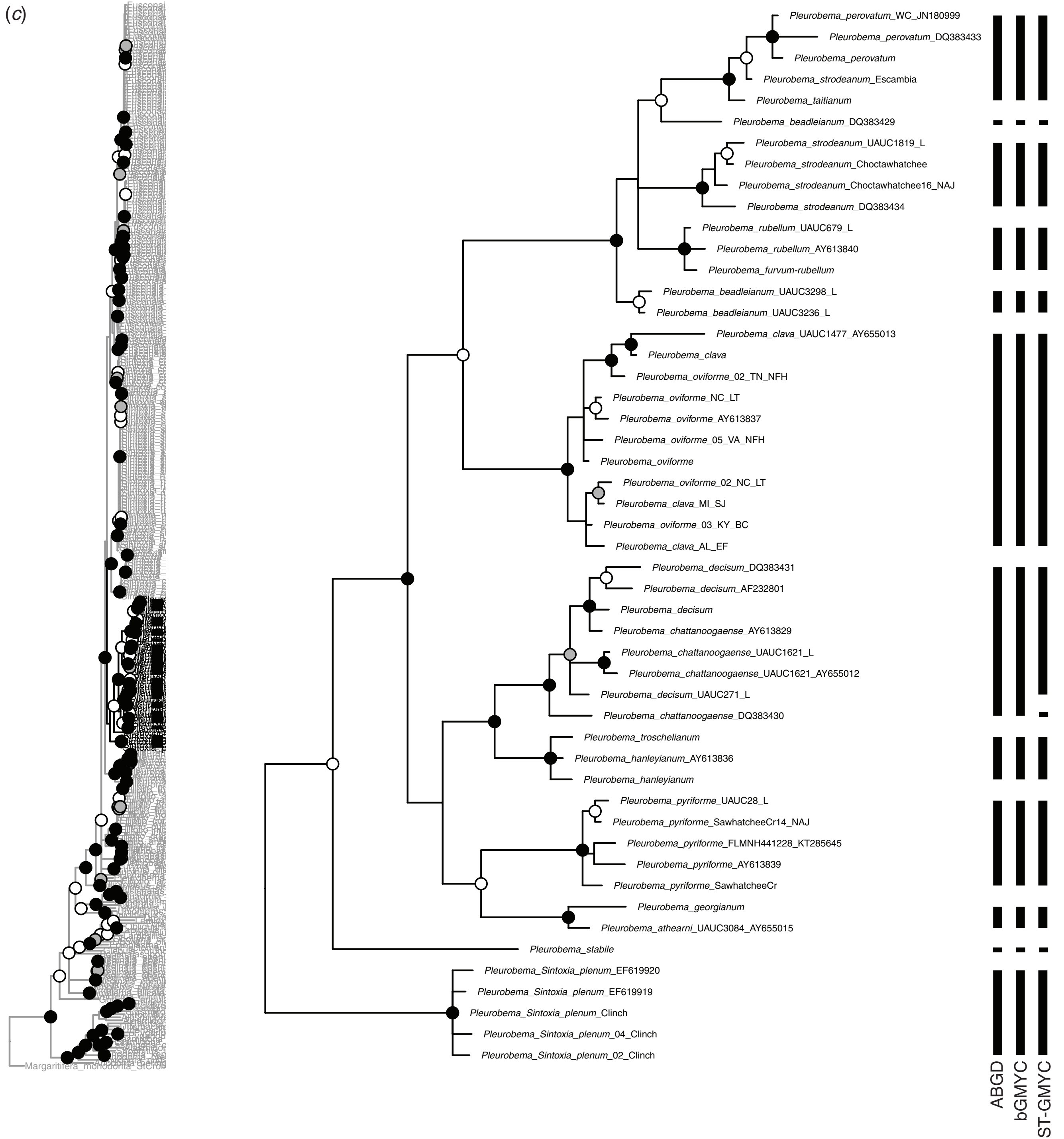

Fig. 3. (*continued*)

P. plenum, *P. riddellii*, *P. rubrum*, and *P. sintoxia*) without assignment of subgenus *Sintoxia*.

Within *Fusconaia* and *Pleurobema*, phylogenetic relationships among species generally agree with current taxonomy. However, we found several species to be conspecific, where molecular data did not clearly distinguish among currently recognised species.

Fusconaia cerina (Conrad, 1838) and *Fusconaia flava* (Rafinesque, 1820)

Fusconaia cerina and *F. flava* were genetically indistinguishable, and *cox1* haplotypes were shared between the species. The currently recognised distribution of *F. cerina* is restricted to Gulf Coast drainages east of the Mississippi River (Vidrine 1993; Williams *et al*. 2008), while *F. flava* occurs in the Mississippi River system, Great Lakes drainages, and Gulf Coast drainages west of the Mississippi River (Howells *et al*. 1996; Williams *et al*. 2008; Watters *et al*. 2009). The type locality for *F. cerina* is 'waters of Louisiana near New Orleans' and *F. flava* was described from 'small creeks flowing into the Green River, KY'. Our analyses included *F. cerina* from Twelvemile Creek (a tributary of the Tickfaw River, LA; Lake Pontchartrain drainage) and *F. flava* from the Green River, KY (a tributary of the Ohio River) (Tables S1 and S2). The two nominal taxa are likely conspecific because recognition of these taxa as separate species (and subsequently their identifications) is based primarily on geographic distribution, and there are only minor morphological differences.

Pleurobema rubrum (Rafinesque, 1820) and *Pleurobema sintoxia* (Rafinesque, 1820)

Pleurobema rubrum and *P. sintoxia* were not genetically differentiated. In contrast to *F. cerina* and *F. flava*, *P. rubrum* and *P. sintoxia* co-occur in many localities and show distinctly different shell morphologies. However, morphological lines can be blurred and identifications of the two species in sympatry are problematic in some drainages (e.g. Green River, KY). *Pleurobema rubrum* often has triangular shells with long, nearly vertical or posteriorly slanted anterior margin, while *P. sintoxia* has triangular to oval shells with a short, straight or broadly rounded anterior margin (Watters *et al*. 2009). Because mussel species can exhibit clinal morphological variation within river drainages (Ortmann 1920; Inoue *et al*. 2013), such differences may be the result of phenotypic plasticity within a single species. These inferences are congruent with previous findings of phenotypic plasticity in freshwater mussels (Burdick and White 2007; Campbell and Lydeard 2012*b*; Jones *et al*. 2015).

The type locality for *P. rubrum* is 'Dans le Kentuky' [River], and the type locality for *P. sintoxia* 'Dans l'Ohio' [River] (Rafinesque 1820). Our samples included *P. rubrum* from the Green River, KY, and *P. sintoxia* from tributaries of the Ohio River including the Green River, KY, and French Creek and Little Mahoning River, PA (Table S1). For *P. rubrum* and *P. sintoxia*, the potential for clinal variation and high morphological variation in sympatry, combined with lack of genetic differentiation between the two species at mtDNA, suggests that they are conspecific, although analyses of additional lines of evidence (e.g. life history, ecology, and additional nuclear genetic markers – see below) are warranted.

Fusconaia askewi (Marsh, 1896), *Fusconaia chunii* (Lea, 1861) and *Fusconaia lananensis* (Frierson, 1901)

Fusconaia askewi, *F. chunii*, and *F. lananensis* were not resolved as mutually monophyletic. *Fusconaia askewi* and *F. lananensis* were genetically indistinguishable (i.e. they shared haplotypes), while *F. chunii* possessed a unique *cox1* haplotype. *Fusconaia askewi* occurs in the Sabine and Neches river drainages (the type locality is Village Creek, a tributary of the Neches River), while *F. lananensis* is endemic to tributaries of the Angelina River (a tributary of the Neches River), and *F. chunii* is endemic to the Trinity River drainage. Recognition of these species has been based primarily on shell morphology and geographic distribution (Howells *et al*. 2012). Interestingly, previous researchers (Vidrine 1993; Howells *et al*. 1996) synonymised *F. chunii* with *F. flava* based on shell morphology. Our results suggest that *F. askewi* and *F. lananensis* are different morphotypes of a single species, and the subtle but significant genetic structure of *F. chunii* (genetic divergence = 1.3% at *cox1*) likely indicates recent evolutionary divergence. As such, *F. chunii* may not be conspecific with *F. askewi*/*F. lananensis* or *F. flava*.

Pleurobema conspecifics

We observed that several species within *Pleurobema* were indistinguishable using molecular data and inferred that the following are likely conspecific: *P. hanleyianum* and *P. troschelianum*; *P. chattanoogaense* and *P. decisum*; *P. furvum* and *P. rubellum*; *P. clava* and *P. oviforme*; and *P. perovatum*, *P. strodeanum*, and *P. taitianum*. Previously, Williams *et al*. (2008) synonomised *P. chattanoogaense* with *P. decisum* and *P. furvum* with *P. rubellum*, which the results of this study support. Williams *et al*. (2008) also synonomised *P. troschelianum* with *P. georgianum*, but our analyses show *P. troschelianum* to be more closely related to *P. hanleyianum*. Furthermore, Ortmann (1925) stated that morphologies of *P. clava* and *P. oviforme* may be clinal variation of a single species and conspecific. However, the current study had small sample sizes for some of *Pleurobema* species due to their rarity or protected conservation statuses; thus, taxa that were not supported as separate species might be the result of limited sample sizes. We recommend careful re-examination of these taxa before synonomy by increasing the sample sizes and adding more molecular markers.

Hidden diversity in *Fusconaia* and *Pleurobema*

DNA barcoding and other molecular phylogenetic techniques provide a more quantitative and less subjective way to improve species identification, assess species boundaries, and estimate the number of putative species (Pons *et al*. 2006). The results of our study underscore this point as we identified three unrecognised putative species. A previous phylogenetic study included two *F*. '*flava*' sequences from the Neosho and Osage rivers (Ff8 and Ff9 from Burdick and White 2007) and Campbell and Lydeard (2012*b*) suggested that this distinct lineage may

be attributable to the nomen *Fusconaia hebetata* (Conrad, 1854). Our additional samples of *F.* cf. *flava* from the Illinois River, AR, clustered with the *Ff8*/*Ff9* clade and suggested *F.* '*flava*' from the Ozark region (Springfield Plateau in particular) to be a distinct taxon. Unfortunately, the type locality for *F. hebetata* is 'From Missouri' with no additional information, and the type specimen has been lost. Our *F.* cf. *flava* specimens are conchologically similar to *Fusconaia flava sampsoniana* (Frierson, 1927) described from the Elk River, MO (a tributary of the Neosho River). Because of phenotypic plasticity in shell morphology, further examinations including molecular identification of available types or topotypic material from dried tissue or shell fragments in addition to morphological analyses are required to elucidate the species identity of *F.* cf. *flava* from the Ozarks.

Similarly, two of three delimitation analyses showed a distinct lineage of *F.* cf. *flava* from the Cossatot and Little rivers. This finding possibly represents an unrecognised or previously undescribed species. The Ozarks and Ouachita Mountains Interior Highlands is physiographically distinct from nearby ecoregions and known to harbour many endemic aquatic species (e.g. crayfishes: Crandall and Templeton 1999; fishes: Berendzen *et al*. 2003; Morrison *et al*. 2006; mussels: Inoue *et al*. 2014). Physiographic isolation between the Interior Highlands and the Mississippi Embayment by past geological and climatic changes likely separated *F.* cf. *flava* in the Cossatot and Little Rivers from the more widespread *F. flava*.

Similar divergence within the *P. riddellii* complex likely occurred. All delimitation analyses showed that *Pleurobema* cf. *riddellii* from the Ouachita River drainage is phylogenetically distinct from populations in the Red River and west Gulf Coast drainages, suggesting a potential unrecognised species within *P. riddellii*. Although we were unable to obtain topotypic *P. riddellii* from the Trinity River in Dallas, TX, individuals from the Sabine, Neches, and Little (a tributary of the Red River) Rivers placed within a single clade, indicating that *P. riddellii* only occurs west of the Red River drainage. Additional samples from the Red, Ouachita, and Mississippi River drainages in Arkansas and Louisiana are needed to assess the potential new species.

Finally, two *Pleurobema* species initially identified as *P. beadleianum* and *P. strodeanum* each split into two clades, which were allopatric in distribution (Fig. 3C). One *P. beadleianum* (DQ383429) was collected from Twelvemile Creek (Lake Pontchartrain drainage in Louisiana), whereas the remainder of the specimens were collected from the Pearl River, LA (Eastern Gulf Coast drainage). Similarly, one *P. strodeanum* in the *P. perovatum*/*P. strodeanum*/*P. taitianum* species clade was collected from the Escambia River, FL, whereas the remaining specimens were from the Choctawhatchee River, FL. Such geographic isolation may have led to significant genetic differentiation within the species. Further investigation, adding more samples and greater spatial coverage, is required to elucidate the taxonomic identity of these species.

Applications of molecular species delimitation

Although there has been much debate regarding the validity of molecular species delimitation using a single locus (Lohse 2009; Taylor and Harris 2012), genetic distance and Yule-coalescent methods with single locus data have been used widely to delimit putative species (Fujisawa and Barraclough 2013). In general, the robustness of molecular delimitation methods depends on sampling of individuals per species, geographic/spatial coverage, and number and resolving power of genetic markers. For example, simulated and empirical studies have demonstrated that increasing sample size and spatial coverage per species improves the model's ability to detect a threshold in branching rates between Yule and coalescent processes, leading to more accurate estimates of species-level diversity (Bergsten *et al*. 2012; Reid and Carstens 2012; Fujisawa and Barraclough 2013). Previous systematic studies of *Fusconaia* and *Pleurobema* have relied on small sample sizes to draw inferences for these genera (means of 2.0–6.7 individuals per species) (Burdick and White 2007; Burlakova *et al*. 2012; Campbell and Lydeard 2012*b*). In this study, we examined almost twice as many individuals per species (average 12.6 individuals per species) and only 19% were represented by singletons. This larger sample size in combination with broader geographic coverage means that our results are robust, even though we used only a single locus (see below).

Despite the increase in sample size, the phylogenies and species delimitations presented in this study were based solely on the mtDNA *cox1* gene tree. It is well recognised that a multilocus coalescent approach provides a more robust estimation of species boundaries because it accounts for gene tree discordance, incomplete lineage sorting, and other confounding factors that create problems using a single-locus approach (Fujita *et al*. 2012; Fontaneto *et al*. 2015). Previous studies using nuclear DNA sequences (e.g. *ITS1*) have successfully delineated species boundaries within mussel taxa (Jones *et al*. 2006; Inoue *et al*. 2014; Pfeiffer *et al*. 2016; Perkins *et al*. 2017). However, such efforts for Pleurobemini have been largely unsuccessful, where *ITS1* did not contain phylogenetically meaningful resolution (Campbell *et al*. 2008; Campbell and Lydeard 2012*a*). Additional hypervariable genetic markers (e.g. microsatellites, restriction-associated DNA sequences – RADseq), as well as increased spatial coverage and sample size per species could be another option in addition to the multilocus approach, especially for questions related to fine-scale phylogenetic patterns. Ideally, researchers should use several approaches to develop multiple independent lines of evidence before taxonomic changes are formalised.

Species identification of freshwater mussels, particularly Pleurobemini species, is challenging due to morphological convergence and phenotypic plasticity of shell morphology. A previous study found that misidentification rates for Pleurobemini species were high relative to other mussel taxa (33% false positive and 34% false negative identification, respectively: Shea *et al*. 2011). The same study concluded that error rates were highest for tribes containing the most speciose genera (e.g. *Elliptio* spp.), suggesting that misidentifications are likely high among congeners and taxa that were overdescribed. Because species identification and discovery of new species for freshwater mussels often are based on external morphological characteristics, phenotypic plasticity and evolutionary convergence in shell morphologies has led to rampant species misidentifications and over- and underestimates of species richness.

Our study shows the presence of previously unrecognised species and potential synonomies within Pleurobemini. As of April 2017, 27 species within the Pleurobemini were listed under the US *Endangered Species Act*, and many have varying conservation statuses among states. Understanding current taxonomic status and systematics for these problematic taxa is important for assessing conservation status and developing viable and effective conservation strategies for threatened and endangered species.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Berendzen, P. B., Simons, A. M., and Wood, R. M. (2003). Phylogeography of the northern hogsucker, *Hypentelium nigricans* (Teleostei: Cypriniformes): genetic evidence for the existence of the ancient Teays River. *Journal of Biogeography* **30**, 1139–1152. doi:10.1046/j.1365-2699.2003.00888.x

Bergsten, J., Bilton, D. T., Fujisawa, T., Elliott, M., Monaghan, M. T., Balke, M., Hendrich, L., Geijer, J., Herrmann, J., Foster, G. N., Ribera, I., Nilsson, A. N., Barraclough, T. G., and Vogler, A. P. (2012). The effect of geographical scale of sampling on DNA barcoding. *Systematic Biology* **61**, 851–869. doi:10.1093/sysbio/sys037

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K., and Das, I. (2006). Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22**, 148–155. doi:10.1016/j.tree.2006.11.004

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M. A., Rambaut, A., and Drummond, A. J. (2014). BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Computational Biology* **10**, e1003537. doi:10.1371/journal.pcbi.1003537

Burdick, R. C., and White, M. M. (2007). Phylogeography of the Wabash pigtoe, *Fusconaia flava* (Rafinesque, 1820) (Bivalvia: Unionidae). *The Journal of Molluscan Studies* **73**, 367–375. doi:10.1093/mollus/eym033

Burlakova, L. E., Campbell, D. C., Karatayev, A. Y., and Barclay, D. (2012). Distribution, genetic analysis and conservation priorities for rare Texas freshwater molluscs in the genera *Fusconaia* and *Pleurobema* (Bivalvia: Unionidae). *Aquatic Biosystems* **8**, 12. doi:10.1186/2046-9063-8-12

Campbell, D. C., and Lydeard, C. (2012*a*). The genera of Pleurobemini (Bivalvia: Unionidae: Ambleminae). *American Malacological Bulletin* **30**, 19–38. doi:10.4003/006.030.0102

Campbell, D. C., and Lydeard, C. (2012*b*). Molecular systematics of *Fusconaia* (Bivalvia: Unionidae: Ambleminae). *American Malacological Bulletin* **30**, 1–17. doi:10.4003/006.030.0101

Campbell, D. C., Serb, J. M., Buhay, J. E., Roe, K. J., Minton, R. L., and Lydeard, C. (2005). Phylogeny of North American amblemines (Bivalvia: Unionoida): prodigious polyphyly proves pervasive across genera. *Invertebrate Biology* **124**, 131–164. doi:10.1111/j.1744-7410.2005.00015.x

Campbell, D. C., Johnson, P. D., Williams, J. D., Rindsberg, A. K., Serb, J. M., Small, K. K., and Lydeard, C. (2008). Identification of 'extinct' freshwater mussel species using DNA barcoding. *Molecular Ecology Resources* **8**, 711–724. doi:10.1111/j.1755-0998.2008.02108.x

Crandall, K. A., and Templeton, A. R. (1999). The zoogeography and centers of origin of the crayfish subgenus *Procericambarus* (Decapoda: Cambaridae). *Evolution* **53**, 123–134. doi:10.1111/j.1558-5646.1999.tb05338.x

Ezard, T., Fujisawa, T., and Barraclough, T. (2013). SPLITS: SPecies' LImits by Threshold Statistics. Version 1.0. R Package. Available at: http://r-forge.r-project.org/projects/splits/.

Fontaneto, D., Flot, J.-F., and Tang, C. Q. (2015). Guidelines for DNA taxonomy, with a focus on the meiofauna. *Marine Biodiversity* **45**, 433–451. doi:10.1007/s12526-015-0319-7

Fujisawa, T., and Barraclough, T. G. (2013). Delimiting species using single-locus data and the generalized mixed Yule coalescent approach: a revised method and evaluation on simulated data sets. *Systematic Biology* **62**, 707–724. doi:10.1093/sysbio/syt033

Fujita, M. K., Leaché, A. D., Burbrink, F. T., McGuire, J. A., and Moritz, C. (2012). Coalescent-based species delimitation in an integrative taxonomy. *Trends in Ecology & Evolution* **27**, 480–488. doi:10.1016/j.tree.2012.04.012

Gangloff, M. M., Williams, J. D., and Feminella, J. W. (2006). A new species of freshwater mussel (Bivalvia: Unionidae), *Pleurobema athearni*, from the Coosa River drainage of Alabama, USA. *Zootaxa* **1118**, 43–56.

Graf, D. L., and Cummings, K. S. (2007). Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). *The Journal of Molluscan Studies* **73**, 291–314. doi:10.1093/mollus/eym029

Haag, W. R. (2012). 'North American Freshwater Mussels: Natural History, Ecology, and Conservation.' (Cambridge University Press: Cambridge.)

Haag, W. R., and Williams, J. D. (2014). Biodiversity on the brink: an assessment of conservation strategies for North American freshwater mussels. *Hydrobiologia* **735**, 45–60. doi:10.1007/s10750-013-1524-7

Hannibal, H. (1912). A synopsis of the recent and tertiary freshwater Mollusca of the Californian Province, based upon an ontogenetic classification. *Proceedings of the Malacological Society of London* **10**, 112–211.

Helaers, R., and Milinkovitch, M. C. (2010). MetaPIGA v2.0: maximum likelihood large phylogeny estimation using the metapopulation genetic algorithm and other stochastic heuristics. *BMC Bioinformatics* **11**, 379. doi:10.1186/1471-2105-11-379

Howells, R. G., Neck, R. W., and Murray, H. D. (1996). 'Freshwater Mussels of Texas.' (Texas Park and Wildlife Department: Austin, TX.)

Howells, R. G., Randklev, C. R., and Ford, N. B. (2012). Taxonomic status of pigtoe unionids in Texas. *Ellipsaria* **14**, 11–15.

Huson, D. H., and Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* **23**, 254–267. doi:10.1093/molbev/msj030

Inoue, K., Hayes, D. M., Harris, J. L., and Christian, A. D. (2013). Phylogenetic and morphometric analyses reveal ecophenotypic plasticity in freshwater mussels *Obovaria jacksoniana* and *Villosa arkansasensis* (Bivalvia: Unionidae). *Ecology and Evolution* **3**, 2670–2683. doi:10.1002/ece3.649

Inoue, K., McQueen, A. L., Harris, J. L., and Berg, D. J. (2014). Molecular phylogenetics and morphological variation reveal recent speciation in freshwater mussels of the genera *Arcidens* and *Arkansia* (Bivalvia: Unionidae). *Biological Journal of the Linnean Society. Linnean Society of London* **112**, 535–545. doi:10.1111/bij.12282

Jones, J. W., Neves, R. J., Ahlstedt, S. A., and Hallerman, E. M. (2006). A holistic approach to taxonomic evaluation of two closely related endangered freshwater mussel species, the oyster mussel *Epioblasma capsaeformis* and tan riffleshell *Epioblasma florentina walkeri* (Bivalvia: Unionidae). *The Journal of Molluscan Studies* **72**, 267–283. doi:10.1093/mollus/eyl004

Jones, J. W., Johnson, N. A., Grobler, P. J., Schilling, D. E., Neves, R. J., and Hallerman, E. M. (2015). Endangered rough pigtoe pearlymussel: assessment of phylogenetic status and genetic differentiation of two disjunct populations. *Journal of Fish and Wildlife Management* **6**, 338–349. doi:10.3996/052013-JFWM-036

Kass, R. E., and Raftery, A. E. (1995). Bayes factors. *Journal of the American Statistical Association* **90**, 773–795. doi:10.1080/01621459.1995.10476572

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**, 772–780. doi:10.1093/molbev/mst010

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120. doi:10.1007/BF01731581

Labarca, C., and Paigen, K. (1980). A simple, rapid, and sensitive DNA assay procedure. *Analytical Biochemistry* **102**, 344–352. doi:10.1016/0003-2697(80)90165-7

Lohse, K. (2009). Can mtDNA barcodes be used to delimit species? A response to Pons *et al.* (2006). *Systematic Biology* **58**, 439–442. doi:10.1093/sysbio/syp039

Lopes-Lima, M., Froufe, E., Do, V. T., Ghamizi, M., Mock, K. E., Kebapci, U., Klishko, O., Kovitvadhi, S., Kovitvadhi, U., Paulo, O. S., Pfeiffer, J. M. III, Raley, M., Riccardi, N., Sereflisan, H., Sousa, R., Teixeira, A., Varandas, S., Wu, X., Zanatta, D. T., Zieritz, A., and Bogan, A. E. (2017). Phylogeny of the most species-rich freshwater bivalve family (Bivalvia: Unionida: Unionidae): defining modern subfamilies and tribes. *Molecular Phylogenetics and Evolution* **106**, 174–191. doi:10.1016/j.ympev.2016.08.021

Miller, M. A., Pfeiffer, W., and Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In 'Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA'. (Ed. Institute of Electrical and Electronics Engineers, IEEE.) pp. 1–8. (Curran Associates: New York.)

Morrison, C. L., Lemarié, D. P., Wood, R. M., and King, T. L. (2006). Phylogeographic analyses suggest multiple lineages of *Crystallaria asprella* (Percidae: Etheostominae). *Conservation Genetics* **7**, 129–147. doi:10.1007/s10592-005-5681-8

Nylander, J., Ronquist, F., Huelsenbeck, J., and Nieves-Aldrey, J. (2004). Bayesian phylogenetic analysis of combined data. *Systematic Biology* **53**, 47–67. doi:10.1080/10635150490264699

Ortmann, A. E. (1920). Correlation of shape and station in fresh-water mussels (Naiades). *Proceedings of the American Philosophical Society* **59**, 268–312.

Ortmann, A. E. (1925). The naiad-fauna of the Tennessee River system below Walden Gorge. *American Midland Naturalist* **9**, 321–372. doi:10.2307/2992763

Perkins, M. A., Johnson, N. A., and Gangloff, M. M. (2017). Molecular systematics of the critically-endangered North American spinymussels (Unionidae: *Elliptio* and *Pleurobema*) and description of *Parvaspina* gen. nov. *Conservation Genetics* **18**, 745–757. doi:10.1007/s10592-017-0924-z

Pfeiffer, J. M. III, Johnson, N. A., Randklev, C. R., Howells, R. G., and Williams, J. D. (2016). Generic reclassification and species boundaries in the rediscovered freshwater mussel '*Quadrula*' *mitchelli* (Simpson in Dall, 1896). *Conservation Genetics* **17**, 279–292. doi:10.1007/s10592-015-0780-7

Pons, J., Barraclough, T., Gomez-Zurita, J., Cardoso, A., Duran, D., Hazell, S., Kamoun, S., Sumlin, W., and Vogler, A. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* **55**, 595–609. doi:10.1080/10635150600852011

Puillandre, N., Strong, E. E., Bouchet, P., Boisselier, M. C., Couloux, A., and Samadi, S. (2009). Identifying gastropod spawn from DNA barcodes: possible but not yet practicable. *Molecular Ecology Resources* **9**, 1311–1321. doi:10.1111/j.1755-0998.2009.02576.x

Puillandre, N., Lambert, A., Brouillet, S., and Achaz, G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* **21**, 1864–1877. doi:10.1111/j.1365-294X.2011.05239.x

R Core Team (2015). R: a language and environment for statistical computing. Available at: http://www.R-project.org/.

Rafinesque, C. S. (1820). Monographie des coquilles bivalves fluvutules de la Riviére Ohio, contenant douze genres et soixante-huit espéces. *Annales generales des sciences Physiques, a Bruxelles* **5**, 287–322.

Rambaut, A., and Drummond, A. J. (2009). Tracer v1.5. Available at: http://tree.bio.ed.ac.uk/software/tracer/.

Reid, N. M., and Carstens, B. C. (2012). Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology* **12**, 196. doi:10.1186/1471-2148-12-196

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., and Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542. doi:10.1093/sysbio/sys029

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 8014–8018. doi:10.1073/pnas.81.24.8014

Serb, J. M., Buhay, J. E., and Lydeard, C. (2003). Molecular systematics of the North American freshwater bivalve genus *Quadrula* (Unionidae: Ambleminae) based on mitochondrial ND1 sequences. *Molecular Phylogenetics and Evolution* **28**, 1–11. doi:10.1016/S1055-7903(03)00026-5

Shea, C. P., Peterson, J. T., Wisniewski, J. M., and Johnson, N. A. (2011). Misidentification of freshwater mussel species (Bivalvia: Unionidae): contributing factors, management implications, and potential solutions. *Journal of the North American Benthological Society* **30**, 446–458. doi:10.1899/10-073.1

Tanabe, A. S. (2011). Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. *Molecular Ecology Resources* **11**, 914–921. doi:10.1111/j.1755-0998.2011.03021.x

Taylor, H. R., and Harris, W. E. (2012). An emergent science on the brink of irrelevance: a review of the past 8 years of DNA barcoding. *Molecular Ecology Resources* **12**, 377–388. doi:10.1111/j.1755-0998.2012.03119.x

Turgeon, D. D., Quinn, J. F., Jr, Bogan, A. E., Coan, E. V., Hochberg, F. G., Jr, Lyons, W. G., Mikkelsen, P. M., Neves, R. J., Roper, C. F. E., Rosenberg, G., Roth, B., Scheltema, A., Thompson, F. G., Vecchione, M., and Williams, J. D. (1998). Common and Scientific Names of Aquatic Invertebrates from the United States and Canada: Mollusks.' 2nd edn. Special Publication No. 26. (American Fisheries Society: Bethesda, MD.)

Via, S., Gomulkiewicz, R., De Jong, G., Scheiner, S. M., Schlichting, C. D., and Tienderen, P. V. (1995). Adaptive phenotypic plasticity: consensus and controversy. *Trends in Ecology & Evolution* **10**, 212–217. doi:10.1016/S0169-5347(00)89061-8

Vidrine, M. F. (1993). 'The Historical Distributions of Freshwater Mussels in Louisiana.' (Gail Q. Vidrine Collectibles: Eunice, LA.)

Watters, G. T. (1994). Form and function of unionoidean shell sculpture and shape (Bivalvia). *American Malacological Bulletin* **11**, 1–20.

Watters, G. T., Hoggarth, M. A., and Stansbery, D. H. (2009). 'The Freshwater Mussels of Ohio.' (Ohio State University: Columbus, OH.)

Williams, J. D., Warren, M. L. Jr, Cummings, K. S., Harris, J. L., and Neves, R. J. (1993). Conservation status of freshwater mussels of the United States and Canada. *Fisheries (Bethesda, Md.)* **18**, 6–22. doi:10.1577/1548-8446(1993)018<0006:CSOFMO>2.0.CO;2

Williams, J. D., Bogan, A. E., and Garner, J. T. (2008). 'Freshwater Mussels of Alabama and the Mobile Basin in Georgia, Mississippi & Tennessee.' (The University of Alabama Press: Tuscaloosa, AL.)

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