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Revisiting the North American freshwater mussel genus *Quadrula* sensu lato (Bivalvia Unionidae): Phylogeny, taxonomy and species delineation

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Abstract

Freshwater mussels (Bivalvia, Unionidae) have suffered strong declines over the last century. High morphological plasticity of Unionidae causes disturbances in their systematics and taxonomy, hampering conservation efforts. Species that have historically been placed under the North American genus *Quadrula* have suffered from numerous taxonomic and species delineation problems since its inception. Four genera are presently recognized within *Quadrula* sensu lato, that is, *Cyclonaias*, *Quadrula*, *Theliderma* and *Tritogonia*, but their phylogenetic basis remains incompletely tested. In the present study, we reconstructed several two-marker (mtDNA cytochrome *c* oxidase subunit I—COI and NADH dehydrogenase subunit 1—ND1) phylogenies with newly collected specimens and all previously available sequences covering most species within this group. We then delineated the species within the group using an integrative approach with the application of molecular statistical methods, morphometric (Fourier Shape) analyses and geographic distribution data. Four clades corresponding to these genera were consistently recovered in all phylogenies. To validate the generic status of these clades, molecular analyses were complemented with morphological, anatomical and ecological data compiled from the literature. Several revisions are here proposed to the current systematics and taxonomy of these genera, including the synonymization of *Cyclonaias asperata* under *Cyclonaias kieneriana*; the inclusion of *Quadrula apiculata* and *Quadrula rumphiana* under *Quadrula quadrula*; the placement of *Quadrula nobilis* under *Tritogonia*; and finally the separation of the Mobile River basin populations of *Theliderma metanevra* as a new species, that is, *Theliderma johnsoni* n. sp. The conservation implications of the proposed changes are then discussed.

1 | INTRODUCTION

Conservation programs and strategies are largely based on species as conservation units, making species delineation extremely important as a basic conservation tool (Prié, Puillandre, & Bouchet, 2012). However, taxon-based conservation strategies dedicated to the freshwater mussel family Unionidae, one of the world's most endangered taxa, are hindered by phylogenetic and taxonomic uncertainties (Lopes-Lima et al., 2017). This is especially true within the most species-rich Unionidae subfamily, the North American Ambleminae. Across the most recent systematics studies, the Ambleminae is divided in five tribes (Pfeiffer et al., 2019). However, polyphyly and inappropriate species boundaries have been revealed in some of these tribes, including the Quadrulini (Lydeard, Minton, & Williams, 2000; Pfeiffer et al., 2016; Serb, Buhay, & Lydeard, 2003). The quadruline freshwater mussels are distinctive animals producing thick quadrate shells, some of which are heavily sculptured. Shell morphology is highly variable within some species from this group, hindering unambiguous species identification or generic assignment. As shell morphology has been the original basis for Quadrulini systematics and taxonomy to date, the systematics and composition of this tribe have suffered a series of changes since its first description in the early 1900s (see Supporting Information Appendix S1 for an extensive taxonomic history of the Quadrulini). From the beginning of the 20th century, species that had been historically placed within the genus *Quadrula* sensu lato have been divided into four main species groups, that is, the *Quadrula* sensu stricto, the *pustulosa*, the *metanevra* and the *Tritogonia* species groups (Supporting Information Appendix S1). A molecular phylogeny of these taxa by Serb et al. (2003) largely confirmed these groupings and recovered four clades: *Quadrula* sensu strictu, the *pustulosa* species group, the *metanevra* species group and a fourth clade including *Tritogonia verrucosa* and *Quadrula nobilis*. Although these four clades are commonly referred to as genera in regional checklists (Howells 2013; Parmalee & Bogan 1998; Williams, Bogan, & Garner, 2008) the molecular, morphological and ecological evidence supporting these groups remains limited.

The present study is focused on re-examining the phylogeny, systematics and taxonomy of *Quadrula* sensu lato, here defined as including the species from the genera *Quadrula*, *Theliderma*, *Cyclonaias* and *Tritogonia* (Williams et al., 2017). In detail, this study aims to: (a) estimate the phylogenetic relationships of specimens collected in Texas with all published Quadrulini sequences, using a two-marker approach (COI and ND1); (b) perform a comparative shell morphometry evaluation to complement the molecular results; (c) define species boundaries with a taxonomic revision of all analysed taxa; (d) test the four classical generic constructs and their evolutionary significance; and (e) describe the conservation implications of the obtained results.

2 | MATERIALS AND METHODS

2.1 | Sample collection and materials examined

Specimens of quadruline mussels were collected from 50 sites across the state of Texas during 2003–2011 (Figure 1). A total of 89 specimens were collected and placed in 99% ethanol for molecular analyses. Voucher specimens were labelled and deposited in the SUNY Buffalo State College Great Lakes Center collections, Buffalo, New York (BSGLC). The field work was carried out with an appropriate Scientific Research Permit SPR-0503-300 issued by the Texas Parks and Wildlife Department. Additionally, dry shell specimens of the target nominal species were selected for morphometry from specimens deposited at the North Carolina Museum of Natural Sciences (NCMS) and BSGLC (See Supporting Information Table S1 for the examined lot numbers).

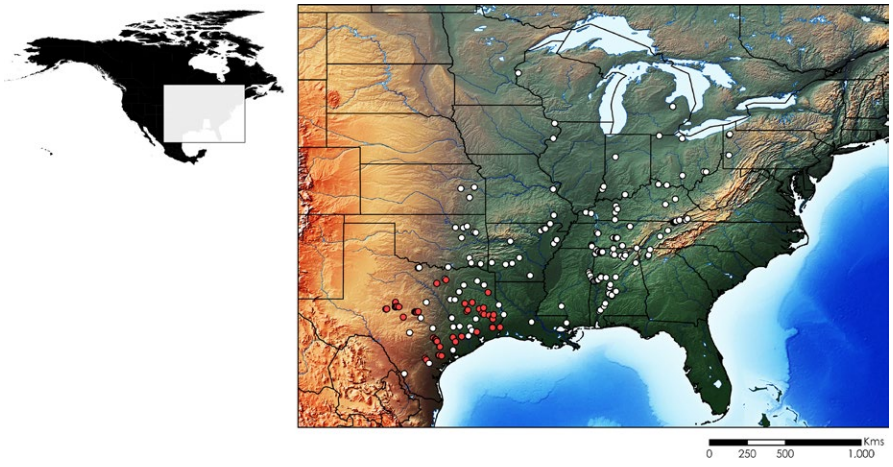

FIGURE 1 Map of all sampling sites for the present study; both tissue and shell materials in red; only shell materials in white [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 List of newly sequenced specimens for Cytochrome c oxidase subunit I (COI) and NADH dehydrogenase subunit 1 (ND1) datasets; nominal taxa, new identification, site, main basin, and COI and ND1 Haplotype number and Genbank references.

TAXON	NEW ID	RIVER	BASIN	GB (COI)	GB (ND1)
Quadrula petrina	*Cyclonaias necki*	San Marcos	S. Antonio/Guadalupe	MG969422	MK503297
Quadrula petrina	*Cyclonaias necki*	San Marcos	S. Antonio/Guadalupe	MG969423	MK503316
Quadrula petrina	*Cyclonaias necki*	San Marcos	S. Antonio/Guadalupe	MG969424	MK503317
Quadrula petrina	*Cyclonaias necki*	San Marcos	S. Antonio/Guadalupe	MG969425	MK503318
Quadrula petrina	*Cyclonaias petrina*	Concho	Colorado	MG969416	MK503293
Quadrula petrina	*Cyclonaias petrina*	Concho	Colorado	MG969417	MK503294
Quadrula petrina	*Cyclonaias petrina*	Concho	Colorado	MG969418	MK503295
Quadrula petrina	*Cyclonaias petrina*	San Saba	Colorado	MG969419	MK503311
Quadrula petrina	*Cyclonaias petrina*	San Saba	Colorado	MG969420	MK503312
Quadrula petrina	*Cyclonaias petrina*	San Saba	Colorado	MG969421	MK503313
Quadrula aurea	*Cyclonaias pustulosa*	San Marcos	S. Antonio/Guadalupe	MK503268	MK503296
Quadrula aurea	*Cyclonaias pustulosa*	San Antonio	S. Antonio/Guadalupe	MK503269	MK503298
Quadrula aurea	*Cyclonaias pustulosa*	San Antonio	S. Antonio/Guadalupe	MK503270	MK503299
Quadrula aurea	*Cyclonaias pustulosa*	San Antonio	S. Antonio/Guadalupe	MK503271	MK503300
Quadrula aurea	*Cyclonaias pustulosa*	San Antonio	S. Antonio/Guadalupe	-	MK503301
Quadrula aurea	*Cyclonaias pustulosa*	Guadalupe	S. Antonio/Guadalupe	MK503272	MK503302
Quadrula aurea	*Cyclonaias pustulosa*	Nueces	Nueces	MK503281	MK503314
Quadrula aurea	*Cyclonaias pustulosa*	Nueces	Nueces	MK503282	MK503315
Quadrula aurea	*Cyclonaias pustulosa*	Guadalupe	S. Antonio/Guadalupe	MK503283	MK503319
Quadrula aurea	*Cyclonaias pustulosa*	Guadalupe	S. Antonio/Guadalupe	MK503284	MK503320
Quadrula houstonensis	*Cyclonaias pustulosa*	Colorado	Colorado	MK503273	MK503303
Quadrula houstonensis	*Cyclonaias pustulosa*	Colorado	Colorado	MK503274	MK503304
Quadrula houstonensis	*Cyclonaias pustulosa*	Colorado	Colorado	MK503275	MK503305
Quadrula mortoni	*Cyclonaias pustulosa*	Sandy Creek	Neches	MK503276	MK503306
Quadrula mortoni	*Cyclonaias pustulosa*	Village Creek	Neches	MK503278	MK503308
Quadrula mortoni	*Cyclonaias pustulosa*	Trinity	Trinity	MK503286	MK503322
Quadrula mortoni	*Cyclonaias pustulosa*	Trinity	Trinity	MK503287	MK503323
Quadrula mortoni	*Cyclonaias pustulosa*	Trinity	Trinity	MK503288	MK503324
Quadrula nobilis	*Tritogonia nobilis*	Neches	Neches	MK503279	MK503309
Quadrula nobilis	*Tritogonia nobilis*	Neches	Neches	MK503280	MK503310
Quadrula nobilis	*Tritogonia nobilis*	Trinity	Trinity	MK503285	MK503321

(Continues)

TABLE 1 (Continued)

TAXON	NEW ID	RIVER	BASIN	GB (COI)	GB (ND1)
Quadrula quadrula	*Quadrula quadrula*	Ohio	Ohio	MK503267	MK503291
Theliderma johnsoni	*Theliderma metanevra*	Alabama	Alabama	MK503289	-
Pleurobema riddellii	*Pleurobema riddellii*	Village Creek	Neches	MK503277	MK503307
Pleurobema oviforme	*Pleurobema oviforme*	Little Tennessee	Mississippi	-	MK503292
Anodonta nuttalliana	*Anodonta nuttalliana*	John Day	Columbia	MK503266	MK503290

TABLE 2 List of morphological, anatomical and behavioural characters of *Cyclonaias*, *Quadrula*, *Theliderma* and *Tritogonia* as recognized in the present study

					Mantle displays (magazines)					
	Sexual dimorphism	**Shell Sulcus**	**Periostracal chevrons**	**Posterior ridge**	**Morphology**	**Size**	**Location (apertures)**	**Reflexive release**	**Hosts**	**GLN**
Cyclonaias	No	No	No	Low rounded	Stomate-shaped	Small	Excurrent	Yes	Ictaluridae (71%) Centrarchidae (24%) Acipenseridae (5%)	0.05–0.09
Quadrula	No	Yes	No	Well developed	Conical (knob-like)[a]	Large[a]	Excurrent[a]	No*	Ictaluridae (67%) Centrarchidae (33%)	0.005–0.009
Theliderma	No	No[b]	Yes	Low rounded to prominent	Variable shape	Small	Excurrent	Yes	Cyprinidae (72%) Centrarchidae (14%) Percidae (14%)	0.03–0.04
Tritogonia	Yes	Yes	No	Well developed	Slug-shaped*	Large*	Both*	No*	Ictaluridae	0.009

Notes. GLN: mean glochidial size index.

[a]Only analysed in one species. [b]For most species.

2.2 | Sequencing, alignments and phylogenetic analyses

A total of 31 quadruline specimens, including all nominal taxa across the state of Texas, were selected for molecular analyses (Table 1). For each sample, genomic DNA extraction (Froufe et al., 2014), amplification and bidirectional sequencing were carried out for the F-type mtDNA cytochrome *c* oxidase subunit I (COI) and NADH dehydrogenase subunit 1 (ND1) genes. For COI, the primers LCO_22me and HCO_700dy (Walker et al., 2006) were used with an annealing temperature of 50°C and polymerase chain reaction (PCR) conditions as described in Froufe et al. (2014). ND1 was amplified using the PCR conditions and primers (Leu-uurF and LoGlyR) of Serb et al. (2003). Sequences were obtained with the BigDye sequencing protocol (Applied Biosystems 3730xl) by Macrogen Inc., Korea. Forward and reverse sequences were edited and assembled using ChromasPro 1.7.4 (Technelysium, Tewantin, Australia). All new sequences have been deposited in GenBank (Table 1 and Supporting Information Tables S2 and S3).

Three datasets were constructed as follows: one for COI, another for ND1 and a third concatenating COI and ND1. The COI and ND1 datasets included all newly sequenced individuals and all Quadrulini sequences available in GenBank database for each gene (Supporting Information Tables S2–S4). The COI + ND1 dataset included all individuals sequenced for both COI and ND1 plus GenBank Quadrulini specimens with sequences available for the two genes (Supporting Information Table S4). For each of the three datasets, sequences of additional specimens were downloaded from Genbank and/or newly sequenced as outgroup (details in Supporting Information Tables S2–S4). The three datasets were aligned with the MAFFT multiple sequence alignment algorithm (Katoh & Standley, 2013). Each individual gene alignment was then restricted to its unique haplotypes, retrieved using DnaSP v5.1.0.1 (Librado & Rozas, 2009).

Phylogenetic analyses were then performed on the three datasets using Bayesian inference (BI) and maximum likelihood (ML). For the BI analyses, the best-fit models of nucleotide substitution were selected using JModelTest 2.1.10 (Darriba, Taboada, Doallo, & Posada, 2012) under the Bayesian information criterion. For each individual gene dataset, a three partition scheme was applied, one per gene codon, with the following selected models: COI (GTR + I + G, HKY, HKY + G), and ND1 (HKY + G, HKY + G, GTR + I + G). For the COI + ND1 dataset, a six partition scheme was applied for the three codons of both COI and ND1 with the same models selected for the individual gene datasets. BI analyses were performed in MrBayes v3.2.6 (Ronquist et al., 2012) implemented in CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010). BI analyses were initiated with program-generated trees and four Markov chains with default incremental heating. Two independent runs of 30×10^6 generations were sampled at intervals of 1,000 generations producing a total of 30,000 trees. Burn-in was determined upon convergence of log likelihood and parameter values using Tracer 1.6 (Rambaut, Suchard, Xie, & Drummond, 2014).

For the ML analyses, the same partitioning scheme was applied for each dataset with the same model (GTR + G) for all partitions, and sequences were then analysed in RaxML 8.2.10 HPC Black Box (Stamatakis, 2014) with 1,000 bootstrap replicates. Haplotype networks were calculated using TCS 1.21 (Clement, Posada, & Crandall, 2000) with a threshold of 95%.

2.3 | Molecular based species delineation methods

Five distinct molecular methods were applied to determine the number of molecular operational taxonomic units (MOTUs). All methods were applied to the COI, ND1 and

TABLE 3 Results of repeatability clade analysis (RCA) of main clades corresponding to the preferred topology

Clades	Analyses	COI + ND1	COI	ND1
Quadrulini	BI	**100**	**100**	
	ML	**74**	55	
Quadrula sensu lato	BI	**100**	**100**	**100**
	ML	**98**	**93**	**90**
Cyclonaias	BI	**100**	**95**	**98**
	ML	**83**	35	68
Quadrula s.s.	BI	**100**	**100**	**100**
	ML	**100**	**99**	**99**
Theliderma	BI	**100**	**100**	89
	ML	**100**	**99**	**72**
Tritogonia	BI	**100**	**100**	**100**
	ML	**100**	**98**	**87**
Cyclonaias infucata + *Cyclonaias kleiniana* + *Cyclonaias kieneriana*	BI	65	**97**	
	ML	55	37	
Cyclonaias petrina + *Cyclonaias nodulata* + *Cyclonaias necki*	BI	**99**	**99**	**100**
	ML	**84**	51	**96**
Cyclonaias pustulosa group	BI	**100**	**100**	89
	ML	**99**	64	45

Notes. In bold values higher than 95% (Bayesian Inference) and 70% (Maximum Likelihood).

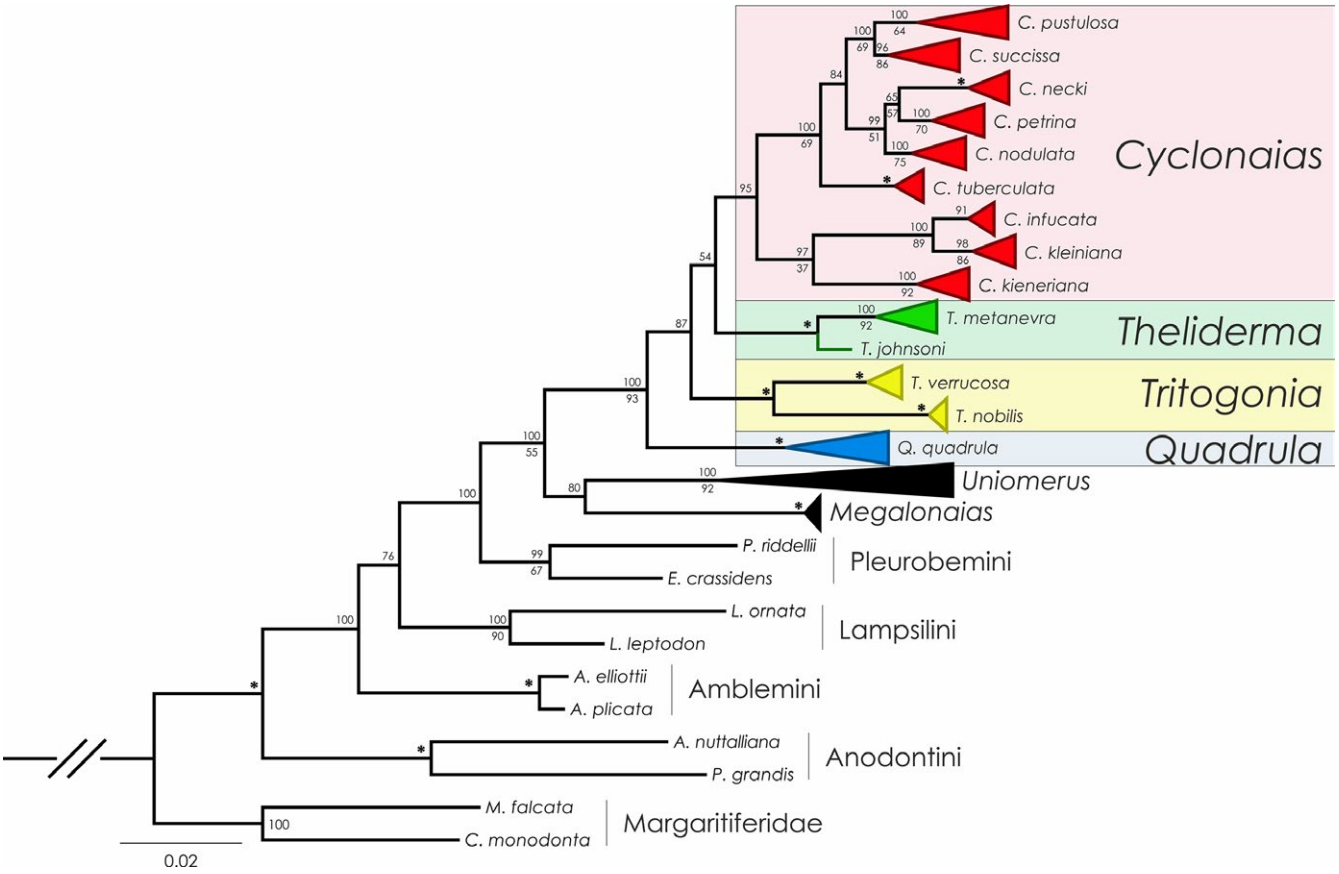

FIGURE 2 Bayesian consensus tree inferred from the cytochrome *c* oxidase subunit I (COI) gene fragment. The values above and below the nodes indicate Bayesian posterior probability (bpp) percentage and maximum likelihood bootstrap values (bs), respectively. Values over 95% are represented by an asterisk, and those <50% are not shown for clarity [Colour figure can be viewed at wileyonlinelibrary.com]

concatenated (COI + ND1) datasets, with the exception of the BIN system that relies only on COI. The first two are distance based, that is, the BIN system implemented in BOLD (Ratnasingham & Hebert, 2013) and the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012). For the BINs system, the COI dataset without the outgroups was analysed with the Cluster Sequences tool implemented in BOLD 4 (http://v4.boldsystems.org) (Ratnasingham & Hebert, 2013). The ABGD species delineation tool was applied to all three datasets without outgroup using its online version (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with the default settings and the Kimura-2-parameter distance matrix (Puillandre et al., 2012).

Two tree-based molecular species delineation methods were applied to all datasets, that is, the single threshold Generalized Mixed Yule Coalescent (GMYC) model (Fujisawa & Barraclough, 2013) and the Bayesian implementation of the Poisson Tree Processes model (bPTP) (Zhang, Kapli, Pavlidis, & Stamatakis, 2013). For the GMYC method, a Bayesian ultrametric phylogenetic tree was first generated in BEAST 2.4.6 (Bouckaert et al., 2014) with the previously selected models for each partition and four independent runs of 20×10^6 Markov chain Monte Carlo (MCMC) generations, sampled every 1×10^3 generations. Convergence of the parameters was evaluated using Tracer 1.6 software (Rambaut et al., 2014). The consensus tree was annotated using TreeAnnotator 2.4.6 (Bouckaert et al., 2014). The consensus tree was loaded into the R software package "Species Limits by Threshold Statistics" (Ezard, Fujisawa, & Barraclough, 2009) in R 3.2.0 (R Core Group available via http://www.r-project.org) and analysed using the single threshold model. For the bPTP, the BI phylogenetic trees previously obtained were used as input trees in the bPTP web server (available at: http://species.h-its.org/) with 1×10^6 iterations of MCMC and 20% burn-in. Finally, a 95% statistical parsimony connection limit was used, by using TCS 1.21 (Clement et al., 2000). Sequence divergences (uncorrected *p*-distance) were assessed using MEGA 7 (Kumar, Stecher, & Tamura, 2016).

2.4 | Morphometry

For a detailed analysis of inter- and intraspecific variation in shell shape within the quadruline genera *Cyclonaias*, *Quadrula* and *Theliderma*, we used Fourier Shape Analysis, as developed and explained by Crampton and Haines (1996).

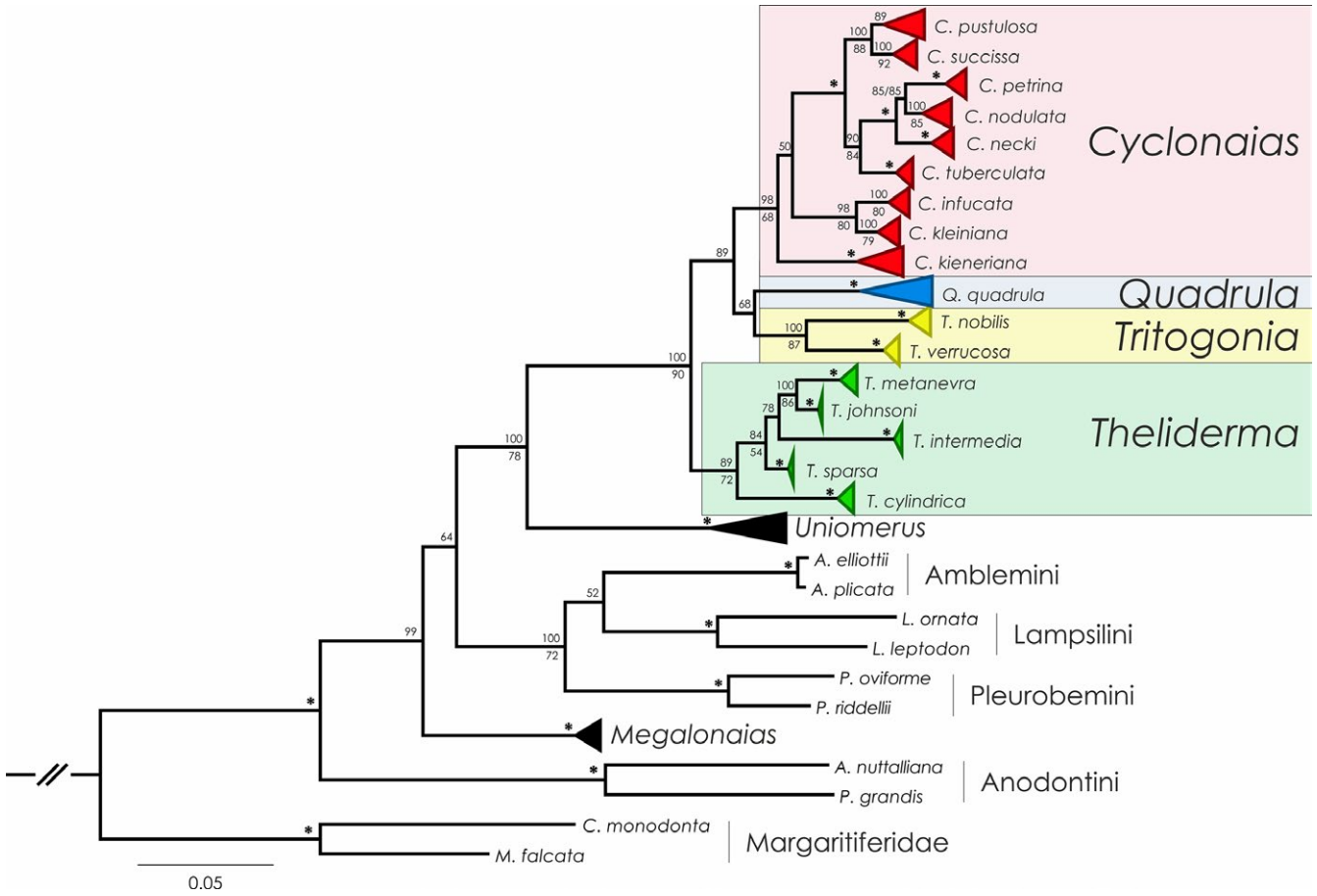

FIGURE 3 Bayesian consensus tree inferred from the NADH dehydrogenase subunit 1 (ND1) gene fragment. The values above and below the nodes indicate Bayesian posterior probability (bpp) percentage and maximum likelihood bootstrap values (bs), respectively. Values over 95% are represented by an asterisk, values below 50% are not shown for clarity [Colour figure can be viewed at wileyonlinelibrary.com]

This method decomposes xy-coordinates of a shell outline into a number of harmonics, each of which is in turn explained by two Fourier coefficients. Xy-coordinates of the sagittal shell outline of 1,222 specimens from BSGLC and NCMS collections (739 specimens of *Cyclonaias* spp., 254 specimens of *Quadrula* spp. and 229 specimens of *Theliderma* spp.; Supporting Information Table S1) were obtained from digital photographs using the program IMAGEJ (Rasband, 2008) and subjected to fast Fourier transformation using the program HANGLE, applying a smoothing normalization of 3 to eliminate high-frequency pixel noise. Preliminary analysis indicated that the first 10 harmonics described the outlines with sufficiently high precision. Discarding of the first harmonic, which does not contain any shape information, resulted in a set of 18 Fourier coefficients per individual. Outlines of all specimens within each of the three genera were then rotated to maximum overlap by program HTREE, resulting in the final set of 18 Fourier coefficients per individual.

For visual examination of variation in shell shape within and between true and nominal species, principal component analysis were performed on the 18 Fourier coefficients of (a) all true species (recognized by the molecular species delineation methods, see results) of *Cyclonaias*, including a maximum of 50 specimens per species; (b) all nominal species of *Cyclonaias pustulosa*; (c) only *Cyclonaias kieneriana* and *Cyclonaias kleiniana*; (d) all nominal species of *Quadrula*; (e) all true species (recognized by the molecular species delineation methods, see results) of *Theliderma*; and (f) only *Theliderma metanevra* and *Theliderma johnsoni* n. sp. (see Supporting Information Appendix S2 for a detailed description of *T. johnsoni* n. sp.). Synthetic outlines of extreme and average shell shapes were drawn using program HCURVE as explained in Crampton and Haines (1996).

We assessed the rate of accurate identification of true and nominal species based on shell shape using linear discriminant analysis (LDA) on the 18 Fourier coefficients. To test for statistically significant differences in sagittal shell shape between species, multivariate analyses of variance (MANOVA) were run on the 18 Fourier coefficients. Pairwise Hotelling's post hoc tests were performed to identify significant differences between each pair of true/nominal species. Statistical analyses were performed in PAST v.3 (Hammer & Harper, 2006).

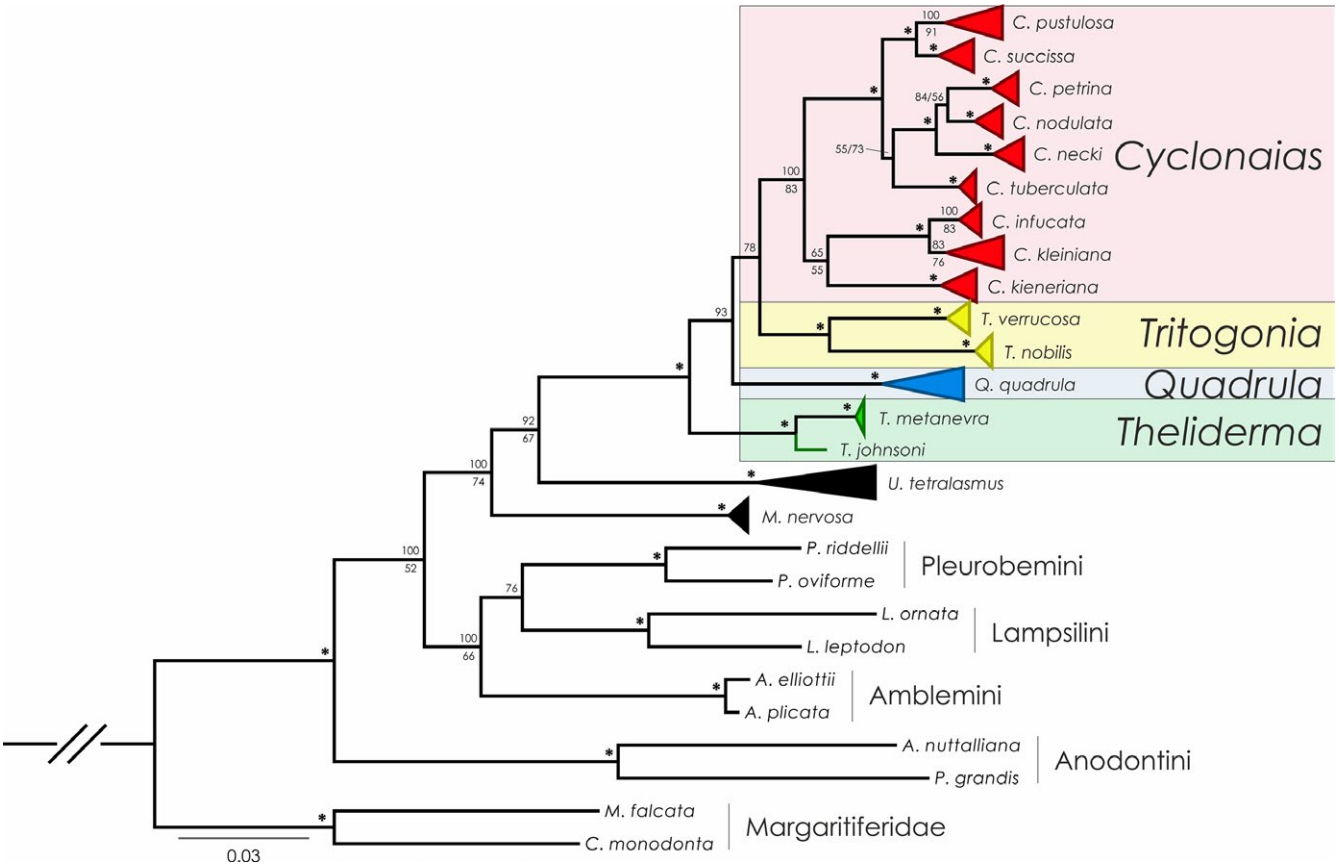

FIGURE 4 Bayesian consensus tree inferred from the NADH dehydrogenase subunit 1 (ND1) and cytochrome *c* oxidase I (COI) gene fragments concatenated dataset. The values above and below the nodes indicate Bayesian posterior probability (bpp) percentage and maximum likelihood bootstrap values (bs), respectively. Values over 95% are represented by an asterisk, values below 50% are not shown for clarity [Colour figure can be viewed at wileyonlinelibrary.com]

2.5 | Ecological, morphological and anatomical traits

An extensive bibliographic review of selected ecological, morphological and anatomical traits was accomplished for all species within *Quadrula* s.l. (Table 2; Supporting Information Table S5).

3 | RESULTS

3.1 | Alignments and phylogenetic analyses

The COI dataset spanned 582 nucleotides (nt) and included 289 unique haplotypes (232 polymorphic and 192 parsimony informative sites). The ND1 dataset covered 619 bp with 339 unique haplotypes (297 polymorphic and 257 parsimony informative sites). Finally, the combined COI + ND1 dataset was 1,192 nt long and included 325 individual sequences (501 polymorphic and 427 parsimony informative sites). No insertions or deletions, and no stop codons were observed in any of the datasets after translating all sequences to amino acids.

The results of the BI and ML phylogenetic analyses for the three datasets presented similar topologies (Table 3), thus only BI phylogenetic trees are shown in Figures 2–4. In the COI phylogeny, the Quadrulini clade is monophyletic and well supported in the BI analyses. Within the Quadrulini clade, the *Megalonaias* + *Uniomerus* clade is sister to a clade including three well-supported subclades corresponding to the genera *Quadrula*, *Tritogonia*, and *Theliderma*, and a clade including all *Cyclonaias* sequences (Figure 2).

The ND1 phylogeny recovered similar phylogenetic patterns to that obtained with COI. However, in these analyses, the Quadrulini is not monophyletic, with the remaining Ambleminae tribe clades, that is, Amblemini, Pleurobemini and Lampsilini clustering within the Quadrulini tribe clade (Figure 3). The *Uniomerus* clade is sister to a clade containing the four remaining Quadrulini genera (i.e., *Quadrula*, *Tritogonia*, *Theliderma* and *Cyclonaias*; Figure 3). While *Cyclonaias*, *Quadrula* and *Tritogonia* are well supported, *Theliderma* has a low support value (Figure 3). The COI + ND1 phylogeny shows Quadrulini as monophyletic with *Uniomerus* being

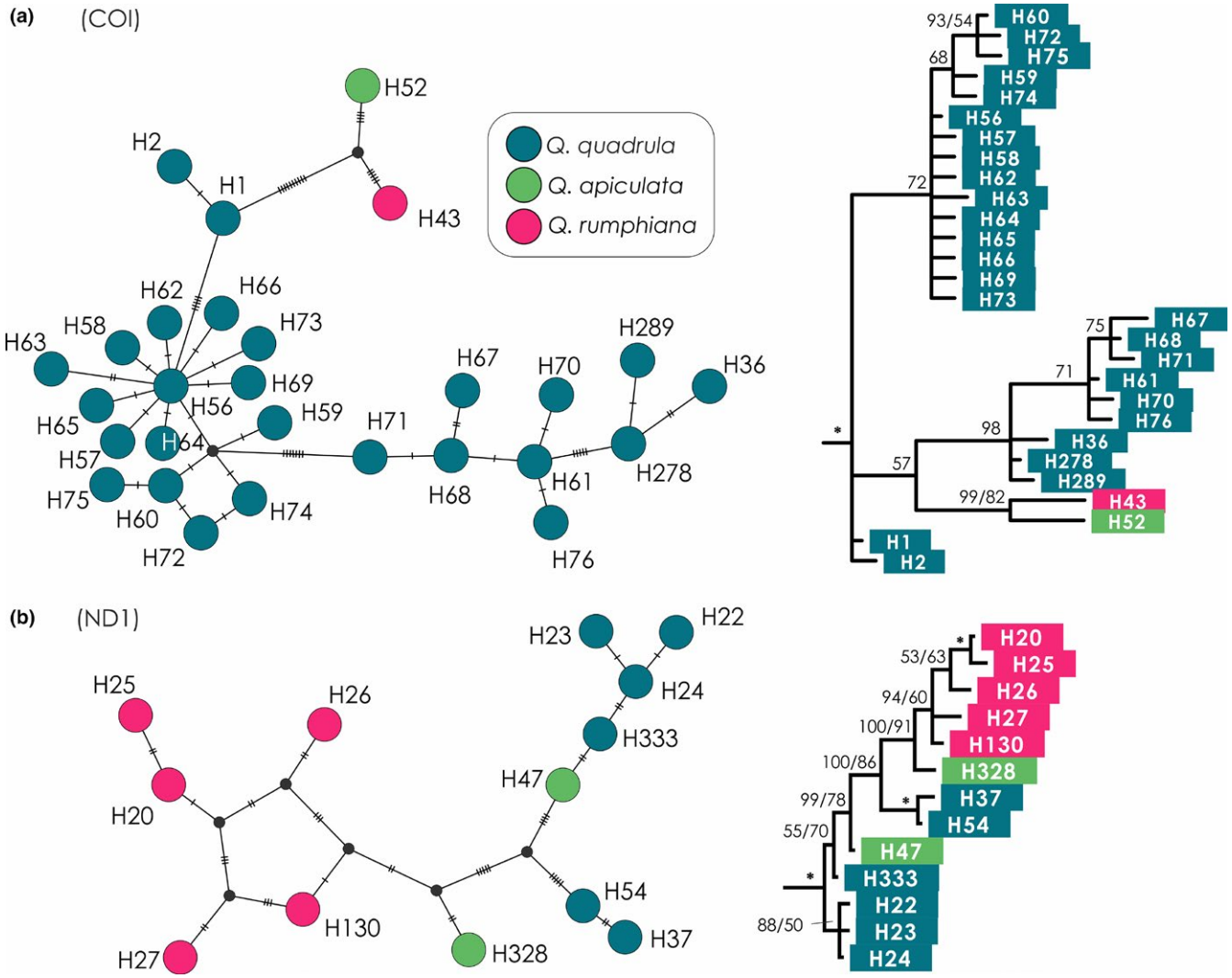

FIGURE 5 Haplotype (TCS) networks and uncollapsed *Quadrula* clade from Figures 2 and 3, showing the relationships of nominal species within the *Quadrula quadrula* group for (a) cytochrome *c* oxidase I (COI) and (b) NADH dehydrogenase subunit 1 (ND1) [Colour figure can be viewed at wileyonlinelibrary.com]

sister to a clade comprising four well-supported clades (*Quadrula*, *Tritogonia*, *Theliderma* and *Cyclonaias*; Figure 4).

3.1.1 | Cyclonaias

Within *Cyclonaias*, the clade labelled *C. pustulosa* includes specimens originally identified as *Cyclonaias aurea*, *Cyclonaias houstonensis*, *Cyclonaias mortoni*, *C. pustulosa* and *Cyclonaias refulgens*.

3.1.2 | Quadrula

All sequences from the nominal species *Quadrula quadrula*, *Quadrula apiculata* and *Quadrula rumphiana* cluster within the *Q. quadrula* clade in all phylogenies (Figures 2–4). However, both nominal species *Q. apiculata* and *Q. rumphiana* were found to be nested within *Q. quadrula* (Figures 2–4). Both the COI and ND1 95% threshold haplotype networks of the *Q. quadrula* clade reveal a low number of mutations among the nominal taxa *Q. quadrula*, *Q. apiculata* and *Q. rumphiana* (Figure 5a,b).

3.1.3 | Theliderma

Not many COI sequences of *Theliderma* are represented in the COI dataset, and therefore in the COI and COI + ND1 phylogenies (Figures 2 and 4). Nevertheless, in these phylogenies two distinct clades were obtained in sequences from specimens of *T. metanevra*: one corresponding to specimens from the Tennessee basin, and the other with specimens from the Mobile basin (Figures 2 and 4). The ND1 phylogeny is better represented with all species recognized to date except for *Theliderma stapes* (Figure 3).

TABLE 4 Pairwise genetic distance matrixes of nominal quadruline species of the genera *Cyclonaias*, *Quadrula*, *Theliderma*, and *Tritogonia*, using the original nominal taxa

	Within groups		Between groups									
	COI	ND1	*Cyclonaias asperata*	*Cyclonaias kieneriana*	*Cyclonaias kleiniana*	*Cyclonaias infucata*	*Cyclonaias nodulata*	*Cyclonaias petrina*	*Cyclonaias necki*	*Cyclonaias pustulosa*	*Cyclonaias aurea*	*Cyclonaias houstonensis*
Cyclonaias asperata	0.012	0.012		0.012	0.082	0.094	0.093	0.102	0.094	0.082	0.082	0.078
Cyclonaias kieneriana	—	—			0.081	0.094	0.089	0.101	0.093	0.081	0.082	0.077
Cyclonaias kleiniana	0.012	0.011	0.080	—		0.035	0.099	0.094	0.099	0.083	0.085	0.083
Cyclonaias infucata	0.006	0.007	0.082	—	0.032		0.097	0.092	0.097	0.087	0.090	0.085
Cyclonaias nodulata	0.006	0.009	0.077	—	0.088	0.083		0.038	0.040	0.063	0.063	0.064
Cyclonaias petrina	0.007	0.006	0.076	—	0.095	0.090	0.028		0.047	0.063	0.062	0.064
Cyclonaias necki	0.007	0.007	0.077	—	0.094	0.084	0.041	0.039		0.064	0.067	0.066
Cyclonaias pustulosa	0.010	0.011	0.076	—	0.092	0.085	0.052	0.053	0.051		0.017	0.012
Cyclonaias aurea	0.011	0.012	0.078	—	0.092	0.083	0.050	0.051	0.051	0.014		0.018
Cyclonaias houstonensis	0.007	0.008	0.075	—	0.088	0.081	0.058	0.059	0.055	0.014	0.017	
Cyclonaias mortoni	0.013	0.012	0.075	—	0.086	0.079	0.052	0.054	0.055	0.020	0.019	0.020
Cyclonaias refulgens	0.015	0.010	0.074	—	0.091	0.084	0.052	0.052	0.052	0.014	0.014	0.017
Cyclonaias succissa	0.011	0.011	0.081	—	0.094	0.085	0.048	0.044	0.054	0.036	0.033	0.041
Cyclonaias tuberculata	0.006	0.006	0.078	—	0.088	0.090	0.050	0.056	0.062	0.058	0.056	0.064
Quadrula quadrula	0.014	0.012	0.112	—	0.110	0.103	0.096	0.097	0.098	0.108	0.104	0.112
Quadrula apiculata	—	0.018	0.105	—	0.096	0.096	0.093	0.089	0.095	0.100	0.099	0.103
Quadrula rumphiana	—	0.010	0.105	—	0.099	0.095	0.093	0.089	0.095	0.097	0.092	0.100
Theliderma cylindrica	—	0.010	—	—	—	—	—	—	—	—	—	—
Theliderma intermedia	—	0.003	—	—	—	—	—	—	—	—	—	—
Theliderma metanevra	0.017	0.021	0.091	—	0.092	0.096	0.094	0.093	0.090	0.084	0.087	0.086
Theliderma sparsa	—	0.002	—	—	—	—	—	—	—	—	—	—
Tritogonia verrucosa	0.007	0.008	0.096	—	0.105	0.093	0.102	0.104	0.098	0.105	0.107	0.104
Tritogonia nobilis	0.009	0.011	0.105	—	0.118	0.107	0.108	0.101	0.106	0.102	0.102	0.102

Notes. Left: mean uncorrected *p*-distance within putative species for cytochrome oxidase subunit I (COI) and for NADH dehydrogenase, subunit 1 (ND1) genes. Right: mean uncorrected *p*-distance among putative species of COI (below the diagonal) and ND1 (above the diagonal) genes.

3.1.4 | Tritogonia

The sequences of specimens originally identified as *Q. nobilis* cluster together with those from *T. verrucosa* in all phylogenies forming a well-supported clade here assigned to *Tritogonia* (Figures 2–4).

3.2 | Genetic divergence and species delineation methods

3.2.1 | Cyclonaias

Pairwise uncorrected *p*-distance values among six of the nominal *Cyclonaias* species, *C. pustulosa*, *C. aurea*, *C. houstonensis*, *C. mortoni* and *C. refulgens* were low (≤2% for both COI and ND1: Table 4).

Of the 14 putative *Cyclonaias* species, only nine were recognized as MOTUs based on a consensus of all species delineation methods, applied on the COI, ND1 and COI + ND1 datasets (Table 5). The pairwise uncorrected *p*-distance between these recognized *Cyclonaias* MOTUs varied between 2.8% (COI)/3.1% (ND1) and 11.2% (COI)/10.2% (ND1; Table 6). The uncorrected *p*-distance within each of the recognized MOTUs was ≤1.2% for COI and ≤1.6% for ND1 (Table 6).

3.2.2 | Quadrula

The pairwise uncorrected *p*-distance among all nominal *Quadrula* species varied from 1.4% (COI)/1.7% (ND1) to 3.4% (COI)/2.7% (ND1; Table 4). Taking into account the three datasets, only a single MOTU was consensually recognized for the *Quadrula* genus (Table 5) with a within *p*-distance value of 1.7% for COI and 1.9% for ND1 (Table 6).

3.2.3 | Theliderma

The pairwise uncorrected *p*-distance among all the nominal *Theliderma* species ranged between 4.0% and 10.6% for ND1

Cyclonaias mortoni	*Cyclonaias refulgens*	*Cyclonaias succissa*	*Cyclonaias tuberculata*	*Quadrula quadrula*	*Quadrula apiculata*	*Quadrula rumphiana*	*Theliderma cylindrica*	*Theliderma intermedia*	*Theliderma metanevra*	*Theliderma sparsa*	*Tritogonia verrucosa*	*Tritogonia nobilis*
0.086	0.080	0.083	0.094	0.101	0.107	0.114	0.112	0.143	0.111	0.105	0.114	0.115
0.085	0.079	0.083	0.096	0.101	0.109	0.111	0.116	0.143	0.111	0.106	0.111	0.114
0.090	0.084	0.092	0.088	0.109	0.116	0.121	0.110	0.143	0.117	0.105	0.112	0.123
0.092	0.088	0.095	0.093	0.108	0.110	0.117	0.115	0.139	0.116	0.110	0.107	0.125
0.062	0.059	0.064	0.055	0.123	0.129	0.134	0.129	0.144	0.121	0.113	0.118	0.126
0.061	0.058	0.064	0.065	0.127	0.131	0.136	0.125	0.140	0.121	0.110	0.122	0.130
0.066	0.062	0.070	0.059	0.127	0.131	0.134	0.127	0.147	0.126	0.115	0.116	0.126
0.019	0.012	0.033	0.054	0.108	0.112	0.116	0.121	0.136	0.115	0.106	0.105	0.119
0.020	0.014	0.031	0.051	0.107	0.111	0.115	0.118	0.136	0.119	0.107	0.106	0.118
0.020	0.013	0.029	0.052	0.103	0.107	0.111	0.116	0.134	0.114	0.105	0.101	0.118
	0.017	0.030	0.050	0.111	0.115	0.119	0.126	0.137	0.118	0.107	0.106	0.118
0.020		0.027	0.049	0.108	0.113	0.116	0.120	0.137	0.116	0.106	0.104	0.116
0.037	0.035		0.053	0.109	0.113	0.122	0.124	0.144	0.126	0.113	0.110	0.119
0.055	0.058	0.053		0.115	0.117	0.120	0.127	0.146	0.126	0.113	0.116	0.121
0.109	0.108	0.100	0.098		0.017	0.027	0.104	0.139	0.116	0.108	0.109	0.105
0.100	0.100	0.092	0.085	0.034		0.020	0.109	0.143	0.117	0.112	0.111	0.107
0.097	0.097	0.088	0.084	0.034	0.015		0.112	0.145	0.119	0.117	0.110	0.116
—	—	—	—	—	—	—		0.106	0.086	0.079	0.122	0.126
—	—	—	—	—	—	—	—		0.081	0.073	0.135	0.137
0.088	0.088	0.095	0.083	0.101	0.090			0.096		0.040	0.115	0.126
—	—	—	—	—	—	—	—	—	—		0.105	0.106
0.105	0.106	0.100	0.098	0.114	0.116	—	—	0.116	—	0.096		0.093
0.106	0.102	0.099	0.114	0.110	0.116	—	—	0.114	—	0.114	0.085	

(Table 4). The higher within *p*-distance recorded value was reached for *T. metanevra*, 1.7% for COI and 2.1% for ND1 (Table 4).

All originally described *Theliderma* species are here recognized as MOTUs with *T. metanevra* being further divided in two distinct MOTUs, that is, *T. metanevra* for specimens from the Tennessee River basin and *T. johnsoni* n. sp. from the Mobile River basin (Table 5). The *p*-distance values among the recognized *Theliderma* MOTUs varied between 3.5% and 10.1% for ND1 (Table 6). The *p*-distance within each of the recognized MOTUs was ≤0.9% for ND1 (Table 6).

3.2.4 | Tritogonia

Our analyses revealed a complete consensus of two individual MOTUs within the *Tritogonia* genus (Table 5). The two recognized MOTUs *T. verrucosa* and *Tritogonia nobilis* exhibited high interspecific *p*-distance divergence, 8.5% (COI)/9.3% (ND1), and low intraspecific *p*-distance <0.9% for COI and <1.1% ND1 (Table 6).

3.3 | Morphometry

3.3.1 | Cyclonaias

Linear discriminant analysis on the 18 Fourier coefficients extracted through Fourier Shape Analysis for all *Cyclonaias* species recognized in this study assigned 75% of individuals to the correct species (Figure 6a). Species that are particularly difficult to separate by shell shape are *C. kieneriana* and *C. pustulosa* (16% misidentifications), and *Cyclonaias infucata* and *C. kleiniana* (10%). In addition, most true species differed significantly from each other in their shell shape as approximated by 18 Fourier coefficients, with the exception of *C. infucata* and *C. kleiniana* (MANOVA, pairwise Hotelling's test $p = 0.742$), and *C. infucata* and *Cyclonaias necki* (MANOVA, pairwise Hotelling's test $p = 0.138$).

The proportion of *C. pustulosa* specimens correctly identified to the original nominal species within the *C. pustulosa* complex exceeded that of *Cyclonaias* specimens correctly identified to species level (see above), with 80% correct

TABLE 5 Results of molecular species delineation methods

	COI					ND1				COI + ND1				CONSENSUS
	BOLD	ABGD	TCS (95%)	bPTP	GMYC	ABGD	TCS (95%)	bPTP	GMYC	ABGD	TCS (95%)	bPTP	GMYC	MOTUs
Cyclonaias														
Cyclonaias asperata	✓	✓	✓	✓	✓	✗	✗	✗	✗	✓	✓	✓	✓	✗
Cyclonaias kieneriana	—	—	—	—	—	✓	✓	✓	✓	—	—	—	—	✓
Cyclonaias infucata	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cyclonaias kleiniana	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cyclonaias nodulata	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cyclonaias petrina	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cyclonaias necki	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cyclonaias pustulosa	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cyclonaias aurea	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
Cyclonaias houstonensis	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✓	✗
Cyclonaias mortoni	✗	✗	✗	✗	✓	✗	✗	✗	✓	✓	✓	✗	✓	✗
Cyclonaias refulgens	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
Cyclonaias succissa	✓	✓	✓	✓	✓	✗	✓	✗	✓	✓	✓	✓	✓	✓
Cyclonaias tuberculata	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Quadrula														
Quadrula quadrula clade 1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Quadrula quadrula clade 2	✗	✗	✗	✓	✗	✗	✗	✗	✗	✗	✓	✗	✓	✗
Quadrula quadrula clade 3	✓	✓	✗	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗
Quadrula apiculata	✓	✓	✓	✓	✓	✗	✗	✗	✗	✗	✗	✗	✓	✗
Quadrula rumphiana	✗	✓	✗	✗	✗	✓	✓	✗	✗	✗	✗	✗	✓	✗
Theliderma														
Theliderma cylindrica	—	—	—	—	—	✓	✓	✓	✓	—	—	—	—	✓
Theliderma intermedia	—	—	—	—	—	✓	✓	✓	✓	—	—	—	—	✓
Theliderma metanevra	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Theliderma johnsoni n. sp.	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Theliderma sparsa	—	—	—	—	—	✓	✓	✓	✓	—	—	—	—	✓
Tritogonia														
Tritogonia verrucosa	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Tritogonia nobilis	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Notes. ✓: recognized as a molecular operational taxonomic unit (MOTU); ✗: not recognized as a MOTU; —: not analysed.

identifications (Figure 6b). All nominal species differed significantly from each other in their shell shape as approximated by 18 Fourier coefficients (MANOVA, pairwise Hotelling's tests $p < 0.05$).

Using only the nominal species *C. kieneriana* and *Cyclonaias asperata*, the Fourier coefficients differed significantly between *C. kieneriana* and its synonym *C. asperata* (MANOVA: $F_{18,82} = 2.094$, $p = 0.013$), and 95% of specimens were classified correctly based on shell shape through LDA (Figure 6c).

3.3.2 | Quadrula

Fourier coefficients differed significantly between the nominal species of *Quadrula* (MANOVA, pairwise Hotelling's tests $p < 0.05$; Figure 6c). Seventy-six per cent of specimens were assigned to the correct nominal species, with 21% and 11% of misidentifications between *Q. apiculata* versus *Q. quadrula* and *Q. rumphiana*, respectively.

3.3.3 | Theliderma

Within the genus *Theliderma*, 91% of specimens were identified to the correct species (as they are here recognized) by LDA of Fourier coefficients (Figure 6e). *Theliderma cylindrica*, characterized by its typical elongated-rectangular shape, was 100% correctly identified. Considerable difficulties in separation by shell shape were present for *Theliderma sparsa* versus *T. johnsoni* n. sp. (21% misidentifications) and *T. metanevra* (13%), respectively. Most true *Theliderma* species pairs differed significantly from each other in their shell shape with the exception of *T. sparsa* versus *T. johnsoni* n. sp. (MANOVA, pairwise Hotelling's test: $p = 0.525$), *T. sparsa* versus *T. metanevra* ($p = 0.227$) and *T. stapes* and *T. johnsoni* n. sp. ($p = 0.427$; *p*-value could not be computed for the pair *T. sparsa* vs. *T. stapes* due to low replicate number).

When including the whole *Theliderma* dataset in LDA, only 5% of specimens of the pair *T. metanevra*/*T. johnsoni* n. sp. were assigned to the wrong clade (Figure 6e). When using only the *T. metanevra* dataset, 11% of specimens were misidentified (Figure 6f), though Fourier coefficients were significantly different between the two species (MANOVA: $F_{18,46} = 3.097$, $p = 0.001$).

3.4 | Diagnostic characters of the classical genera within *Quadrula* s.l

Species within *Quadrula* and *Tritogonia* share a number of ecological and morphological traits but are distinct from those within *Cyclonaias* and *Theliderma* (Table 2; Supporting Information Table S5). *Quadrula* and *Tritogonia* species exhibit a marked sulcus that is absent in *Cyclonaias* and *Theliderma*, with the exception of *T. sparsa* and *T. stapes* that may display shallow sulci (Table 2; Supporting Information Table S5). *Quadrula* and *Tritogonia* glochidial size index is ten times smaller than in *Cyclonaias* and *Theliderma* (Table 2; Supporting Information Table S5). *Quadrula* and *Tritogonia* also seem to share similar morphological and behavioural patterns of the mantle displays, also known as mantle magazines. While *Quadrula* and *Tritogonia* seem to exhibit large mantle displays with a non-reflexive glochidia release strategy when disturbed, *Cyclonaias* and *Theliderma* mantle displays are small and more inconspicuous and immediatelly expell their glochidial content when physically disturbed (Table 2; Supporting Information Table S5). However, some caution has to be taken when interpreting this data since mantle displays were only studied in a small number of species.

Within *Quadrula* s.l. some of the analysed characters are exclusive and can be used to recognize some of the classical recognized genera *Cyclonaias*, *Quadrula*, *Theliderma* and *Tritogonia* (Table 2; Supporting Information Table S5).

The presence of dark chevrons imprinted in the periostracum of shells is a trait that is exclusive of *Theliderma* species and can be used to recognize the genus within Quadrulini (Table 2; Supporting Information Table S5).

The stomate-shaped morphology of the mantle displays seems to be a diagnostic character for *Cyclonaias*, but laboratory studies on *C. asperata* (= *C. kieneriana*) did not observe any mantle display for this species (Haag & Staton, 2003).

Theliderma hosts are mainly composed of small cyprinids while catfishes are the main hosts of the other three *Quadrula* s.l. genera (Table 2; Supporting Information Table S5). The mantle displays and glochidia of *Theliderma* are smaller than those of *Cyclonaias* (Table 2; Supporting Information Table S5).

Tritogonia verrucosa and *T. nobilis* are sexually dimorphic in shell shape, a trait that is unique within the Quadrulini and therefore diagnostic of the genus (Table 2; Supporting Information Table S5). In addition, the mantle display mechanism of *T. verrucosa*, which involves the mantle to completely cover both the incurrent and excurrent aperture, is very distinct from those of all of the other *Quadrula* s.l. species (Supporting Information Table S5). However, this trait needs to be verified for *T. nobilis* in order to be considered diagnostic of the genus.

4 | DISCUSSION

4.1 | Phylogenetic relationships within *Quadrula* and generic support

The three BI and ML phylogenies (COI, ND1, and COI + ND1) obtained in the present study revealed a

TABLE 6 Pairwise genetic distance matrixes of quadruline species of the genera *Cyclonaias*, *Quadrula*, *Theliderma*, and *Tritogonia*, as recognized in the present study

	Within groups		Between groups						
	COI	ND1	*Cyclonaias kieneriana*	*Cyclonaias infucata*	*Cyclonaias kleiniana*	*Cyclonaias nodulata*	*Cyclonaias petrina*	*Cyclonaias necki*	*Cyclonaias pustulosa*
Cyclonaias kieneriana	0.012	0.012		0.094	0.082	0.093	0.102	0.094	0.082
Cyclonaias infucata	0.006	0.007	0.082		0.035	0.097	0.092	0.097	0.089
Cyclonaias kleiniana	0.012	0.011	0.080	0.032		0.099	0.094	0.099	0.085
Cyclonaias nodulata	0.006	0.009	0.077	0.083	0.088		0.038	0.04	0.063
Cyclonaias petrina	0.007	0.006	0.076	0.090	0.095	0.028		0.047	0.062
Cyclonaias necki	0.007	0.007	0.077	0.084	0.094	0.041	0.039		0.065
Cyclonaias pustulosa	0.016	0.016	0.076	0.082	0.089	0.052	0.053	0.052	
Cyclonaias succissa	0.011	0.011	0.081	0.085	0.094	0.048	0.044	0.054	0.036
Cyclonaias tuberculata	0.006	0.006	0.078	0.090	0.088	0.050	0.056	0.062	0.057
Quadrula quadrula	0.017	0.019	0.112	0.103	0.109	0.096	0.096	0.098	0.107
Theliderma cylindrica	—	0.01	—	—	—	—	—	—	—
Theliderma intermedia	—	0.003	—	—	—	—	—	—	—
Theliderma metanevra	0.009	0.005	0.090	0.095	0.091	0.093	0.094	0.090	0.086
Theliderma johnsoni	—	0.002	0.093	0.099	0.096	0.095	0.092	0.088	0.088
Theliderma sparsa	—	0.002	—	—	—	—	—	—	—
Tritogonia verrucosa	0.007	0.008	0.096	0.093	0.105	0.102	0.104	0.098	0.105
Tritogonia nobilis	0.009	0.011	0.105	0.107	0.118	0.108	0.101	0.106	0.103

Notes. Left: mean uncorrected *p*-distance within species for cytochrome oxidase subunit I (COI) and for NADH dehydrogenase, subunit 1 (ND1) genes. Right: mean uncorrected *p*-distance among species of COI (below the diagonal) and ND1 (above the diagonal) genes.

well-supported *Quadrula* sensu lato clade subdivided into four clades (mainly in the BI analyses), corresponding to the genera *Quadrula*, *Cyclonaias*, *Theliderma*, and *Tritogonia* (Figures 2–4; Table 3). Furthermore, taxa in these clades exhibit coherent combinations of traits that in our opinion support the validity of their generic status as recently recognized by Williams et al. (2017) (Figures 2–4; Tables 3 and 5, Supporting Information Table S5).

The current molecular phylogenies cannot strongly support any suprageneric relationships (probably due to insufficient genetic marker representation) within *Quadrula* s.l. However, the morphological and ecological data here presented suggest common evolutionary origins for the genera *Quadrula* and *Tritogonia*, and for *Cyclonaias* and *Theliderma* (Table 2; Supporting Information Table S5). While *Quadrula* and *Tritogonia* include large reflexive mantle displays, miniaturized shell glochidia, and marked shell sulci, *Cyclonaias* and *Theliderma* species have small non-reflexive mantle displays, larger glochidia, and absent or shallow shell sulci (Table 2; Supporting Information Table S5).

The series of traits shared by *Quadrula* and *Tritogonia* are likely associated with maximizing attachment success to their main hosts, the catfishes (Table 2). These traits include large conspicuous mantle displays that do not respond to mechanical disturbance (but probably to another type of stimulus, for example, chemical, that might capitalize on the acute olfactory sense of their hosts) and miniaturized glochidia. *Tritogonia* species are the only *Quadrula* s.l. species that exhibit marked shell sexual dimorphism. This is probably a result of the presence of mantle displays that completely cover the incurrent and excurrent apertures

Cyclonaias succissa	*Cyclonaias tuberculata*	*Quadrula quadrula*	*Theliderma cylindrica*	*Theliderma intermedia*	*Theliderma metanevra*	*Theliderma johnsoni*	*Theliderma sparsa*	*Tritogonia verrucosa*	*Tritogonia nobilis*
0.083	0.095	0.107	0.112	0.143	0.111	0.111	0.105	0.114	0.115
0.095	0.093	0.112	0.115	0.139	0.117	0.115	0.11	0.107	0.125
0.092	0.088	0.115	0.11	0.143	0.118	0.116	0.105	0.112	0.123
0.064	0.055	0.128	0.129	0.144	0.123	0.117	0.113	0.118	0.126
0.064	0.065	0.131	0.125	0.14	0.125	0.114	0.11	0.122	0.13
0.07	0.059	0.13	0.127	0.147	0.129	0.12	0.115	0.116	0.126
0.031	0.052	0.112	0.121	0.136	0.119	0.112	0.106	0.105	0.118
	0.053	0.114	0.124	0.144	0.129	0.118	0.113	0.11	0.119
0.053		0.117	0.127	0.146	0.126	0.126	0.113	0.116	0.121
0.100	0.097		0.108	0.141	0.122	0.109	0.112	0.11	0.11
—	—	—		0.106	0.088	0.082	0.079	0.122	0.126
—	—	—	—		0.084	0.076	0.073	0.135	0.137
0.096	0.083	0.102	—	—		0.035	0.042	0.117	0.129
0.094	0.085	0.094	—	—	0.032		0.036	0.109	0.121
—	—	—	—	—	—	—		0.105	0.106
0.100	0.098	0.114	—	—	0.096	0.097	—		0.093
0.099	0.114	0.110	—	—	0.115	0.107	—	0.085	

of females, resulting in a distortion of their shells (Table 2, Supporting Information Table S5). On the other hand, a specialization in attracting small cyprinids and percids seems to have driven reproductive behaviour and morphology in *Theliderma* towards females that are generally completely buried with only the mantle display being visible (Sietman, Davis, & Hove, 2012). The displays of *Theliderma* are also more conspicuously displayed during the day favouring the visual predatory habits of cyprinids, which is in contrast to the other three *Quadrula* s.l. genera who are generally displaying at night when feeding activity in catfishes is highest (Hove *et al.* 2011). *Theliderma* species are unique within Quadrulini in the production of mucoid conglutinates (Haag, 2012) and by presenting dark chevrons in the shells periostracum (Table 2; Supporting Information Table S5). The glochidia of *Theliderma* are also much bigger than those of *Tritogonia* and *Quadrula* and more similar in size to most of the other species within the Ambleminae (Table 2; Barnhart, Haag, & Roston, 2008). The large size of *Theliderma* glochidia can be related to the much lower fecundity of this genus when compared with the other *Quadrula* s.l. genera (Haag, 2012). *Cyclonaias* presents a set of reproductive features that are similar to those in *Theliderma* species. However, glochidial size in *Cyclonaias* is always larger than in *Theliderma,* and *Cyclonaias* exhibit a prevalence to catfish hosts rather than cyprinids and percids (Table 2). Adaptation to catfish hosts again is likely associated with the unique stomate-shaped mantle displays exhibited by *Cyclonaias* species (Table 2). The miniaturized glochidia shared by *Quadrula* and *Tritogonia* probably represent a derivation from the more primitive glochidial size of most amblemines (Barnhart et al., 2008). On the other hand, preference for and related adaptations to catfish hosts seem to be ancestral for the

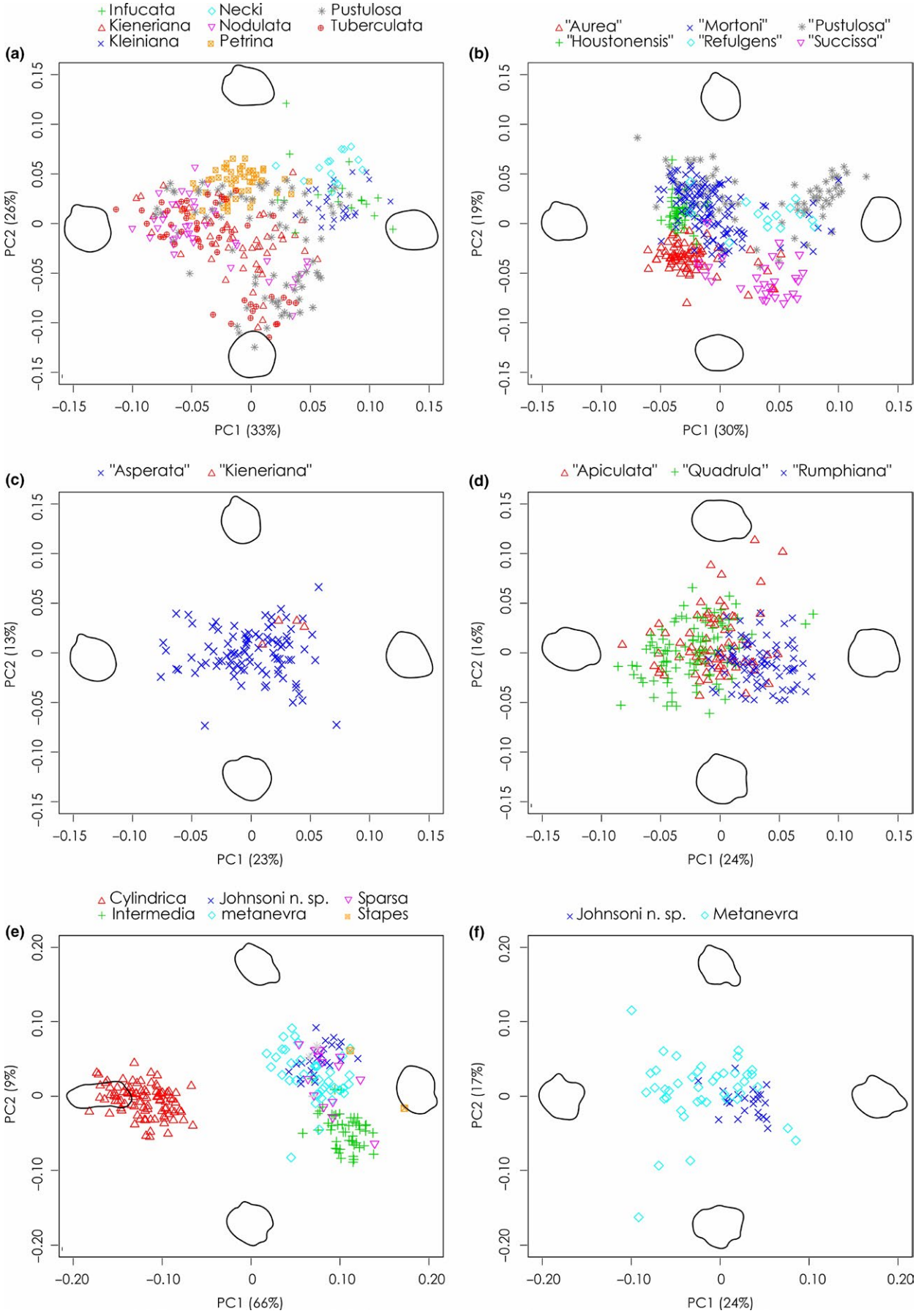
(a)
Infucata
Kieneriana
Kleiniana
Necki
Nodulata
Petrina
Pustulosa
Tuberculata
PC2 (26%)
PC1 (33%)
(b)
"Aurea"
"Houstonensis"
"Mortoni"
"Refulgens"
"Pustulosa"
"Succissa"
PC2 (19%)
PC1 (30%)
(c)
"Asperata"
"Kieneriana"
PC2 (13%)
PC1 (23%)
(d)
"Apiculata"
"Quadrula"
"Rumphiana"
PC2 (16%)
PC1 (24%)
(e)
Cylindrica
Intermedia
Johnsoni n. sp.
metanevra
Sparsa
Stapes
PC2 (9%)
PC1 (66%)
(f)
Johnsoni n. sp.
Metanevra
PC2 (17%)
PC1 (24%)

FIGURE 6 Shell outline principal component scores for the first two PC axes obtained from 18 Fourier coefficients of (a) all true species (recognized by molecular species delineation methods; see results) of *Cyclonaias*, including a maximum of 50 specimens per species; (b) all nominal species of *Cyclonaias pustulosa*; (c) only *Cyclonaias kieneriana* and *Cyclonaias asperata*; (d) all nominal species of *Quadrula*; (e) all true species (recognized by molecular species delineation methods; see results) of *Theliderma*; and (f) only *Theliderma metanevra* and *Theliderma johnsoni* n. sp. Synthetic shell outlines of "extreme" morphotypes are displayed with the anterior margin facing to the left and the dorsal margin to the top of the page [Colour figure can be viewed at wileyonlinelibrary.com]

Quadrulini, while preference for small cyprinids and percids in *Theliderma* is probably the derived state. A more robust multi-marker molecular approach is needed in order to get a clearer view on the evolutionary aspects of these interesting adaptations and to resolve the suprageneric relationships among *Quadrula* s.l. genera.

4.2 | Phylogeny and systematics implications within the four *Quadrula* sensu lato genera

4.2.1 | Cyclonaias

The present results confirm the results of a recent study on this genus (Johnson et al., 2018) recognizing nine of the 14 *Cyclonaias* species listed by Williams et al. (2017) as valid species (Table 7). However, we here consider *C. asperata* as a synonym of *C. kieneriana* due to the residual genetic divergence between these two taxa (ND1 *p*-distance <1%) and the fact that *C. kieneriana* (Lea, 1852) has priority over *C. asperata* (Lea, 1861). In contrast, Williams et al. (2017) recognized both species based on their morphological distinctiveness and the fact that molecular evidence for synonymy was based on only one marker (ND1) from a single specimen. However, ND1 has been shown to be a highly representative marker of overall mtDNA evolution in unionoid mussels (Fonseca, Lopes-Lima, Eackles, King, & Froufe, 2016). In addition, divergence between *C. asperata* and *C. kieneriana* sequences was very low. As a result, both ND1 (BI and ML) analyses were unable to resolve both species' phylogenies, and all ND1 species delineation methods were unable to separate the two species (Table 5), indicating that *C. asperata* should be synonymized under *C. kieneriana*. The morphometry results supported the distinct morphology of the two nominal species but very few *C. kieneriana* shells ($n = 4$) were available, preventing a comprehensive analysis (Figure 6c). Although *C. asperata* has been reported from a much wider geographic range than *C. kieneriana*, both species are sympatric in the whole range of *C. kieneriana* suggesting that specimens previously described as *C. kieneriana* are particular smooth forms of the same species (Figure 7).

Until recently, *Cyclonaias archeri* has been considered a subspecies of *C. asperata* (Turgeon et al., 1998). However, since no sequences, tissues or shell specimens of *C. archeri* were available for this study, we rely on Williams et al. (2008, 2017) and recognize this species as separate from *C. asperata*, based on its distinct morphology.

Cyclonaias necki has recently been separated from *Cyclonaias petrina* based on molecular data (COI) and morphology (Burlakova, Karatayev, Froufe, Bogan, & Lopes-Lima, 2018; Johnson et al., 2018). The specific rank of *C. necki* is here confirmed by all species delineation methods used on each of the datasets (Table 5). The shell shape is also significantly different between *C. petrina* and *C. necki* (Figure 6a), confirming observations of Burlakova et al. (2018) and Johnson et al. (2018) that *C. necki* shells are thinner, more compressed and more rectangular in shape with a more distinct and prominent posterior ridge. Distribution ranges of the two species are exclusive, with *C. necki* being present only in the San Antonio/Guadalupe River basins, while *C. petrina* is restricted to the Colorado basin (Figure 8; Burlakova et al., 2018).

The present paper confirms the inclusion of four nominal species, that is, *C. aurea, C. houstonensis*, *C. mortoni*, *C. refulgens*, within *C. pustulosa* (Table 7) and *Cyclonaias succissa*, as a related but distinct species, as proposed by Johnson et al. (2018). None of the phylogenies resolved them as monophyletic, and *p*-distance values among these taxa were very low (Table 4). All nominal species here synonymized with *C. pustulosa* have distinct and exclusive geographic distributions (Figure 9). The molecular results suggest that *C. pustulosa* is divided into several morphotypes each in a distinct geographic area. These morphotypes are clearly visible in the morphometry results and explain why these populations used to be considered distinct species (Figure 7b).

The remaining *Cyclonaias* species recognized in the present study, that is, *C. infucata*, *C. kleiniana*, *C. kieneriana*, *Cyclonaias nodulata* and *Cyclonaias tuberculata*, were always retrieved as well supported, divergent clades (Figures 2–4), and recognized by all species delineation methods (Table 5). Furthermore, the shell shape is different among all of these latter species, except for the pair *C. infucata* and *C. kleiniana*, which might be explained by their closer genetic relationship (Figures 2–4; Table 6).

4.2.2 | Quadrula

In the absence of genetic data and shell materials for *Quadrula couchiana* and *Quadrula fragosa*, the first being most likely extinct (Williams et al., 2017) and the second on the verge of extinction (Sietman, 2003), we make no considerations about their systematics and accept both as valid within the *Quadrula* genus following Williams et al. (2017).

TABLE 7 Historical classification of species formerly assigned to *Quadrula*. * extinct.

Haas (1969a)	Graf & Cummings (2007)	Bogan (2013)	Williams *et al.* (2017)	This study
Quadrula				
Quadrula (Quadrula) quadrula quadrula	*Quadrula quadrula*	*Quadrula quadrula*	*Quadrula quadrula*	1. *Quadrula quadrula*
Quadrula (Quadrula) quadrula apiculata	*Quadrula apiculata*	*Quadrula apiculata*	*Quadrula apiculata*	+ *Quadrula apiculata*
Quadrula (Quadrula) quadrula rumphiana	*Quadrula rumphiana*	*Quadrula rumphiana*	*Quadrula rumphiana*	+ *Quadrula rumphiana*
Quadrula (Pustulosa) couchiana	*Amphinaias couchiana*	*Quadrula couchiana*	*Quadrula couchiana*	2. *Quadrula couchiana**
Quadrula (s.s.) quadrula fragosa	*Quadrula fragosa*	*Quadrula fragosa*	*Quadrula fragosa*	3. *Quadrula fragosa*
Cyclonaias				
Quadrula (Pustulosa) pustulosa keineriana [sic]		*Quadrula kieneriana*	*Cyclonaias kieneriana*	1. *Cyclonaias kieneriana*
Quadrula (Pustulosa) pustulosa pernodosa	*Amphinaias asperata*	*Quadrula asperata*	*Cyclonaias asperata*	+ *Cyclonaias asperata*
Fusconaia succissa succissa	*Quicucina infucata*	*Quadrula infucata*	*Cyclonaias infucata*	2. *Cyclonaias infucata*
Quincuncina securiformis kleiniana		*Quadrula kleiniana*	*Cyclonaias kleiniana*	3. *Cyclonaias kleiniana*
Quadrula (Pustulosa) archeri	*Amphinaias archeri*		*Cyclonaias archeri*	4. *Cyclonaias archeri*
Quadrula (Pustulosa) nodulata	*Amphinaias nodulata*	*Quadrula nodulata*	*Cyclonaias nodulata*	5. *Cyclonaias nodulata*
Quadrula (Pustulosa) petrina	*Amphinaias petrina*	*Quadrula petrina*	*Cyclonaias petrina*	6. *Cyclonaias petrina*
				7. *Cyclonaias necki*
Quadrula (Pustulosa) pustulosa pustulosa	*Amphinaias pustulosa*	*Quadrula pustulosa*	*Cyclonaias pustulosa*	8. *Cyclonaias pustulosa*
Quadrula (Pustulosa) aurea	*Amphinaias aurea*	*Quadrula aurea*	*Cyclonaias aurea*	+ *Cyclonaias aurea*
	Amphinaias houstonensis	*Quadrula houstonensis*	*Cyclonaias houstonensis*	+ *Cyclonaias houstonensis*
Quadrula (Pustulosa) pustulosa mortoni		*Quadrula mortoni*	*Cyclonaias mortoni*	+ *Cyclonaias mortoni*
Quadrula (Pustulosa) pustulosa refulgens	*Amphinaias refulgens*	*Quadrula refulgens*	*Cyclonaias refulgens*	+ *Cyclonaias refulgens*
Fusconaia succissa succissa	*Fusconaia succissa*	*Quadrula succissa*	*Cyclonaias succissa*	9. *Cyclonaias succissa*
Cyclonaias tuberculata tuberculata	*Cyclonaias tuberculata*	*Cyclonaias tuberculata*	*Cyclonaias tuberculata*	10. *Cyclonaias tuberculata*
Theliderma				
Orthonymus cylindricus	*Theliderma cylindrica*	*Quadrula cylindrica*	*Theliderma cylindrica*	1. *Theliderma cylindrica*
Orthonymus intermedius	*Theliderma intermedia*	*Quadrula intermedia*	*Theliderma intermedia*	2. *Theliderma intermedia*
Orthonymus metanevrus metanevrus	*Theliderma metanevra*	*Quadrula metanevra*	*Theliderma metanevra*	3. *Theliderma metanevra*
Orthonymus metanevrus tuberosus	*Theliderma tuberosa*			
				4. *Theliderma johnsoni* n. sp.
	Theliderma sparsa	*Quadrula sparsa*	*Theliderma sparsa*	5. *Theliderma sparsa*
	Theliderma stapes			6. *Theliderma stapes*
Tritogonia				
Tritogonia verrucosa	*Tritogonia verrucosa*	Quadrula verrucosa	*Tritogonia verrucosa*	1. *Tritogonia verrucosa*
Quadrula (Quadrula) quadrula nobilis	*Quadrula nobilis*	*Quadrula nobilis*	*Quadrula nobilis*	2. *Tritogonia nobilis*

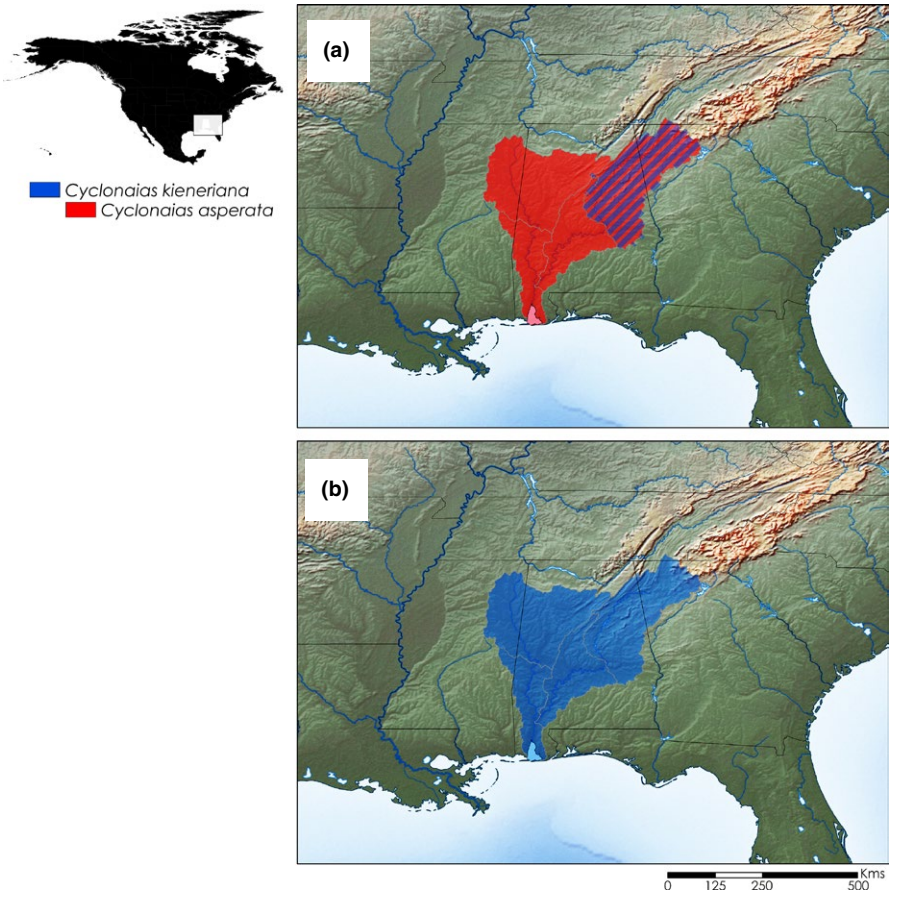

FIGURE 7 Distribution maps of (a) nominal species *Cyclonaias asperata* and *Cyclonaias kieneriana* before the present study and (b) of *C. kieneriana* as proposed in the present study [Colour figure can be viewed at wileyonlinelibrary.com]

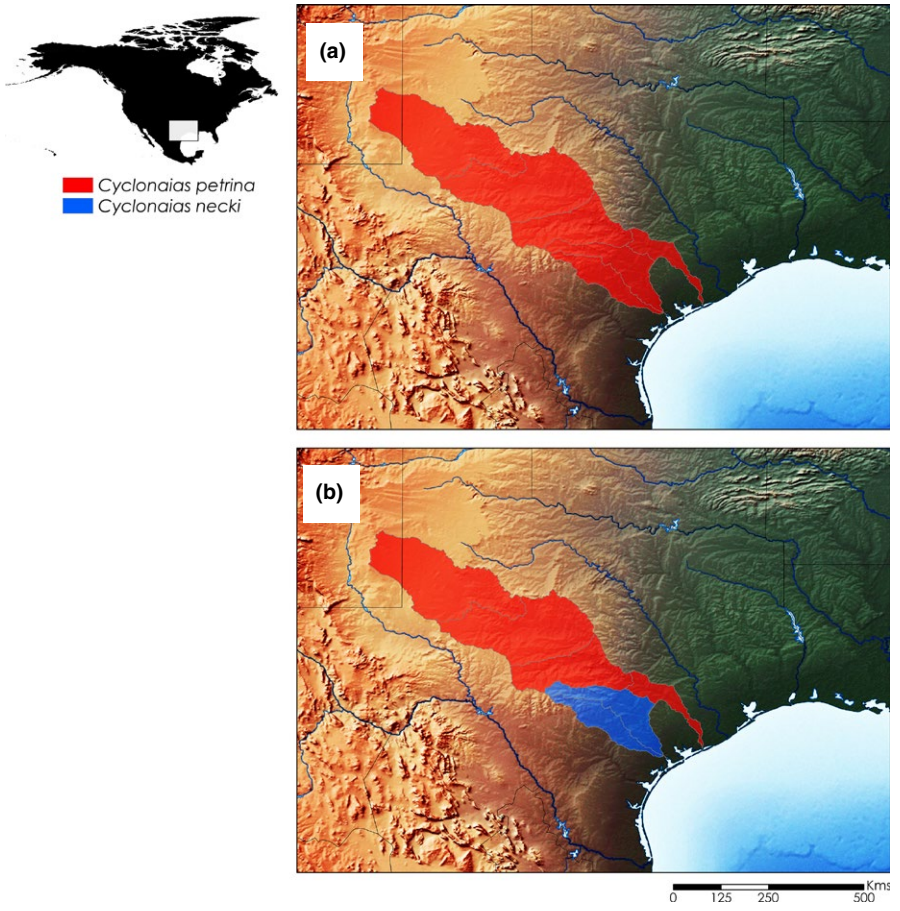

FIGURE 8 Distribution maps of (a) *Cyclonaias petrina* before Burlakova et al. (2018) and (b) of *C. petrina* and *Cyclonaias necki* after Burlakova et al. (2018) and Johnson et al. (2018) findings also supported by the present study [Colour figure can be viewed at wileyonlinelibrary.com]

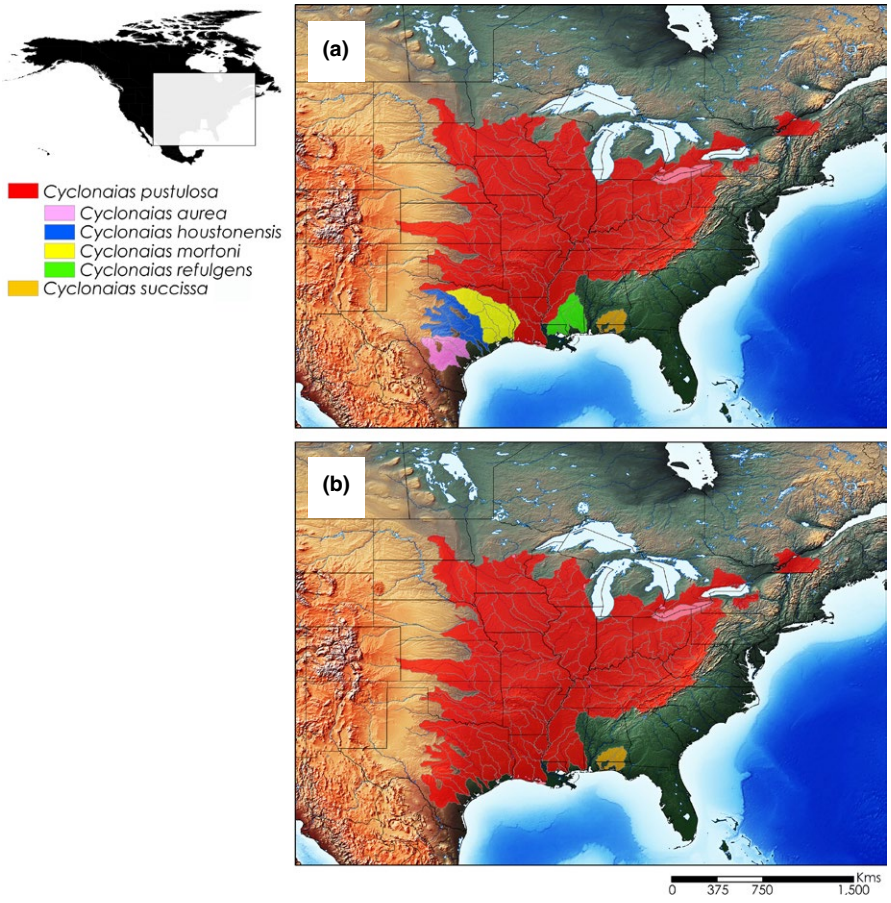

FIGURE 9 Distribution maps of (a) nominal species within the *Cyclonaias pustulosa* group and (b) of *C. pustulosa* and *Cyclonaias succissa* as confirmed by Johnson et al. (2018) and the present study [Colour figure can be viewed at wileyonlinelibrary.com]

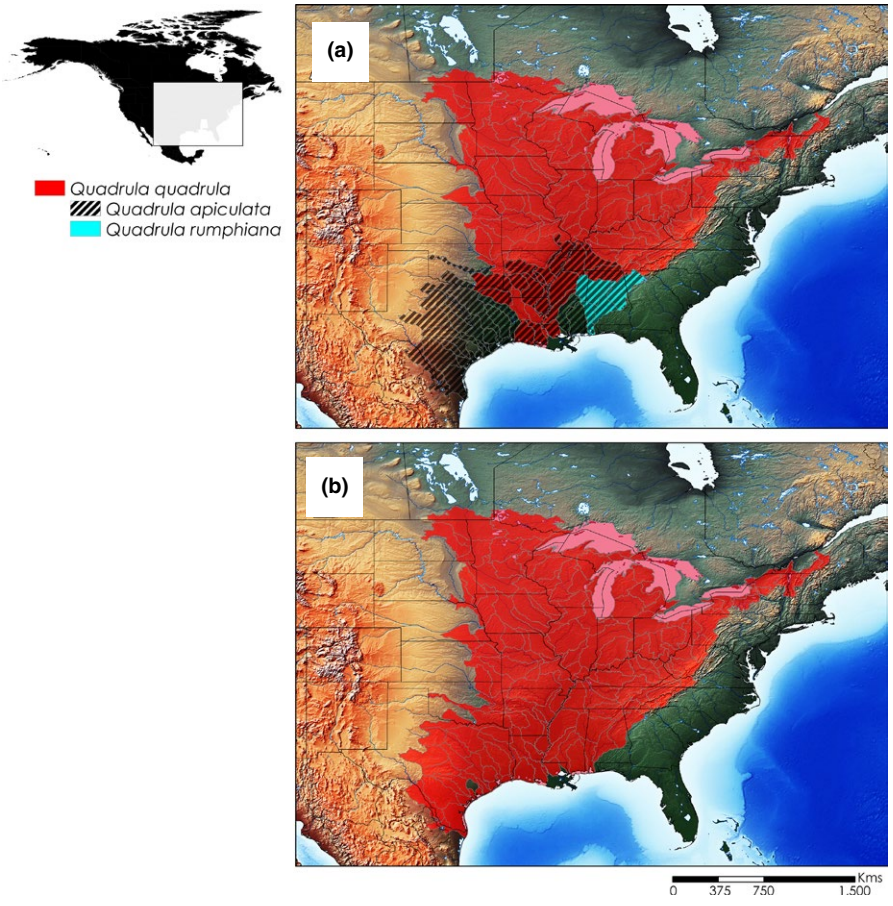

FIGURE 10 Distribution maps of (a) nominal species within the *Quadrula quadrula* group and (b) of *Quadrula quadrula* as proposed in the present study [Colour figure can be viewed at wileyonlinelibrary.com]

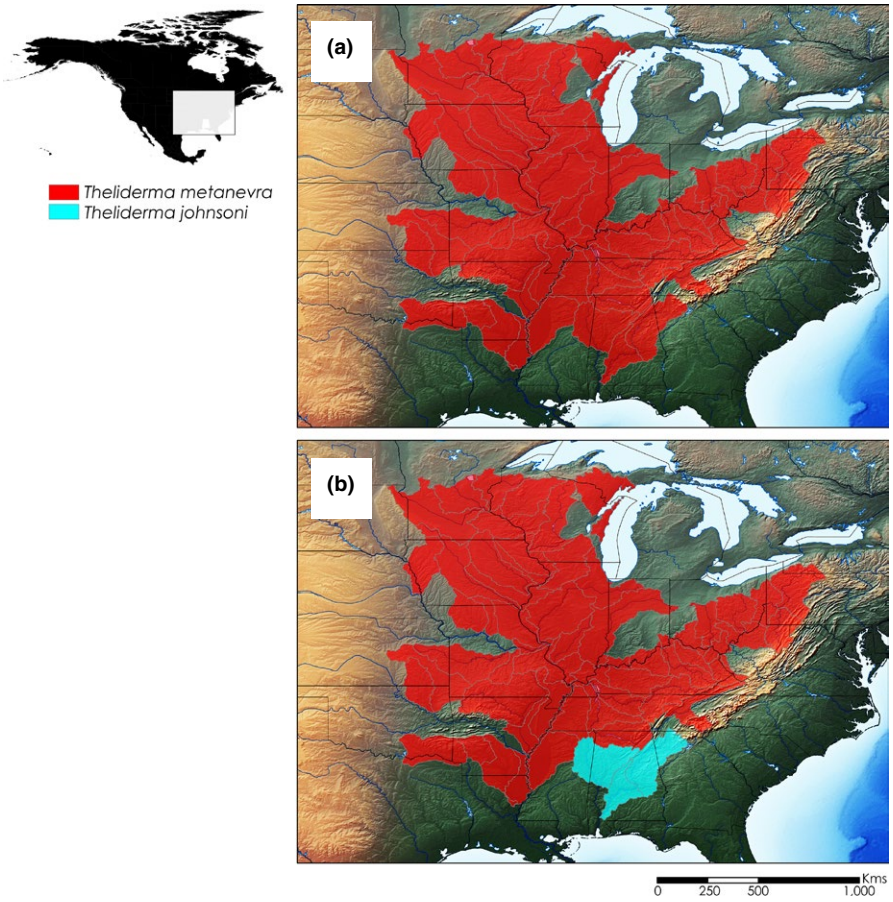

FIGURE 11 Distribution maps of (a) *Theliderma metanevra* before the present study and (b) after the present study divided in *T. metanevra* and *Theliderma johnsoni* n. sp [Colour figure can be viewed at wileyonlinelibrary.com]

We here synonymize *Q. apiculata* and *Q. rumphiana* under *Q. quadrula*. Although only a small number of sequences were available for *Q. apiculata* and *Q. rumphiana*, the level of divergence among these three nominal species is low for both markers (Table 4). Furthermore, in all phylogenies, *Q. quadrula* is paraphyletic, with *Q. apiculata* and *Q. rumphiana* falling inside the clade (Figures 2–4). The level of divergence between these three nominal taxa is actually lower than the divergence between the distinct clades of COI within *Q. quadrula* sensu stricto identified by Mathias, Hoffman, Wilson, and Zanatta (2018) and also retrieved here in the COI phylogeny and haplotype network (Figures 2 and 6a). A specific rank for each of these divergent clades was rejected in that study due to the existence of gene-flow among them as shown by their microsatellite dataset (Mathias et al., 2018). The nominal species *Q. apiculata*, *Q. rumphiana* and *Q. quadrula* sensu stricto presented distinct shell shapes but only 76% of specimens were assigned to the correct nominal species (Figure 6d). The slightly distinct shell morphology again suggests that distinct nominal species were assigned to regional forms despite the relative overlap in distribution range of *Q. apiculata* with both *Q. quadrula* and *Q. rumphiana* (Figure 10) that may also be related to the considerable overlap among shell shape forms (Figure 6d).

4.2.3 | Theliderma

Only two shells and no genetic material were available for *T. stapes*, since the species is very endangered and possibly extinct (NatureServe, 2018). Until new evidence emerges, we therefore accept it as valid within the *Theliderma* genus following Williams et al. (2017). Based on the molecular phylogenies and all species delineation methods, we recognize five additional species within *Theliderma*, that is, *T. cylindrica*, *Theliderma intermedia*, *T. metanevra*, *T. johnsoni* n. sp. and *T. sparsa* (Figures 2–4; Tables 1 and 5). The nominal species *T. metanevra* is here divided in two distinct species, the *T. metanevra* sensu stricto with a Mississippi basin distribution and *T. johnsoni* n. sp. distributed within the Mobile basin (Figure 11). The two species show high genetic divergence (3.2% for COI and 3.5% for ND1; Table 6). They also differ morphologically, presenting distinct shell shape with only 5%–11% of specimens being misidentified by Fourier analysis (Figure 6e,f) as well as other morphological features (see Supporting Information Appendix S2).

4.2.4 | Tritogonia

The position of *T. nobilis* could not be resolved in a previous single marker approach (Serb et al., 2003) but in the

present study, all phylogenies reveal a well-supported clade comprising *T. nobilis* and *T. verrucosa*. We therefore move the nominal species *Q. nobilis* into *Tritogonia* as *T. nobilis*. Until the end of the 20th century, *T. nobilis* was not recognized by most authors as a separate species from *Q. quadrula* (Williams et al., 2008). However, its placement under *Tritogonia* is not new as Simpson (1914) already used this designation. Both *T. nobilis* and *T. verrucosa* exhibit marked sexual dimorphism (Simpson, 1914; Williams et al., 2008), which is a synapomorphy of the genera within the Quadrulini.

4.3 | Conservation implications

4.3.1 | Cyclonaias

As *C. asperata* is here synonymized under *C. kieneriana*, future conservation status assessment of *C. kieneriana* should include the distribution of *C. asperata* sensu stricto (Figure 7), which would be expected to decrease the extinction risk of the species under the currently recognized systematics. The separation of *C. necki* from *C. petrina* will likely increase the extinction risk of both species as their distributions are even smaller than previously believed (Figure 8) but see Johnson et al. (2018) for detailed conservation implications. In contrast, the secure conservation status of *C. pustulosa* (Supporting Information Table S6) is here strengthened by the inclusion of the nominal taxa *C. aurea*, *C. houstonensis*, *C. mortoni* and *C. refulgens* (Figure 9; Table 7). However, due to their genetic uniqueness, the populations from Eastern Texas (originally identified as *C. mortoni*) should be managed independently.

4.3.2 | Quadrula

Synonymization of the nominal species *Q. rumphiana* and *Q. apiculata* under *Q. quadrula* does not affect the conservation status of *Q. quadrula* due the wide distribution areas and low extinction risk of the three forms. That said, subtler potential genetic differences between populations originally assigned to these species are likely to be revealed in future studies applying faster evolving markers.

4.3.3 | Theliderma

The conservation status of *T. metanevra* is currently considered as secure mainly based on the species' wide distribution range. However, considering that the Mobile basin populations in fact represent a separate species (Figure 11), *T. johnsoni* n. sp., the conservation statuses of *T. metanevra* and *T. johnsoni* n. sp. need to be re-assessed separately, and the two species need to be managed independently.

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SUPPORTING INFORMATION

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

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