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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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Running title: Phylogeny of freshwater mussel genus *Quadrula sensu lato*
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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*, i.e. *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. 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, i.e. *Theliderma johnsoni* n. sp.. The conservation implications of the proposed changes are then discussed.

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Introduction

Conservation programs and strategies are largely based on species as conservation units, making species delineation extremely important as a basic conservation tool (Prié *et al.* 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*. 2018). However, polyphyly and inappropriate species boundaries have been revealed in some of these tribes, including the Quadrulini (Lydeard *et al*. 2000; Serb *et al*. 2003, Pfeiffer *et al*. 2016). 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 Supplementary Appendix 1 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, i.e. the *Quadrula sensu stricto*, the *pustulosa*, the *metanevra*, and the *Tritogonia* species groups (Supplementary Appendix 1). 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 (e.g., Parmalee & Bogan 1998; Williams *et al*. 2008; Howells 2013) 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.

Materials & Methods

Sample collection and materials examined

Specimens of quadruline mussels were collected from 50 sites across the state of Texas during 2003-2011 (Fig. 1). A total of 89 specimens were collected and placed in 99% ethanol for molecular analyses. Voucher specimens were labeled 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 Supplementary Table 1 for the examined lot numbers).

Sequencing, alignments and phylogenetic analyses

31 quadruline specimens, including all nominal taxa across the state of Texas, were selected for molecular analyses (Table 2). 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 50ºC 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 2 and Supplementary Tables 2 and 3).

Three datasets were constructed: 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 (Supplementary Tables 2, 3 and 4). The COI+ND1 dataset included all individuals sequenced for both COI and ND1 plus GenBank Quadrulini specimens with sequences available for the two genes (Supplementary Table 4). For each of the three datasets, sequences of additional specimens were downloaded from Genbank and/or newly sequenced as outgroup (details in Supplementary Tables 2, 3, and 4). 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 *et al*. 2012) under the Bayesian information criterion. For each indidivual 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 *et al*. 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 *et al*. 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 analyzed in RaxML 8.2.10 HPC Black Box (Stamatakis 2014) with 1000 bootstrap replicates. Haplotype networks were calculated using TCS 1.21 (Clement *et al*. 2000) with a threshold of 95 %.

Molecular based species deliniation 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 concatenated (COI + ND1) datasets, with the exception of the BIN system that relies only on COI. The first two are distance based, i.e. 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 outgoups was analyzed with the Cluster Sequences tool implemented in BOLD 4 (http://v4.boldsystems.org) (Ratnasingham & Hebert 2013). The Automatic Barcode Gap Discovery (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 (K2P) distance matrix (Puillandre *et al*. 2012).

Two tree-based molecular species deliniation methods were applied to all datasets, i.e, the single threshold Generalized Mixed Yule Coalescent (GMYC) model (Fujisawa & Barraclough 2013) and the Bayesian implementation of the Poisson Tree Processes model (bPTP) (Zhang *et al*. 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 x 10^6 Markov chain Monte Carlo (MCMC) generations, sampled every 1 x 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 'SPLITS' (Species Limits by Threshold Statistics; Ezard *et al*. 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 x 10^6 iterations of Markov chain Monte Carlo (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 *et al*. 2016).

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 & Haines (1996). 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.; Supplementary Table 1) were obtained from digital photographs using the program IMAGEJ (Rasband 2008) and subjected to fast Fourier transformation using the program HANGLE, applying a smoothing normalisation 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 (PCA) was performed on the 18 Fourier coefficients of (A) all true species (recognized by the molecular species deliniation 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 deliniation methods, see results) of *Theliderma*; and (F) only *Theliderma metanevra* and *Theliderma johnsoni* n. sp. (see Supplementary Appendix 2 for a detailed description of *Theliderma johnsoni* n. sp.). Synthetic outlines of extreme and average shell shapes were drawn using program HCURVE as explained in Crampton & 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 posthoc 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).

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 3; Supplementary Table 5)

Results

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 1192 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 4), thus only BI phylogenetic trees are shown in Figs. 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 (Fig. 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, i.e. Amblemini, Pleurobemini, and Lampsilini clustering within the Quadrulini tribe clade (Fig. 3). The *Uniomerus* clade is sister to a clade containing the four remaining Quadrulini genera (i.e., *Quadrula*, *Tritogonia*, *Theliderma*, and

Cyclonaias) (Fig. 3). While *Cyclonaias*, *Quadrula*, and *Tritogonia* are well supported, *Theliderma* has a low support value (Fig. 3). The COI + ND1 phylogeny shows Quadrulini as monophyletic with *Uniomerus* being sister to a clade comprising four well supported clades (*Quadrula*, *Tritogonia*, *Theliderma* and *Cyclonaias*) (Fig. 4).

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

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

Theliderma. Not many COI sequences of *Theliderma* are represented in the COI dataset, and therefore in the COI and COI + ND1 phylogenies (Figs. 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 (Figs. 2 and 4). The ND1 phylogeny is better represented with all species recognized to date except for *T. stapes* (Fig. 3).

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

Genetic divergence and Species delineation methods

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 5).

Of the 14 putative *Cyclonaias* species, only 9 were recognized as Molecular Operational Taxonomic Units (MOTUs) based on a consensus of all species delineation methods, applied on the COI, ND1 and COI+ND1 datasets (Table 6). 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 7). The uncorrected *p*-distance within each of the recognized MOTUs was ≤1.2% for COI and ≤1.6% for ND1 (Table 7).

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 5). Taking into account the three datasets, only a single MOTU was consensually recognized for the *Quadrula* genus (Table 6) with a within *p*-distance value of 1.7% for COI and 1.9% for ND1 (Table 7).

Theliderma. The pairwise uncorrected *p*-distance among all the nominal *Theliderma* species ranged between 4.0% and 10.6% for ND1 (Table 5). The higher within *p*-distance recorded value was reached for *Theliderma metanevra*, 1.7% for COI and 2.1% for ND1 (Table 5).

All originally described *Theliderma* species are here recognized as MOTUs with *T. metanevra* being further divided in two distinct MOTUs, i.e. *T. metanevra* for specimens from the Tennessee River basin and *T. johnsoni* n. sp. from the Mobile River basin (Table 6). The *p*-distance values among the recognized *Theliderma* MOTUs varied between 3.5% and 10.1% for ND1 (Table 7). The *p*-distance within each of the recognized MOTUs was ≤0.9% for ND1 (Table 7).

Tritogonia. Our analyses revealed a complete consensus of two individual MOTUs within the *Tritogonia* genus (Table 6). The two recognized MOTUs *T. verrucosa* and *T. 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 7).

Morphometry

Cyclonaias. LDA 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 (Fig. 6A). Species that are particularly difficult to separate by shell shape are *C. kieneriana* and *C. pustulosa* (16% misidentifications), and *C. 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 *C. 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 identifications (Fig. 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 *C. 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 (Fig. 6C).

Quadrula. Fourier coefficients differed significantly between the nominal species of *Quadrula* (MANOVA, pairwise Hotelling's tests $P<0.05$) (Fig. 6C). Seventy six percent of specimens were assigned to the correct nominal species, with 21% and 11% of misidentifications between *Q. apiculata* vs. *Q. quadrula* and *Q. rumphiana,* respectively.

Theliderma. Within the genus *Theliderma,* 91% of specimens were identified to the correct species (as they are here recognized) by LDA of Fourier coefficients (Fig. 6E). *T. cylindrica,* characterised by its typical elongated-rectangular shape, was 100% correctly identified. Considerable difficulties in separation by shell shape were present for *T. sparsa* vs. *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* vs. *T. johnsoni* n. sp. (MANOVA, pairwise Hotelling's test: P=0.525), *T. sparsa* vs. *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 two *T. metanevra*/*T. johnsoni* n. sp. were assigned to the wrong clade (Fig. 6E). When using only the *T. metanevra* dataset, 11% of specimens were misidentified (Fig. 6F), though Fourier coefficients were significantly different between the two species (MANOVA: $F_{18,46}$=3.097, P=0.001).

Diagnostic characters of the classical genera within Quadrula s.l.

Species within *Quadrula* and *Tritogonia* share a number of ecological and morphological traits but distinct from those within *Cyclonaias* and *Theliderma* (Table 3; Supplementary Table 5). *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 3; Supplementary Table 5). *Quadrula* and *Tritogonia* glochidial size index is ten times smaller than in *Cyclonaias* and *Theliderma* (Table 3; Supplementary Table 5). *Quadrula* and *Tritogonia* also seem to share similar morphological and behavioral 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 3; Supplementary Table 5). 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 3; Supplementary Table 5).

The presence of dark chevrons imprinted in the periostracum of shells is exclusive of *Theliderma* species and can be used to recognize the genus within Quadrulini (Table 3; Supplementary Table 5).

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 3; Supplementary Table 5). The mantle displays and glochidia of *Theliderma* are smaller than those of *Cyclonaias* (Table 3; Supplementary Table 5).

Tritogonia verrucosa and *Tritogonia nobilis* are sexually dimorphic in shell shape, this trait is unique within the Quadrulini and therefore diagnostic of the genus (Table 3; Supplementary Table 5). In addition, the mantle display mechanism of *Tritogonia 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 (Supplementary Table 5). However, this trait needs to be verified for *Tritogonia nobilis* in order to be considered diagnostic of the genus.

Discussion

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 well-supported *Quadrula sensu lato* clade subdivided into four clades (mainly in the BI analyses), corresponding to the genera *Quadrula*, *Cyclonaias*, *Theliderma*, and *Tritogonia* (Figs. 2-4; Table 4). 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) (Figs. 2-4; Table 4, Table 6, Supplementary Table 5).

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 3; Supplementary Table 5). 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 3; Supplementary Table 5).

The series of traits shared by *Quadrula* and *Tritogonia* are likely associated with maximising attachment success to their main hosts, the catfishes (Table 3). These traits include large conspicuous mantle displays that do not respond to mechanical disturbance (but probably to another type of stimulus, e.g. 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 of females, resulting in a distortion of their shells (Table 3, Supplementary Table 5). 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 *et al*. 2011). 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 3; Supplementary Table 5). 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 3; Barnhart *et al*. 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 3). Adaptation to catfish hosts again is likely associated with the unique stomate shaped mantle displays exhibited by *Cyclonaias* species (Table 3). The miniaturized glochidia shared by *Quadrula* and *Tritogonia* seem to reveal this trait to be derived from the more classical 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 Quadrulini, whilst 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.

Phylogeny and systematics implications within the four Quadrula sensu lato genera

Cyclonaias. The present results, confirm the results of a recent study on this genus (Johnson et al. 2018) recognizing 9 of the 14 *Cyclonaias* species listed by Williams *et al*. (2017) as valid species (Table 1). 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 *et al*. 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 both species (Table 6), 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 (Fig. 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 (Fig. 7).

Until recently, *Cyclonaias archeri* has been considered a subspecies of *C. asperata* (e.g. Turgeon *et al*. 1998). However, since no sequences, tissues, or shell specimens of *Cyclonaias 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 *et al*. 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 6). The shell shape is also significantly different between *C. petrina* and *C. necki* (Fig. 6A), confirming observations of Burlakova *et al*. (2018) and Johnson *et al*. (2018) that *Cyclonaias 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 (Fig. 8; Burlakova *et al*. 2018).

The present paper confirms the inclusion of 4 nominal species, i.e. *C. aurea*, *C. houstonensis*, *C. mortoni*, *C. refulgens*, within *C. pustulosa* (Table 1) and *C. 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 5). All nominal species here synonymized with *C. pustulosa* have distinct and exclusive geographic distributions (Fig. 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 (Fig 7b).

The remaining *Cyclonaias* species recognized in the present study, i.e. *C. infucata*, *C. kleiniana*, *C. kieneriana*, *C. nodulata*, and *C. tuberculata*, were always retrieved as well supported, divergent clades (Figs 2-4), and recognized by all species delineation methods (Table 6). 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 (Figs 2-4; Table 7).

Quadrula. In the absence of genetic data and shell materials for *Quadrula couchiana* and *Q. 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).

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 5). Furthermore, in all phylogenies, *Q. quadrula* is paraphyletic, with *Q.*

apiculata and *Q. rumphiana* falling inside the clade (Figs 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 *et al*. (2018) and also retrieved here in the COI phylogeny and haplotype network (Fig. 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 (Fig. 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* (Fig. 10) that may also be related to the considerable overlap among shell shape forms (Fig. 6D).

Theliderma. Only two shells and no genetic material were available for *Theliderma 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*, i.e. *T. cylindrica, T. intermedia, T. metanevra, T. johnsoni* n. sp., and *T. sparsa* (Figs. 2-4; Tables 1 and 5). The nominal species *Theliderma 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 (Fig. 11). The two species show high genetic divergence (3.2% for COI and 3.5% for ND1; Table 7). They also differ morphologically, presenting distinct shell shape with only 5 to 11% of specimens being misidentified by Fourier analysis (Figs. 6E and 6F) as well as other morphological features (see Supplementary Appendix 2).

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 *Quadrula nobilis* into *Tritogonia* as *Tritogonia 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.

Conservation implications

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* (Fig. 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 (Fig. 8) but see Johnson *et al*. (2018) for detailed conservation implications. In contrast, the secure conservation status of *Cyclonaias pustulosa* (Supplementary Table 6) is here strengthened by the inclusion of the nominal taxa *C. aurea, C. houstonensis, C. mortoni, and C. refulgens* (Fig. 9; Table 1). However, due to their genetic uniqueness, the populations from Eastern Texas (originally identified as *C. mortoni*) should be managed independently.

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.

Theliderma. The conservation status of *Theliderma 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 (Fig. 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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Figure legends

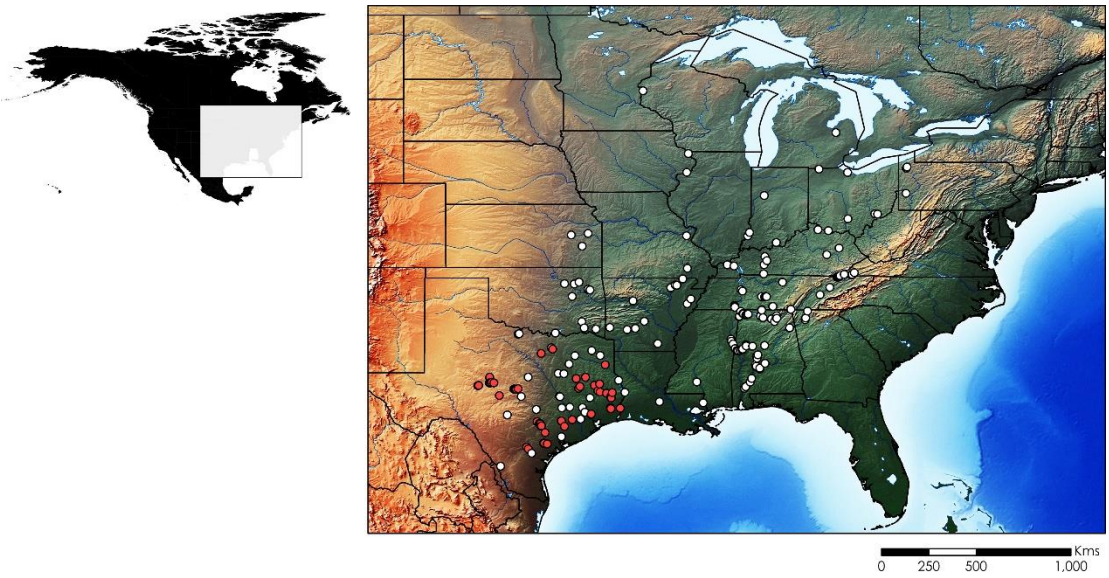

Figure 1. Map of all sampling sites for the present study; both tissue and shell materials in red; only shell materials in white.

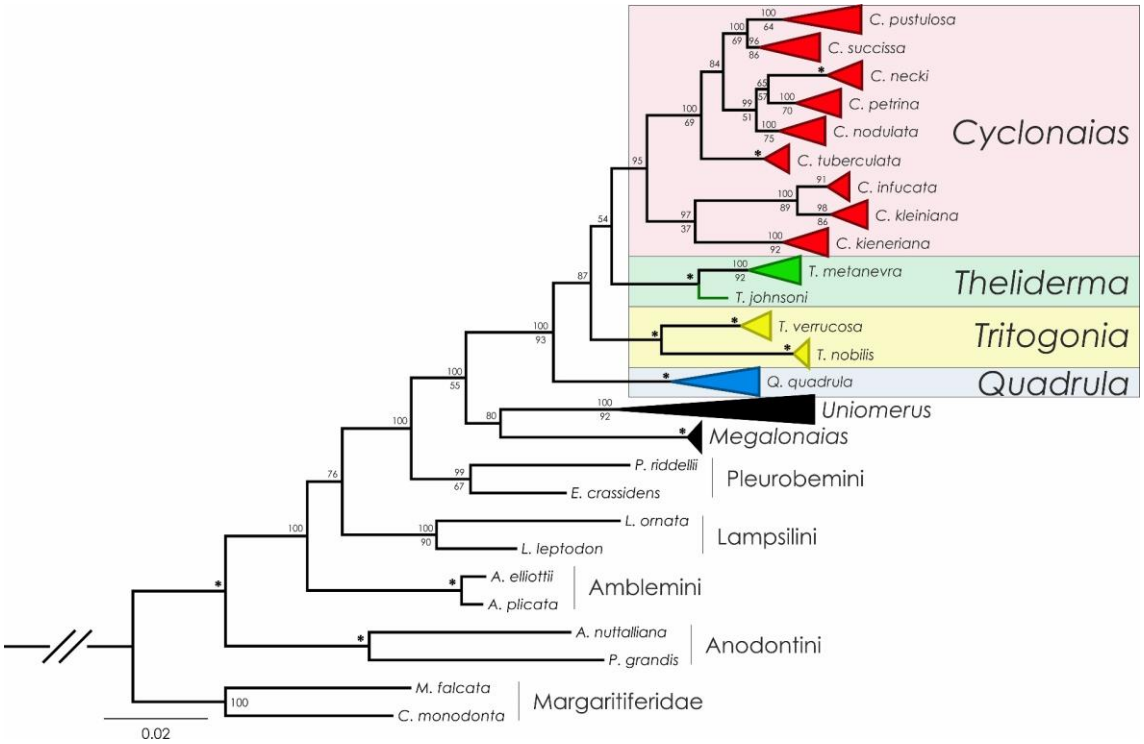

Figure 2. Bayesian consensus tree inferred from the cytochrome c oxidase subunit I (COI) gene fragment. The values above and below the nodes indicate Bayesian posterior probability (bpp) percentage and Maximum Likelihood bootstrap values (bs), respectively. Values over 95% are represented by an asterisk, and those <50% were erased for clarity.

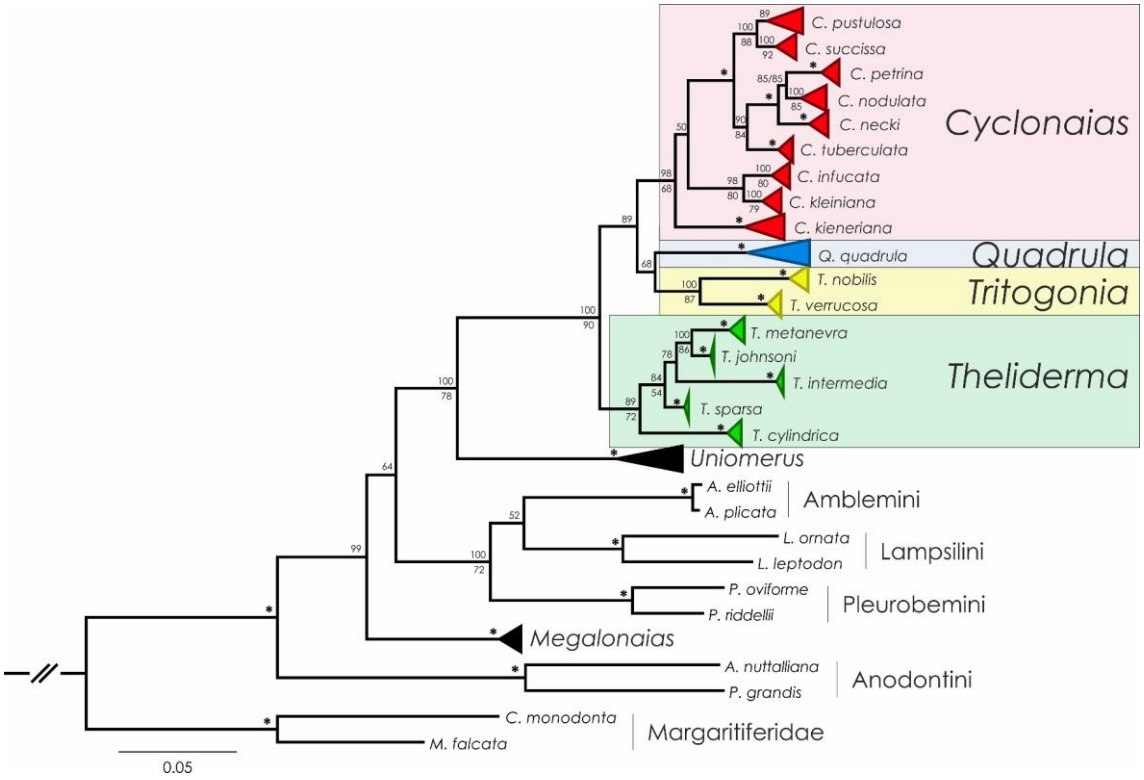

Figure 3. Bayesian consensus tree inferred from the NADH dehydrogenase subunit 1 (ND1) gene fragment. The values above and below the nodes indicate Bayesian posterior probability (bpp) percentage and Maximum Likelihood bootstrap values (bs), respectively. Values over 95% are represented by an asterisk, values below 50% were erased for clarity.

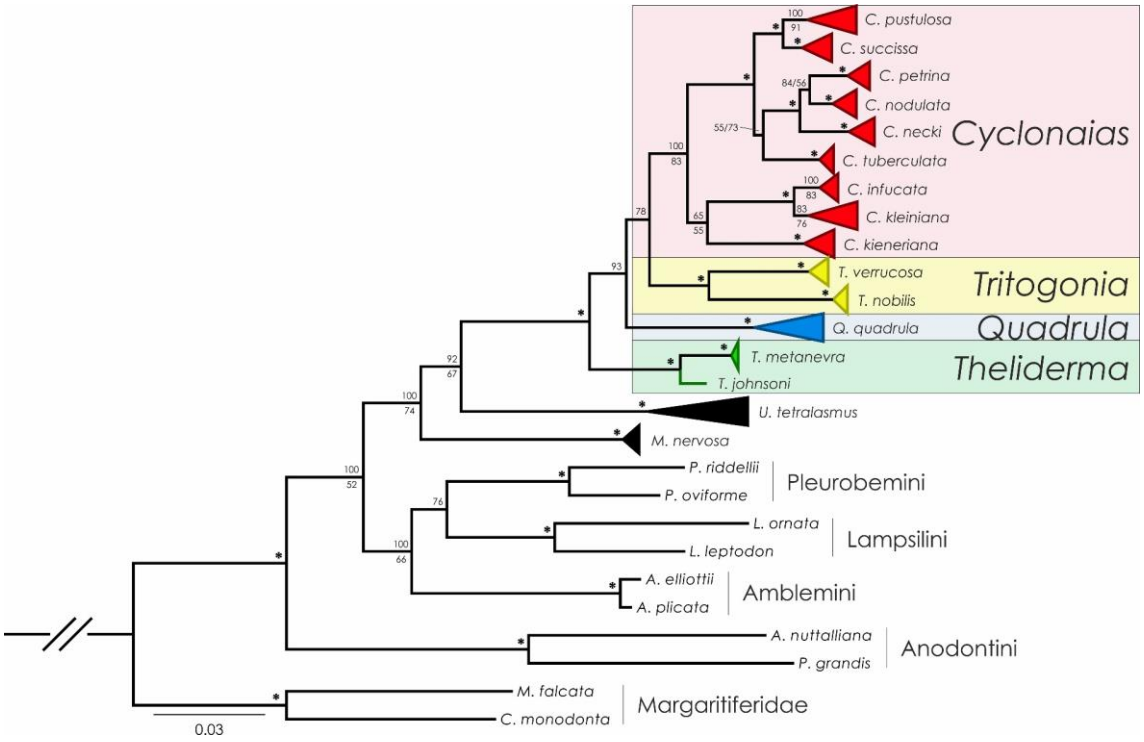

Figure 4. Bayesian consensus tree inferred from the NADH dehydrogenase subunit 1 (ND1) the cytochrome c oxidase I (COI) gene fragments concatenated dataset. The values above and below the nodes indicate Bayesian posterior probability (bpp) percentage and Maximum Likelihood bootstrap values (bs), respectively. Values over 95% are represented by an asterisk, values below 50% were erased for clarity.

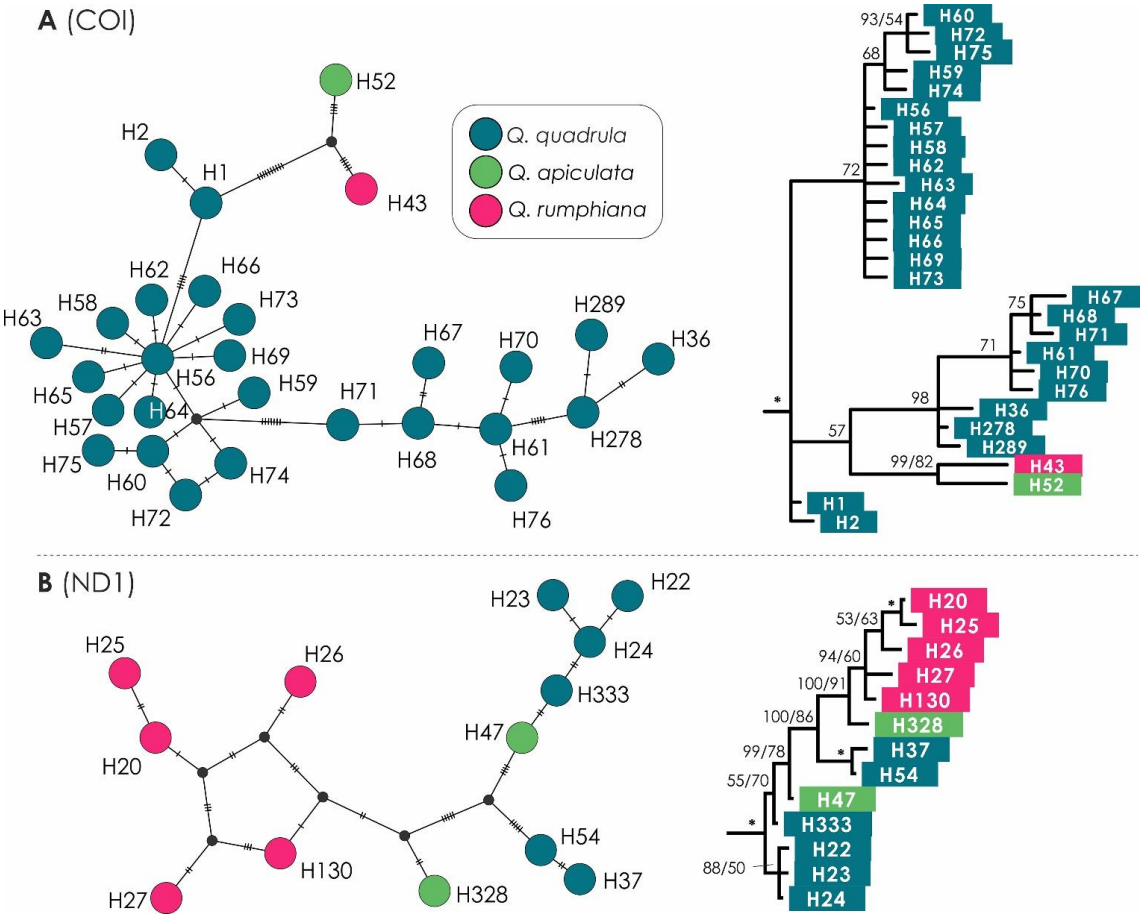

Figure 5. Haplotype (TCS) networks and uncollapsed Quadrula clade from figures 2 and 3, showing the relationships of nominal species within the *Quadrula quadrula* group for A) cytochrome c oxidase I (COI) and B) NADH dehydrogenase subunit 1 (ND1).

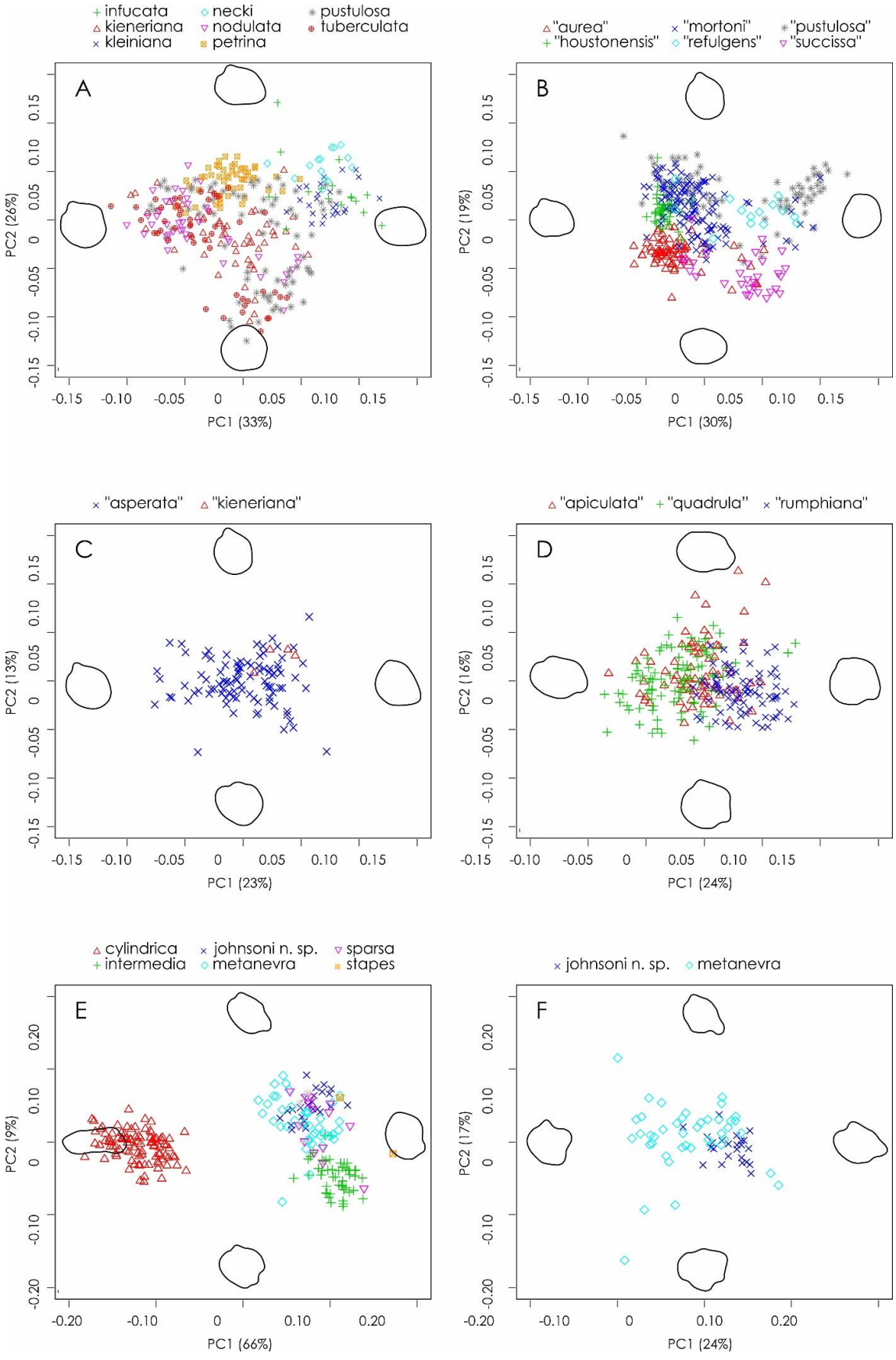
infucata
kieneriana
kleiniana
necki
nodulata
petrina
pustulosa
tuberculata
A
PC2 (26%)
PC1 (33%)
"aurea"
"houstonensis"
"mortoni"
"refulgens"
"pustulosa"
"succissa"
B
PC2 (19%)
PC1 (30%)
"asperata"
"kieneriana"
C
PC2 (13%)
PC1 (23%)
"apiculata"
"quadrula"
"rumphiana"
D
PC2 (16%)
PC1 (24%)
cylindrica
intermedia
johnsoni n. sp.
metanevra
sparsa
stapes
E
PC2 (9%)
PC1 (66%)
johnsoni n. sp.
metanevra
F
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 deliniation 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 deliniation 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.

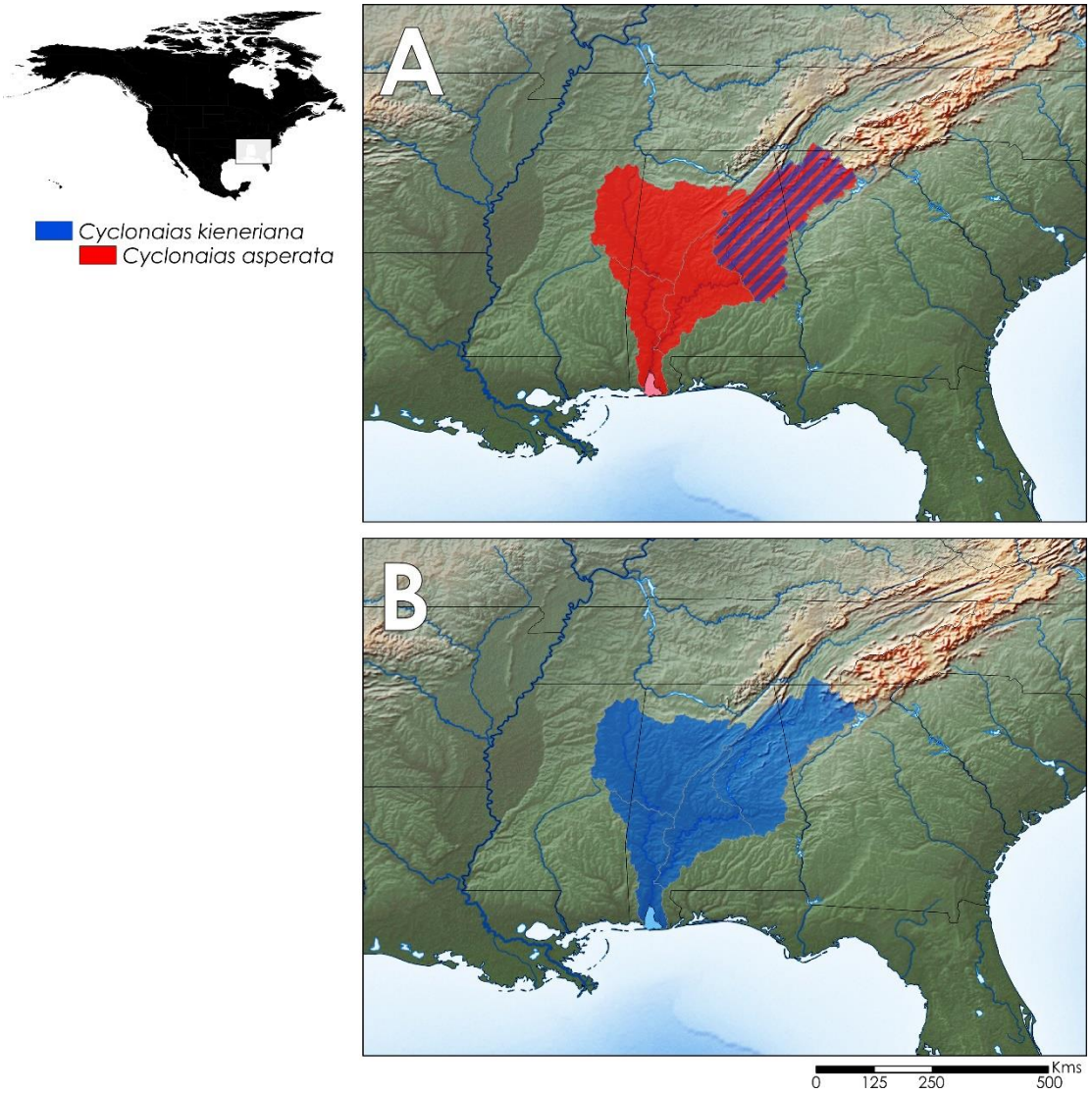

Figure 7. Distribution maps of A) nominal species *Cyclonaias asperata* and *Cyclonaias kieneriana* before the present study and B) of *Cyclonaias kieneriana* as proposed in the present study.

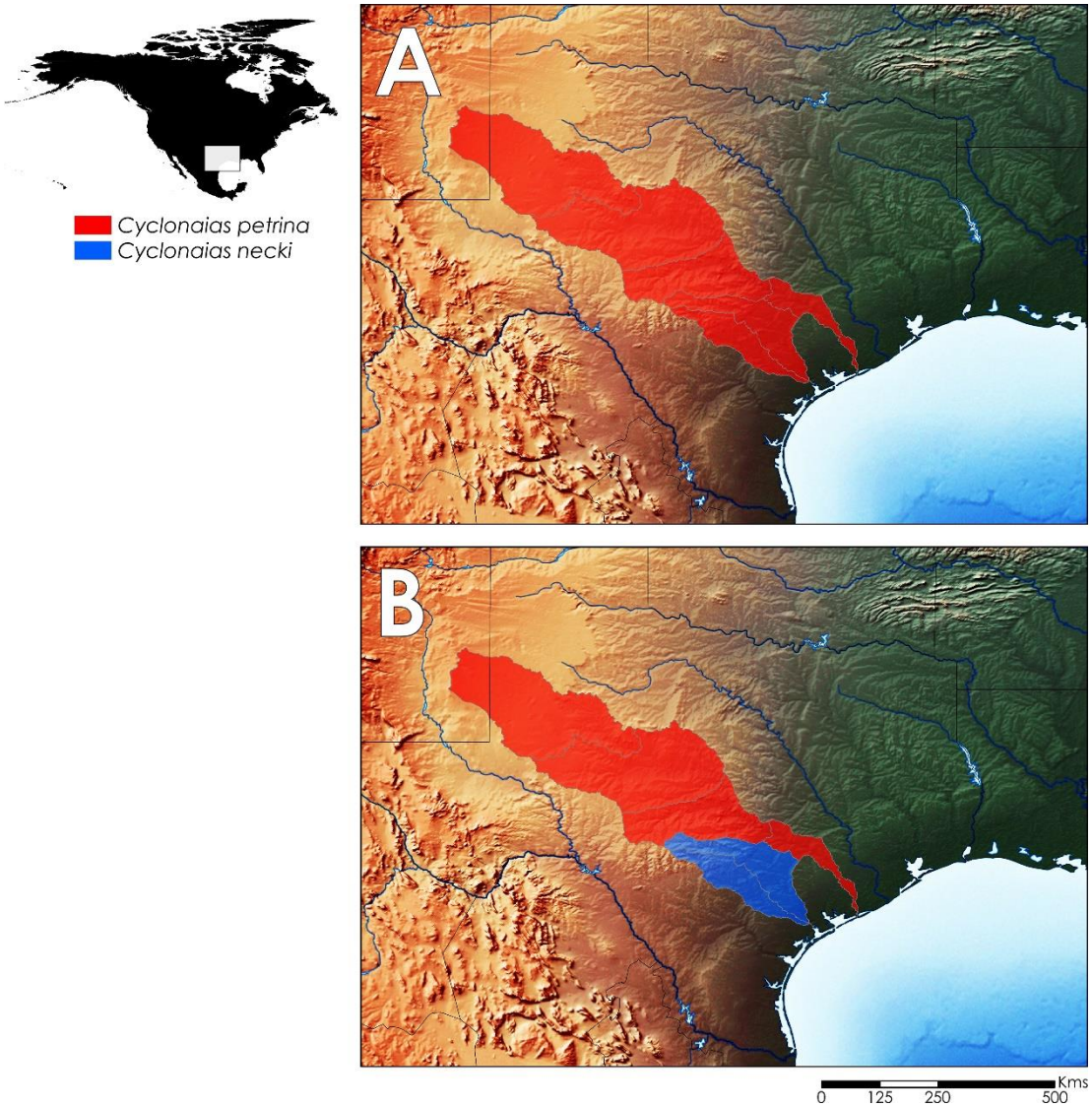

Figure 8. Distribution maps of A) *Cyclonaias petrina* before Burlakova *et al*. (2018) and B) of *C. petrina* and *Cyclonaias necki* after Burlakova *et al*. (2018) and Johnson et al. (2018) findings also supported by the present study.

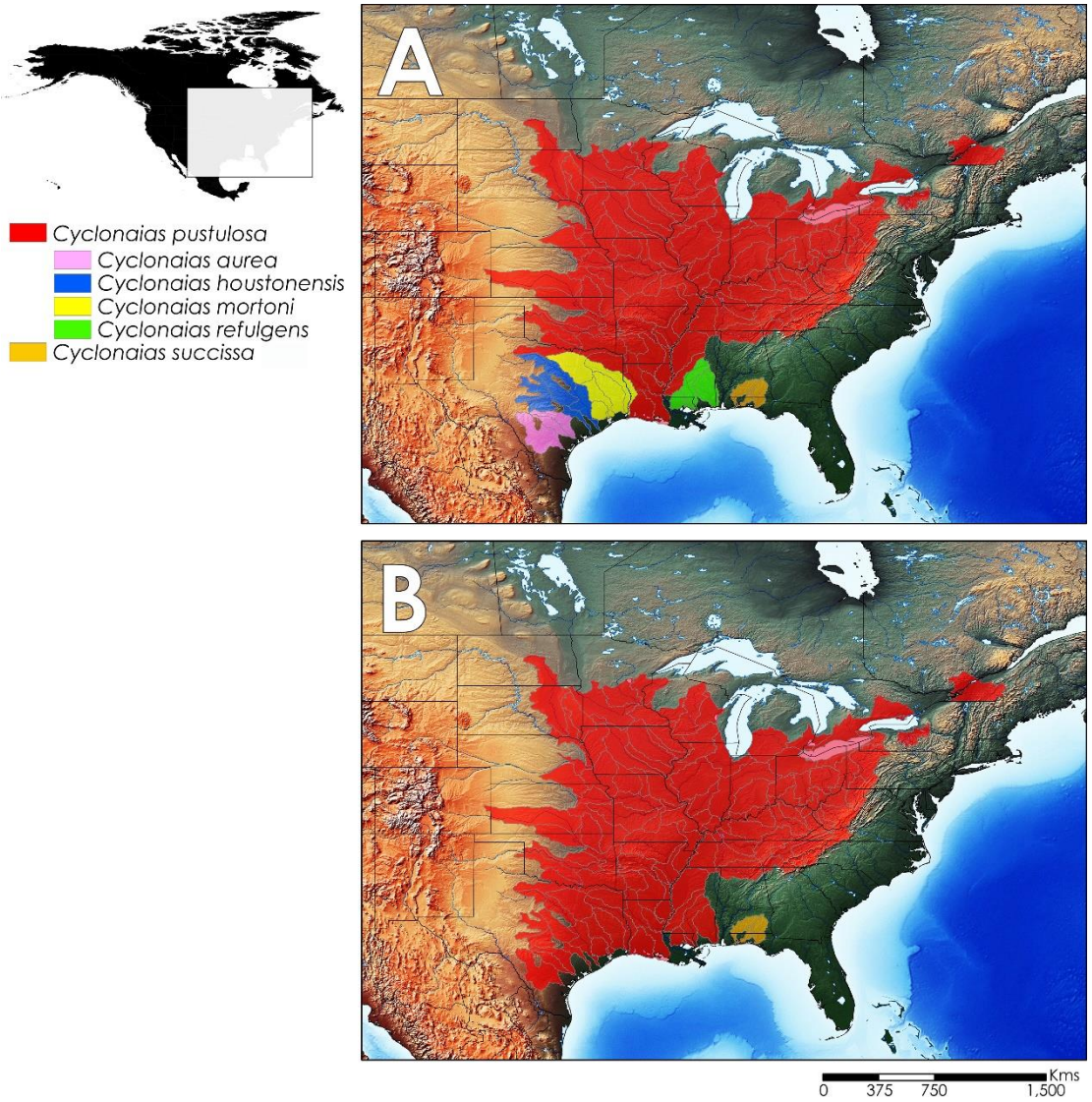

Figure 9. Distribution maps of A) nominal species within the *Cyclonaias pustulosa* group and B) of *Cyclonaias pustulosa* and *Cyclonaias succissa* as confirmed by Johnson et al. (2018) and the present study.

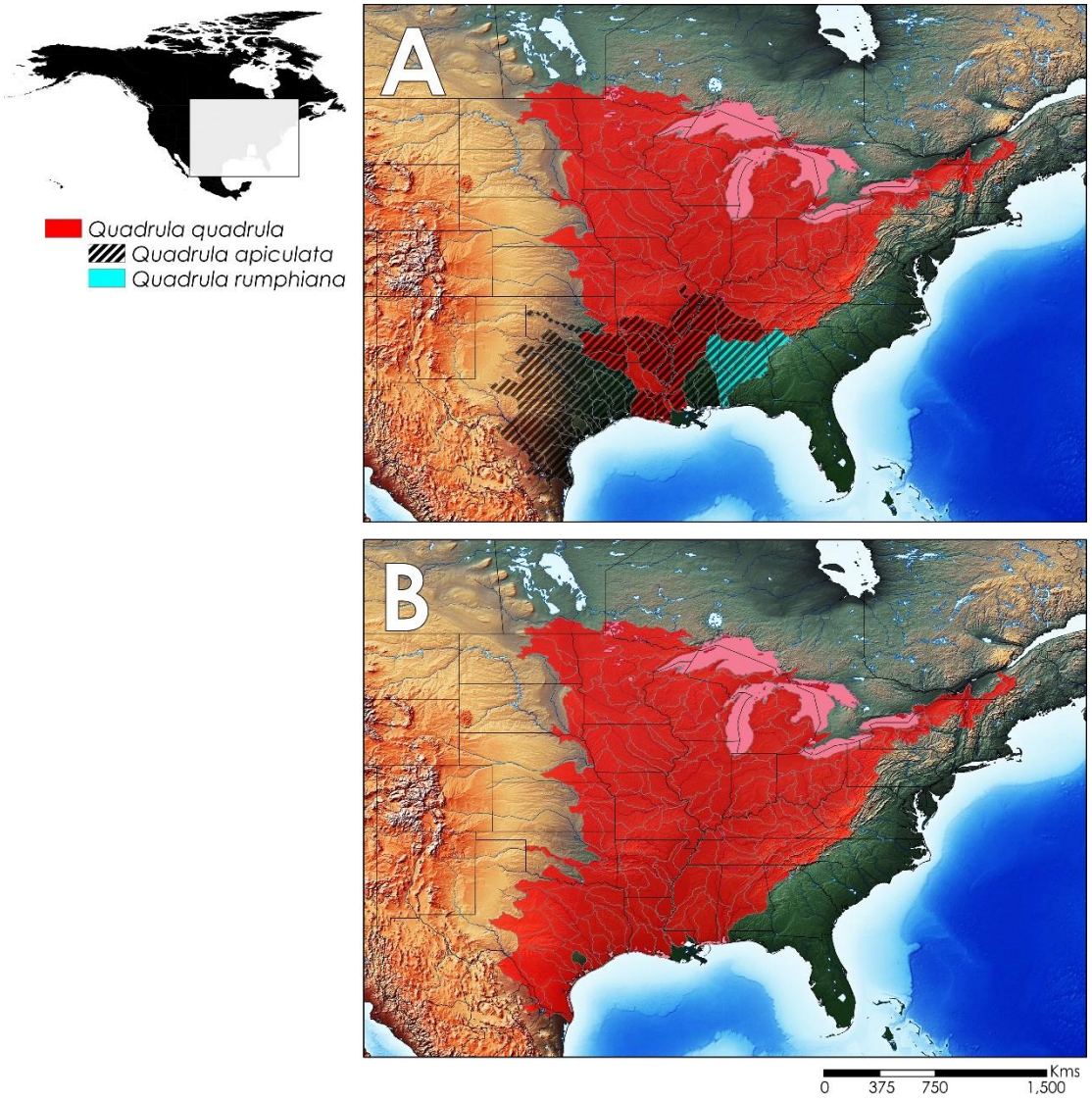

Figure 10. Distribution maps of A) nominal species within the *Quadrula quadrula* group and B) of *Quadrula quadrula* as proposed in the present study.

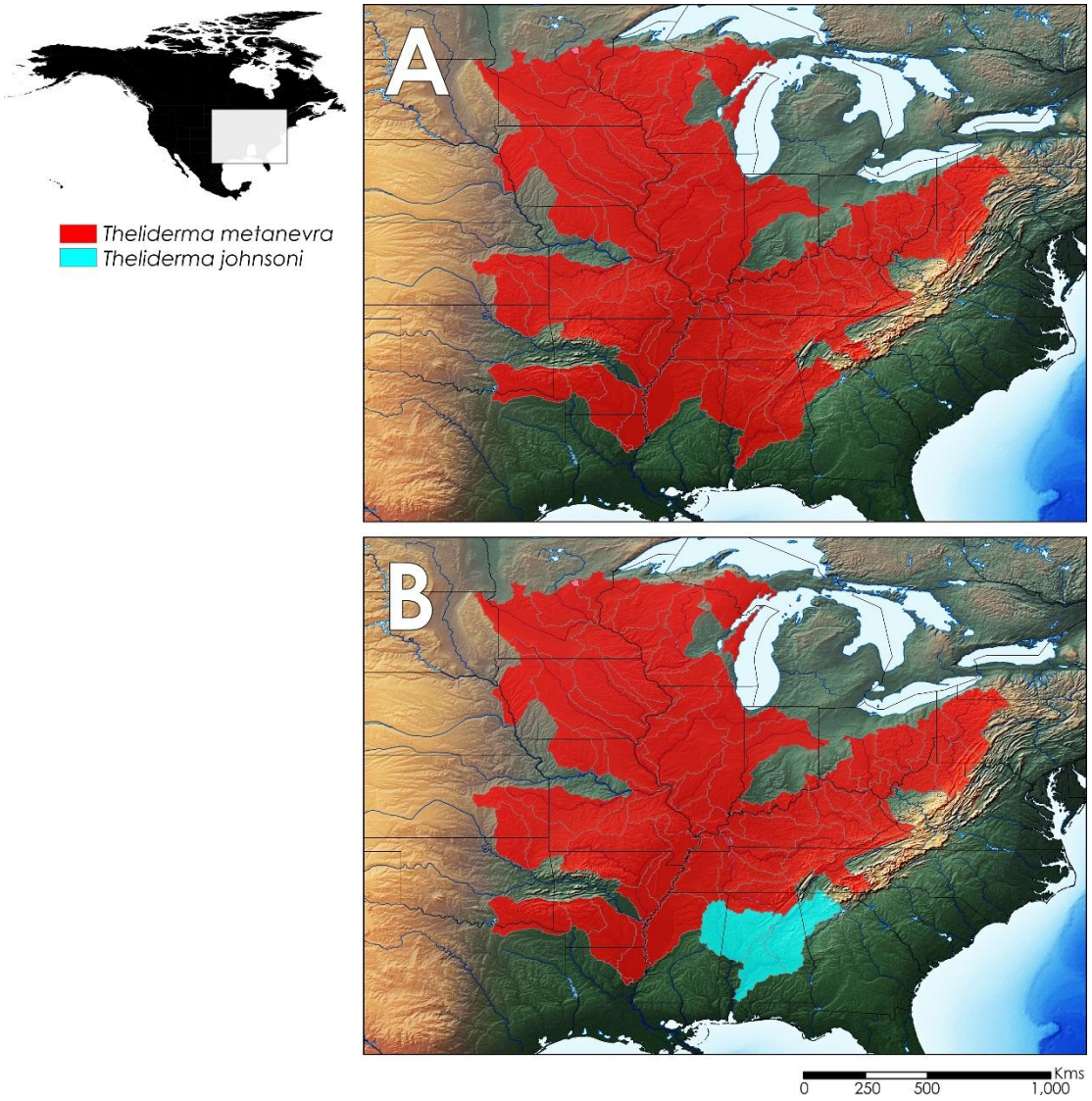

Figure 11. Distribution maps of A) *Theliderma metanevra* before the present study and B) after the present study divided in *T. metanevra* and *T. johnsoni* n. sp.

Haas (1969a)	Graf & Cummings (2007)	Bogan (2010)	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*

Table 1. Historical classification of species formerly assigned to *Quadrula*. * extinct.

Table 2. 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)	HAP (COI)	GB (ND1)	HAP (ND1)
Quadrula petrina	*Cyclonaias necki*	San Marcos	S. Antonio/Guadalupe	MG969422	Hap14	BIV2442	Hap100
Quadrula petrina	*Cyclonaias necki*	San Marcos	S. Antonio/Guadalupe	MG969423	Hap14	BIV2467	Hap143
Quadrula petrina	*Cyclonaias necki*	San Marcos	S. Antonio/Guadalupe	MG969424	Hap26	BIV2468	Hap113
Quadrula petrina	*Cyclonaias necki*	San Marcos	S. Antonio/Guadalupe	MG969425	Hap27	BIV2469	Hap114
Quadrula petrina	*Cyclonaias petrina*	Concho	Colorado	MG969416	Hap11	BIV2438	Hap097
Quadrula petrina	*Cyclonaias petrina*	Concho	Colorado	MG969417	Hap12	BIV2439	Hap098
Quadrula petrina	*Cyclonaias petrina*	Concho	Colorado	MG969418	Hap12	BIV2440	Hap098
Quadrula petrina	*Cyclonaias petrina*	San Saba	Colorado	MG969419	Hap23	BIV2462	Hap097
Quadrula petrina	*Cyclonaias petrina*	San Saba	Colorado	MG969420	Hap23	BIV2463	Hap097
Quadrula petrina	*Cyclonaias petrina*	San Saba	Colorado	MG969421	Hap12	BIV2464	Hap111
Quadrula aurea	*Cyclonaias pustulosa*	San Marcos	S. Antonio/Guadalupe	BIV2441	Hap13	BIV2441	Hap099
Quadrula aurea	*Cyclonaias pustulosa*	San Antonio	S. Antonio/Guadalupe	BIV2443	Hap15	BIV2443	Hap101
Quadrula aurea	*Cyclonaias pustulosa*	San Antonio	S. Antonio/Guadalupe	BIV2444	Hap74	BIV2444	Hap102
Quadrula aurea	*Cyclonaias pustulosa*	San Antonio	S. Antonio/Guadalupe	BIV2445	Hap17	BIV2445	Hap103
Quadrula aurea	*Cyclonaias pustulosa*	San Antonio	S. Antonio/Guadalupe	-	-	BIV2446	Hap102
Quadrula aurea	*Cyclonaias pustulosa*	Guadalupe	S. Antonio/Guadalupe	BIV2447	Hap18	BIV2447	Hap144
Quadrula aurea	*Cyclonaias pustulosa*	Nueces	Nueces	BIV2465	Hap24	BIV2465	Hap102
Quadrula aurea	*Cyclonaias pustulosa*	Nueces	Nueces	BIV2466	Hap25	BIV2466	Hap112
Quadrula aurea	*Cyclonaias pustulosa*	Guadalupe	S. Antonio/Guadalupe	BIV2470	Hap28	BIV2470	Hap115
Quadrula aurea	*Cyclonaias pustulosa*	Guadalupe	S. Antonio/Guadalupe	BIV2471	Hap28	BIV2471	Hap145
Quadrula houstonensis	*Cyclonaias pustulosa*	Colorado	Colorado	BIV2453	Hap19	BIV2453	Hap104
Quadrula houstonensis	*Cyclonaias pustulosa*	Colorado	Colorado	BIV2454	Hap19	BIV2454	Hap105
Quadrula houstonensis	*Cyclonaias pustulosa*	Colorado	Colorado	BIV2455	Hap19	BIV2455	Hap106

Quadrula mortoni	*Cyclonaias pustulosa*	Sandy Creek	Neches	BIV2456	Hap16	BIV2456	Hap107
Quadrula mortoni	*Cyclonaias pustulosa*	Village Creek	Neches	BIV2458	Hap21	BIV2458	Hap109
Quadrula mortoni	*Cyclonaias pustulosa*	Trinity	Trinity	BIV2473	Hap30	BIV2473	Hap117
Quadrula mortoni	*Cyclonaias pustulosa*	Trinity	Trinity	BIV2474	Hap16	BIV2474	Hap118
Quadrula mortoni	*Cyclonaias pustulosa*	Trinity	Trinity	BIV2475	Hap31	BIV2475	Hap118
Quadrula nobilis	*Tritogonia nobilis*	Neches	Neches	BIV2460	Hap22	BIV2460	Hap110
Quadrula nobilis	*Tritogonia nobilis*	Neches	Neches	BIV2461	Hap22	BIV2461	Hap110
Quadrula nobilis	*Tritogonia nobilis*	Trinity	Trinity	BIV2472	Hap29	BIV2472	Hap116

Table 3. List of morphological, anatomical and behavioural characters of *Cyclonaias*, *Quadrula*, *Theliderma* and *Tritogonia* as recognized in the present study. GLN - mean glochidial size index. [1] only analysed in one species, [2] for most species.

	Sexual dimorphism	Shell Sulcus	Periostracal chevrons	Posterior ridge	Mantle displays (magazines)			Reflexive release	Hosts	GLN
					Morphology	Size	Location (apertures)			
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)[1]	Large[1]	Excurrent[1]	NO*	Ictaluridae (67%) Centrarchidae (33%)	0.005-0.009
Theliderma	NO	NO[2]	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

Table 4. Results of Repeatability Clade Analysis (RCA) of main clades corresponding to the preferred topology. In bold values higher than 95% (Bayesian Inference) and 70% (Maximum Likelihood).

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**
C. infucata + C. kleiniana + C. kieneriana	BI	65	**97**	
	ML	55	37	
C. petrina + C. nodulata + C. necki	BI	**99**	**99**	**100**
	ML	**84**	51	**96**
C. pustulosa group	BI	**100**	**100**	89
	ML	**99**	64	45

Table 5. Pairwise genetic distance matrixes of nominal quadruline species of the genera *Cyclonaias*, *Quadrula*, *Theliderma*, and *Tritogonia*, using the original nominal taxa. **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.

	Within groups		**Between goups**																					
	COI	ND1	*C. asperata*	*C. kieneriana*	*C. kleiniana*	*C. infucata*	*C. nodulata*	*C. petrina*	*C. necki*	*C. pustulosa*	*C. aurea*	*C. houstonensis*	*C. mortoni*	*C. refulgens*	*C. succissa*	*C. tuberculata*	*Q. quadrula*	*Q. apiculata*	*Q. rumphiana*	*T. cylindrica*	*T. intermedia*	*T. metanevra*	*T. sparsa*	*T. verrucosa*	*T. nobilis*
C. asperata	0.012	0.012		0.012	0.082	0.094	0.093	0.102	0.094	0.082	0.082	0.078	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
C. kieneriana	----	----			0.081	0.094	0.089	0.101	0.093	0.081	0.082	0.077	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
C. kleiniana	0.012	0.011	0.080	----		0.035	0.099	0.094	0.099	0.083	0.085	0.083	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
C. infucata	0.006	0.007	0.082	----	0.032		0.097	0.092	0.097	0.087	0.090	0.085	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
C. nodulata	0.006	0.009	0.077	----	0.088	0.083		0.038	0.040	0.063	0.063	0.064	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
C. petrina	0.007	0.006	0.076	----	0.095	0.090	0.028		0.047	0.063	0.062	0.064	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
C. necki	0.007	0.007	0.077	----	0.094	0.084	0.041	0.039		0.064	0.067	0.066	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
C. pustulosa	0.010	0.011	0.076	----	0.092	0.085	0.052	0.053	0.051		0.017	0.012	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
C. aurea	0.011	0.012	0.078	----	0.092	0.083	0.050	0.051	0.051	0.014		0.018	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
C. houstonensis	0.007	0.008	0.075	----	0.088	0.081	0.058	0.059	0.055	0.014	0.017		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
C. mortoni	0.013	0.012	0.075	----	0.086	0.079	0.052	0.054	0.055	0.020	0.019	0.020		0.017	0.030	0.050	0.111	0.115	0.119	0.126	0.137	0.118	0.107	0.106	0.118
C. refulgens	0.015	0.010	0.074	----	0.091	0.084	0.052	0.052	0.052	0.014	0.014	0.017	0.020		0.027	0.049	0.108	0.113	0.116	0.120	0.137	0.116	0.106	0.104	0.116
C. succissa	0.011	0.011	0.081	----	0.094	0.085	0.048	0.044	0.054	0.036	0.033	0.041	0.037	0.035		0.053	0.109	0.113	0.122	0.124	0.144	0.126	0.113	0.110	0.119
C. tuberculata	0.006	0.006	0.078	----	0.088	0.090	0.050	0.056	0.062	0.058	0.056	0.064	0.055	0.058	0.053		0.115	0.117	0.120	0.127	0.146	0.126	0.113	0.116	0.121
Q. quadrula	0.014	0.012	0.112	----	0.110	0.103	0.096	0.097	0.098	0.108	0.104	0.112	0.109	0.108	0.100	0.098		0.017	0.027	0.104	0.139	0.116	0.108	0.109	0.105
Q. apiculata	----	0.018	0.105	----	0.096	0.096	0.093	0.089	0.095	0.100	0.099	0.103	0.100	0.100	0.092	0.085	0.034		0.020	0.109	0.143	0.117	0.112	0.111	0.107
Q. rumphiana	----	0.010	0.105	----	0.099	0.095	0.093	0.089	0.095	0.097	0.092	0.100	0.097	0.097	0.088	0.084	0.034	0.015		0.112	0.145	0.119	0.117	0.110	0.116
T. cylindrica	----	0.010	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----		0.106	0.086	0.079	0.122	0.126
T. intermedia	----	0.003	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----		0.081	0.073	0.135	0.137
T. metanevra	0.017	0.021	0.091	----	0.092	0.096	0.094	0.093	0.090	0.084	0.087	0.086	0.088	0.088	0.095	0.083	0.101	0.090			0.096		0.040	0.115	0.126
T. sparsa	----	0.002	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----		0.105	0.106
T. verrucosa	0.007	0.008	0.096	----	0.105	0.093	0.102	0.104	0.098	0.105	0.107	0.104	0.105	0.106	0.100	0.098	0.114	0.116	----	----	0.116	----	0.096		0.093
T. nobilis	0.009	0.011	0.105	----	0.118	0.107	0.108	0.101	0.106	0.102	0.102	0.102	0.106	0.102	0.099	0.114	0.110	0.116	----	----	0.114	----	0.114	0.085	

	COI					ND1				COI + ND1				CONSENSUS MOTUs
	BOLD	ABGD	TCS (95%)	bPTP	GMYC	ABGD	TCS (95%)	bPTP	GMYC	ABGD	TCS (95%)	bPTP	GMYC	
Cyclonaias														
C. asperata	✓	✓	✓	✓	✓	✗	✗	✗	✗	✓	✓	✓	✓	✗
C. kieneriana	-	-	-	-	-	✓	✓	✓	✓	-	-	-	-	✓
C. infucata	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. kleiniana	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. nodulata	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. petrina	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. necki	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. pustulosa	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. aurea	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
C. houstonensis	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✓	✗
C. mortoni	✗	✗	✗	✗	✓	✗	✗	✗	✓	✓	✓	✗	✓	✗
C. refulgens	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
C. succissa	✓	✓	✓	✓	✓	✗	✓	✗	✓	✓	✓	✓	✓	✓
C. tuberculata	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Quadrula														
Q. quadrula clade 1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Q. quadrula clade 2	✗	✗	✗	✓	✗	✗	✗	✗	✗	✗	✓	✗	✓	✗
Q. quadrula clade 3	✓	✓	✗	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗
Q. apiculata	✓	✓	✓	✓	✓	✗	✗	✗	✗	✗	✗	✗	✓	✗
Q. rumphiana	✗	✓	✗	✗	✗	✓	✓	✗	✗	✗	✗	✗	✓	✗
Theliderma														
T. cylindrica	-	-	-	-	-	✓	✓	✓	✓	-	-	-	-	✓

T. intermedia	-	-	-	-	-	✓	✓	✓	✓	-	-	-	-	✓
T. metanevra	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T. johnsoni n. sp.	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T. sparsa	-	-	-	-	-	✓	✓	✓	✓	-	-	-	-	✓
Tritogonia														
T. verrucosa	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T. nobilis	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 6. Results of molecular species delineation methods. ✓recognized as a molecular operational taxonomic unit (MOTU); ✘ not recognized as a MOTU; - not analysed.

Table 7. Pairwise genetic distance matrixes of quadruline species of the genera *Cyclonaias*, *Quadrula*, *Theliderma*, and *Tritogonia*, as recognized in the present study. **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.

	Within groups		**Between goups**															
	COI	ND1	*C. kieneriana*	*C. infucata*	*C. kleiniana*	*C. nodulata*	*C. petrina*	*C. necki*	*C. pustulosa*	*C. succissa*	*C. tuberculata*	*Q. quadrula*	*T. cylindrica*	*T. intermedia*	*T. metanevra*	*T. johnsoni*	*T. sparsa*	*T. verrucosa*	*T. nobilis*
C. kieneriana	0.012	0.012		0.094	0.082	0.093	0.102	0.094	0.082	0.083	0.095	0.107	0.112	0.143	0.111	0.111	0.105	0.114	0.115
C. infucata	0.006	0.007	0.082		0.035	0.097	0.092	0.097	0.089	0.095	0.093	0.112	0.115	0.139	0.117	0.115	0.11	0.107	0.125
C. kleiniana	0.012	0.011	0.080	0.032		0.099	0.094	0.099	0.085	0.092	0.088	0.115	0.11	0.143	0.118	0.116	0.105	0.112	0.123
C. nodulata	0.006	0.009	0.077	0.083	0.088		0.038	0.04	0.063	0.064	0.055	0.128	0.129	0.144	0.123	0.117	0.113	0.118	0.126
C. petrina	0.007	0.006	0.076	0.090	0.095	0.028		0.047	0.062	0.064	0.065	0.131	0.125	0.14	0.125	0.114	0.11	0.122	0.13
C. necki	0.007	0.007	0.077	0.084	0.094	0.041	0.039		0.065	0.07	0.059	0.13	0.127	0.147	0.129	0.12	0.115	0.116	0.126
C. pustulosa	0.016	0.016	0.076	0.082	0.089	0.052	0.053	0.052		0.031	0.052	0.112	0.121	0.136	0.119	0.112	0.106	0.105	0.118
C. succissa	0.011	0.011	0.081	0.085	0.094	0.048	0.044	0.054	0.036		0.053	0.114	0.124	0.144	0.129	0.118	0.113	0.11	0.119
C. tuberculata	0.006	0.006	0.078	0.090	0.088	0.050	0.056	0.062	0.057	0.053		0.117	0.127	0.146	0.126	0.126	0.113	0.116	0.121
Q. quadrula	0.017	0.019	0.112	0.103	0.109	0.096	0.096	0.098	0.107	0.100	0.097		0.108	0.141	0.122	0.109	0.112	0.11	0.11
T. cylindrica	----	0.01	----	----	----	----	----	----	----	----	----	----		0.106	0.088	0.082	0.079	0.122	0.126
T. intermedia	----	0.003	----	----	----	----	----	----	----	----	----	----	----		0.084	0.076	0.073	0.135	0.137
T. metanevra	0.009	0.005	0.090	0.095	0.091	0.093	0.094	0.090	0.086	0.096	0.083	0.102	----	----		0.035	0.042	0.117	0.129
T. johnsoni	----	0.002	0.093	0.099	0.096	0.095	0.092	0.088	0.088	0.094	0.085	0.094	----	----	0.032		0.036	0.109	0.121
T. sparsa	----	0.002	----	----	----	----	----	----	----	----	----	----	----	----	----	----		0.105	0.106
T. verrucosa	0.007	0.008	0.096	0.093	0.105	0.102	0.104	0.098	0.105	0.100	0.098	0.114	----	----	0.096	0.097	----		0.093
T. nobilis	0.009	0.011	0.105	0.107	0.118	0.108	0.101	0.106	0.103	0.099	0.114	0.110	----	----	0.115	0.107	----	0.085	