



## Expansion and systematics redefinition of the most threatened freshwater mussel family, the Margaritiferidae

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### ABSTRACT

Two Unionida (freshwater mussel) families are present in the Northern Hemisphere; the Margaritiferidae, representing the most threatened of unionid families, and the Unionidae, which include several genera of unresolved taxonomic placement. The recent reassignment of the poorly studied *Lamprotula rochechouartii* from the Unionidae to the Margaritiferidae motivated a new search for other potential species of margaritiferids from members of *Gibbosula* and *Lamprotula*. Based on molecular and morphological analyses conducted on newly collected specimens from Vietnam, we here assign *Gibbosula crassa* to the Margaritiferidae. Additionally, we reanalyzed all diagnostic characteristics of the Margaritiferidae and examined museum specimens of *Lamprotula* and *Gibbosula*. As a result, two additional species are also moved to the Margaritiferidae, i.e. *Gibbosula confragosa* and *Gibbosula polysticta*. We performed a robust five marker phylogeny with all available margaritiferid species and discuss the taxonomy within the family. The present phylogeny reveals the division of Margaritiferidae into four ancient clades with distinct morphological, biogeographical and ecological characteristics that justify the division of the Margaritiferidae into two subfamilies (Gibbosulinae and Margaritiferinae) and four genera (*Gibbosula*, *Cumberlandia*, *Margaritifera*, and *Pseudunio*). The systematics of the Margaritiferidae family is redefined as well as their distribution, potential origin and main biogeographic patterns.

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## 1. Introduction

### 1.1. *Unionida* freshwater mussels: diversity and conservation status

The Unionida is the only strictly freshwater order of bivalves (Bogan, 2008). It is an old and widespread order with approximately 800 described species in 180 genera (Bogan, 2008). Six families are currently recognized within Unionida, but only the Unionidae and the Margaritiferidae are widespread in the Northern Hemisphere (Bogan, 2008). While the Unionidae is extremely diverse (> 600 species), until the present study, only 12 species in one genus scattered across North America, Europe, North Africa and Asia had been recognized within the Margaritiferidae (Bolotov et al., 2016; Araujo et al., 2017). Additionally, both families are declining globally and are highly endangered, especially the Margaritiferidae, where all species assessed with sufficient data present a near threatened or threatened conservation status (IUCN, 2018).

### 1.2. Taxonomical history of the Margaritiferidae and its diagnostic characters

Until the end of the twentieth century, the taxonomy and systematics of Unionida had been based primarily on conchological and anatomical characters (e.g. Haas, 1969a; Parmalee and Bogan, 1998; Watters et al., 2009). Due to the better availability of Unionida specimens from North America and Europe, those from tropical and the Southern Hemisphere regions were relatively poorly studied (Simpson, 1900, 1914; Ortmann, 1921; McMichael and Hiscock, 1958).

Early systematists encompassed all genera of freshwater mussels, including *Margaritana* (= *Margaritifera*) species, within the family Unionidae (Table 1: Lea, 1836, 1838, 1852, 1870; Simpson, 1900, 1914; Frierson, 1927). However, in the beginning of the twentieth century, Ortmann (1910) determined that some anatomical characters of some genera were distinct and of prime systematic value. This author erected a new taxon, first as a sub-family, Margaritaninae within Unionidae, but immediately after as a separate family, the Margaritanidae (= Margaritiferidae Henderson, 1929, (1910)), both with the genus and species *Margaritana* (= *Margaritifera*) *margaritifera* (Linnaeus, 1758) as the type. As defined by Ortmann (1910, 1911a,b, 1912), the Margaritanidae presented distinct anatomical features from the other Unionidae species, including the lack of discrete apertures separated by mantle fusions, particular gill and marsupium structure, and glochidial (larval) shape (Table 2). Although at first other malacologists did not recognize Margaritiferidae as a separate family (e.g., Simpson, 1914), soon it was accepted by most researchers (e.g. Henderson, 1929), including in the comprehensive classification of the Unionida published by Haas (1969a,b). In this fundamental work, the family Margaritiferidae was recognized with nine taxa (five species and four subspecies) under a single genus, *Margaritifera*, divided in four subgenera: *Margaritifera*, *Cumberlandia*, *Margaritanopsis* and *Pseudunio*.

During the same period, alternative classifications were published (Modell, 1942, 1949, 1964; Starobogatov, 1970, 1995; Bogatov et al., 2003) based only on few conchological characters that proposed a much larger number of taxa in the Margaritiferidae (Table 1). These studies were controversial and subsequently ignored by most malacologists (e.g., Boss, 1982; Smith, 2001, Graf and Cummings, 2007). Since the beginning of this century, the family Margaritiferidae has been consistently restricted to around 12 species (Smith, 2001; Huff et al., 2004; Graf and Cummings, 2006). Smith (2001), based on morphological characters only, divided the Margaritiferidae into three genera: *Pseudunio*, *Margaritifera*, and *Margaritanopsis*. Soon after, a molecular phylogenetic analysis was published using both nuclear and mitochondrial markers on seven Margaritiferidae species (Huff et al., 2004). Although these phylogenetic analyses presented three clear clades, these did not agree with the genera previously defined by Smith (2001), causing Huff et al. (2004) to conclude that the generic name

*Margaritifera* should be considered for all species. In subsequent phylogenetic studies, the Margaritiferidae has been presented consistently as monophyletic, with a marked genetic structure and divided into three to four major clades; however, most authors have chosen not to discuss its generic assignment keeping *Margaritifera* as the single genus (Huff et al., 2004; Graf and Cummings, 2007; Araujo et al., 2017). Nevertheless, many North American researchers continued to recognize *Cumberlandia* as a valid genus (e.g. Watters et al., 2009; Haag, 2012).

Recently, two comprehensive five loci molecular phylogenies on the Margaritiferidae documented several well-supported divergent clades. Bolotov et al. (2016) recognized only three main clades, assigning them as subgenera (*Margaritanopsis*, *Margaritifera*, and *Pseudunio*) of *Margaritifera*, resembling the previous classification by Haas (1969a). Shortly afterwards, Araujo et al. (2017) described five major divergent clades within the Margaritiferidae, but kept them under the same genus (*Margaritifera*).

### 1.3. Biogeography and diversification of the Margaritiferidae

The family Margaritiferidae has a broad but disjunct distribution range in the Northern Hemisphere (Smith, 2001). It presents an enigmatic biogeographic pattern with species aggregations along the western and eastern continental margins and vast distribution gaps in inland areas (e.g., East Europe, Urals and Siberia), possibly reflecting vicariance events driven by plate tectonics (Taylor, 1988; Smith, 2001; Huff et al., 2004). Recently, Bolotov et al. (2016) and Araujo et al. (2017) reviewed available biogeographic schemes explaining the origin and expansion routes of the Margaritiferidae and independently provided new fossil-calibrated evolutionary models. However, the time and place of origin of the entire family remained unclear (Bolotov et al., 2016; Araujo et al., 2017). The phylogenetic models placed the origin of the Margaritiferidae in the mid-Cretaceous (Bolotov et al., 2016) or even in the Late Triassic (Araujo et al., 2017). The strong temporal discordance between these fossil-calibrated phylogenies together with significant topological differences and low support values in several deep nodes suggest that both studies need additional taxon samples. Inclusion of *Pseudunio homsensis* from the Orontes River in Turkey, that had been missing from the previous phylogenetic studies (Bolotov et al., 2016; Araujo et al., 2017), did not help to obtain a fully resolved evolutionary reconstruction for the family, as it appears to be a close relative of *P. auricularius* (Vikhrev et al., 2017). Additionally, previous analyses also lacked Margaritiferidae taxa from eastern China (i.e., between the Indo-China Peninsula and the Amur River; Smith, 2001; Bolotov et al., 2015, 2016). As has already been noted (Smith, 2001; Bolotov et al., 2015), inclusion of newly discovered species from this vast range disjunction is crucial for developing a comprehensive understanding of the biogeography of the Margaritiferidae. Huang et al. (2017) added molecular sequences of *Gibbosula rochechouartii* to the data set of Araujo et al. (2017) and calculated an updated fossil-calibrated phylogeny placing the origin of the Margaritiferidae crown group in the Late Cretaceous but were not able to obtain a well-resolved biogeographic reconstruction.

A large number of fossil specimens assigned to the Margaritiferidae has been recovered in Europe, Middle Asia, China, Mongolia, Siberia, Japan, North America, and Africa (e.g., Henderson, 1935; Modell, 1957; Martinson, 1982; Ma, 1996; Fang et al., 2009; Van Damme et al., 2015; Bolotov et al., 2016; Araujo et al., 2017). However, recent phylogenetic models were calculated using a limited set of fossil calibrations because the true phylogenetic affinities of many fossil taxa remain unclear due to high conchological variability (Bolotov et al., 2016; Araujo et al., 2017; Huang et al., 2017). The high taxonomic diversity of fossil margaritiferids disagrees with the limited number of extant taxa and likely reflects a lack of critical revisions in systematic paleontology rather than multiple extinction events (Schneider and Prieto, 2011; Bolotov et al., 2016; Araujo et al., 2017). Slow substitution rates in the Margaritiferidae (Bolotov et al., 2016) allow us to expect rather

**Table 1**  
Comparison of Margaritiferidae classifications. Fossil genera excluded. (S) synonym. Superscripts: <sup>1</sup>Under tribe Heudeanini; <sup>2</sup>under subfamily Pseudodontinae; <sup>3</sup>under tribe Margaritiferini; <sup>4</sup>under tribe Leguminaini.

	Ortmann (1910)	Ortmann (1911a, b, 1912)	Frierson (1927)	Henderson (1929)	Thiele (1934)	Modell (1942)	Modell (1949)	Modell (1964)	Haas (1969a,b)	Siarobogatov (1970)	Morrison (1975)	Boss (1982)	Siarobogatov (1995)	Smith (2001)	Graf & Cummings (2007, 2017)	Huff et al. (2004)	Bolotov et al. (2016)	Araujo et al. (2017)	This study
<b>Margaritaniidae</b>		✓			✓														
<b>Margaritaninae</b>			✓																
<i>Margaritana</i>	✓	✓	✓		✓														
( <i>Margaritana</i> )																			
<b>Margaritiferidae</b>				✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
<b>Margaritiferinae</b>						✓	✓	✓	✓	✓ <sup>3</sup>	✓	✓	✓	✓	✓	✓	✓	✓	✓
<i>Margaritifera</i>			✓			✓	✓	✓	✓	✓ <sup>3</sup>	✓	✓	✓	✓	✓	✓	✓	✓	✓
( <i>Margaritifera</i> )					S	✓	✓	✓	✓	✓ <sup>1</sup>				S					S
<i>Margaritanopsis</i>						✓	✓	✓	✓	✓ <sup>3</sup>				✓					
( <i>Margaritanopsis</i> )						✓	✓	✓	✓	✓ <sup>3</sup>				S					S
<i>Cumberlandia</i>		✓	✓		✓	✓	✓	✓	✓	✓ <sup>3</sup>				S			S		✓
( <i>Cumberlandia</i> )																			
<i>Potomida</i>					✓														
<i>Pseudunio</i>					✓					✓ <sup>3</sup>									✓
( <i>Pseudunio</i> )					S				✓	✓ <sup>3</sup>				S					✓
<i>Dahurinaia</i>									S	✓ <sup>1</sup>				S					S
<i>Gibbosula</i>										✓ <sup>1</sup>				S					✓
<i>Psychorhynchus</i>						✓		✓ <sup>1</sup>		✓ <sup>1</sup>				S					✓
( <i>Schaltenia</i> )								✓ <sup>1</sup>		✓ <sup>3</sup>									S
<b>Cucumerunioninae</b>						✓		✓		✓									
<i>Cucumerunio</i>						✓		✓		✓									
<i>Virgus</i>						✓		✓		✓									
<b>Heudeaninae</b>						✓		✓		✓									
<i>Heudeana</i>						✓		✓		✓									
<i>Schepmania</i>						✓		✓		✓									
<i>Ctenodesma</i>						✓		✓		✓									
<b>Pseudodontinae</b>						✓		✓		✓									
<i>Pseudodon</i>						✓		✓		✓									
<i>Monodontina</i>						✓		✓		✓									
<i>Nasus</i>						✓		✓		✓									
<b>Compsopseudodon</b>						✓		✓		✓									
<i>Obovatis</i>						✓		✓		✓									
<i>Pseudodontopsis</i>						✓		✓		✓									
<b>Leguminata</b>						✓		✓		✓									
<i>Microcondylaea</i>						✓		✓		✓									
<i>Leptanodonta</i>						✓		✓		✓									
<i>Gonidea</i>						✓		✓		✓									
<b>Arcidopsinae</b>						✓		✓		✓									
<i>Arcidopsis</i>						✓		✓		✓									
<i>Trapezoides</i>						✓		✓		✓									
<i>Salenata</i>						✓		✓ <sup>2</sup>		✓									

**Table 2**  
 Characters used to define and diagnose Margaritiferidae. <sup>1</sup> papillae present only; <sup>2</sup> hinge teeth reduced.

Character	Ortmann (1910)	Ortmann (1911a, b)	Thiele (1934)	Modell (1942, 1949, 1964)	Haas (1969a,b)	Heard and Guckert (1971)	Boss (1982)	Smith (2001)	Graf and Cummings (2006)	Araujo et al. (2017)
1. Diaphragm incomplete formed by gills	✓	✓	✓		✓	✓	✓	On mantle	✓	On mantle
2. Anterior end of inner gills distant from palps	✓	✓	✓		✓	✓	✓	✓		
3. Branchial and anal siphons/apertures ill-defined not closed	✓	✓	✓		✓	✓	✓	✓		
4. Supra anal not separate	✓	✓ <sup>1</sup>	✓	✓(1949)	✓	✓	✓	✓		✓
5. Incurrent aperture with bifid or arborescent papillae	✓	✓	✓	Elongate, unaffected (1949)	✓	✓	✓	✓		✓
6. Gills no water tubes	✓	✓	✓	✓(1949)	✓	✓	✓	✓		✓
7. Gills irregular scattered interlamellar connections	✓	✓	✓	✓(1949)	✓	✓	✓	✓		✓
8. Gills not fused with mantle posteriorly	✓	✓	✓	✓(1949)	✓	✓	✓	✓		✓
9. Marsupium in all four gills	✓	✓	✓	✓(1949)	✓	✓	✓	✓		✓
10. Tachytitic	✓	✓	✓	✓(1949)	✓	✓	✓	✓		✓
11. Glochidia semilunate, hookless, irregular small teeth	✓	✓	✓	✓(1949)	✓	✓	✓	✓		✓
12. Pedal elevators inconspicuous	✓	✓	✓		✓	✓	✓	✓		✓
13. Anus located dorsal edge posterior adductor muscle	✓	✓	✓		✓	✓	✓	✓		✓
14. Shell elongated	✓	✓	✓		✓	✓	✓	✓		✓
15. Umbo low	✓	✓	✓		✓	✓	✓	✓		✓
16. Shell mostly compressed	✓	✓	✓		✓	✓	✓	✓		✓
17. Shell with numerous folds/sculpture including pustules	✓	✓	✓	some	✓	✓	✓	✓		✓
18. Frequently concave ventral margin	✓	✓	✓		✓	✓	✓	✓		✓
19. Shell with naere	✓	✓	✓		✓	✓	✓	✓		✓
20. Umbo sculpture angular un-joined chevron like hooks	✓	✓	✓	✓	✓	✓	✓	✓		✓
21. Umbo sculpture weak concentric	✓	✓	✓		✓	✓	✓	✓		✓
22. Maximum shell length	✓	✓	✓		✓	✓	150 mm	200 mm		✓
23. Umbo cavity shallow	✓	✓	✓		✓	✓	✓	✓		✓
24. Periostracum heavy, blackish	✓	✓(1911c)	✓		✓	✓	✓	✓		✓
25. Shell aragonite	✓	✓	✓		✓	✓	✓	✓		✓
26. Posterior lateral teeth tend to be reduced	✓	✓ <sup>2</sup>	✓		✓	✓	✓	✓		✓
27. Mantle attachment scars	✓	✓	✓		✓	✓	✓	✓		✓
28. Conchiolin one layer	✓	✓	✓		✓	✓	✓	✓		✓
29. Complete hinge teeth present	✓	✓	✓	✓	✓	✓	✓	✓		✓

delayed diversification processes within the family, although the diversification rates in margaritiferids have never been tested to date.

#### 1.4. Historical description and classification of some incertae sedis Unionidae taxa

Although recent phylogenetic works have increased our knowledge on the position of many Unionida genera from the less studied African and Asian countries (e.g. Pfeiffer and Graf, 2013, 2015; Lopes-Lima et al., 2017a; Bolotov et al., 2017a,b), the most comprehensive revision of the Unionidae classification to date placed 42 genera as incertae sedis (Lopes-Lima et al., 2017a). These included *Gibbosula* (Simpson 1900), whose type species was first described and illustrated by Wood (1815) as *Mya crassa* from an unknown locality in China and later classified under *Gibbosula* (i.e. as *Gibbosula crassa*) within the Unionidae by Simpson (1900). A few years later, another specimen was found in southern China and described as a new species, i.e. *Unio (Quadrula) mansuyi* Dautzenberg & Fischer, 1908. Simpson (1914) placed this species under *Quadrula* and did not associate it with *G. crassa*. A third specimen was described in 1928 and added to *Gibbosula* (i.e. *Gibbosula confragosa* Frierson, 1928) based on conchological similarities with *G. crassa*. In his comprehensive classification of the Unionida, Haas (1969a,b) considered that *Gibbosula* had been superfluously created by Simpson and listed it as a synonym of *Lamprotula*, inside the Unionidae. Additionally, Haas (1969a) listed Dautzenberg & Fischer's species *Unio mansuyi* as a synonym of *Lamprotula crassa*.

Simpson (1914) was the first to notice that *G. crassa* presented some typical margaritiferid conchological features (i.e. mantle attachment scars), but due to other distinct characters (e.g., heavy shell, well developed teeth and deep umbo cavity) it was retained within the Unionidae. Later, Morrison (1975) also noted that *Gibbosula* had the same characters now known to characterize the Margaritiferidae. However, this information was overlooked by most malacologists who continued to follow Haas (1969a) and kept *G. crassa* and *G. confragosa* under *Lamprotula* (e.g. Prozorova et al., 2005; Graf and Cummings, 2007). Finally, some authors recently described conchological differences between the two *Gibbosula* species and *Lamprotula*, and recognized *Gibbosula* as a separate genus within Unionidae (He and Zhuang, 2013; Graf and Cummings, 2018). Furthermore, based on conchological similarities, a third species of *Gibbosula* was recently described, i.e. *Gibbosula nanningensis* (Qian et al., 2015).

The genus *Lamprotula* was recently revealed to be polyphyletic and divided into *Lamprotula* s.s. and *Aculamprotula* (Zhou et al., 2007; Pfeiffer and Graf, 2013). These authors also noted that all species of *Lamprotula* should be comprehensively analyzed in order to clarify their status and relationships. For instance, based on molecular analyses, *Lamprotula rochechouartii* has been moved to Margaritiferidae (Huang et al., 2017). In addition, morphological and molecular characteristics of six specimens of *G. crassa* collected from Bang River, Cao Bang Province, Vietnam in 2016, suggested that the species did not belong to the Unionidae but to the Margaritiferidae (Bogan and Do, 2016). The reassignment of these two Asian species (i.e. *L. rochechouartii* and *G. crassa*) from the Unionidae to the Margaritiferidae raises the question whether there are other overlooked species of Margaritiferidae within this group. To address this issue, the congeneric *G. confragosa* and *L. rochechouartii* shell types were here analyzed as well as other types of *Lamprotula* sp. for potentially misplaced margaritiferids.

Under these considerations, the present study aimed to: (i) perform a detailed morphological characterization of collected *G. crassa* specimens, and available museum specimens of all Margaritiferidae, *Lamprotula* and *Gibbosula*; (ii) sequence and characterize the whole F-type mitogenome of *G. crassa*; (iii) produce a robust phylogeny of the Margaritiferidae using five (nuclear and mitochondrial) markers and discuss the systematics and taxonomy within the family; (iv) compare anatomical, conchological and ecological characters within and among all retrieved clades; and (v) describe the potential origin and ancient

radiations of the Margaritiferidae and detect the most probable ancestral geographic areas on the basis of a new multi-locus fossil-calibrated phylogenetic model, using the most complete sampling of taxa to date and an expanded calibration dataset.

## 2. Materials and methods

### 2.1. Sampling and museum specimens

Six specimens of *G. crassa* were collected during a survey in northern Vietnam in the Bang River, Cao Bang Province, Vietnam, in 2016. Specimens were deposited as vouchers at the North Carolina Museum of Natural Sciences, United States of America (NCSM 102193, 102194) and at the Institute of Ecology and Biological Resources, Hanoi, Vietnam (IEBR-FM 01-03). Museum specimens of *Gibbosula*, *Lamprotula* and Margaritiferidae, including the type specimens of *Unio mansuyi* and *G. confragosa*, were analyzed for morphology and/or genetics (Table 3 and Supplementary Table 1). Foot tissue samples were collected and preserved in 96% ethanol for DNA extraction.

### 2.2. DNA extractions, sequencing, assembly and annotation

DNA was extracted from foot samples of two *G. crassa* individuals and other margaritiferid specimens (Table 3) following Froufe et al. (2016). The complete F-type mitogenome of a single *G. crassa* sample was then sequenced and assembled using an established pipeline (Gan et al., 2014). Mitochondrial gene annotations were performed using MITOS (Bernt et al., 2013). The final tRNAs gene limits were rechecked with ARWEN (Laslett and Canbäck, 2008). Finally, in-house scripts were applied to adjust the mtDNA protein-coding limits since MITOS seems to underestimate gene length (for details, go to <https://figshare.com/s/a756ef19cec8f65d506a>). The whole mitogenome sequence has been deposited in GenBank (MH319826). The mitogenome was then visualized using GenomeVx (Conant and Wolfe, 2008) (Supplementary Fig. 1). The mitochondrial 16S rRNA and Cytochrome c Oxidase I (COI), and the nuclear 18S rRNA, 28S rRNA, and Histone 3 (H3) gene fragments were amplified from the extracted gDNAs of both *G. crassa* and the remaining margaritiferid species, following the conditions described in Bolotov et al. (2016) and Araujo et al. (2017).

### 2.3. Phylogenetic analyses

Individual alignments were performed for each of the five markers: COI - 654 bp, 16S - 475 bp, 18S - 1778 bp, 28S - 307 bp, and H3 - 327 bp. Each alignment was constructed with up to two representatives from all available Margaritiferidae species, including GenBank sequences (Table 3). Representative species from each of the families of the Unionida and from *Neotrigonia*, Trigonidae, the marine sister group of the Unionida (Giribet and Wheeler, 2002), were included as outgroups (Table 3). All individual datasets were aligned using the standalone version of GUIDANCE2 (Sela et al., 2015) with the MAFFT multiple sequence global pair alignment algorithm (Kato and Standley, 2013). The following GUIDANCE parameters were used: GUIDANCE score algorithm; 100 bootstrap replicates; and a column cut-off score of 0.8. Substitution saturation tests for all codon positions were accomplished in the protein-coding loci (COI, and H3) as implemented in DAMBE 6 (Xia, 2017). Phylogenetic analyses were then performed by Bayesian Inference (BI) and Maximum Likelihood (ML) on 13 partitioned datasets from a single marker to a combination of markers as follows: (1) combined dataset 1: COI (3 codons) + 16S + 18S + 28S + H3 (3 codons); (2) combined dataset 2: COI + 16S + 18S + 28S + H3; (3) mtDNA 1: COI (3 codons) + 16S; (4) mtDNA 2: COI + 16S; (5) COI (3 codons); (6) COI; (7) 16S; (8) nDNA: 18S + 28S + H3 (3 codons); (9) nDNA: 18S + 28S + H3; (10) 28S; (11) 18S; (12) H3 (3 codons); and (13) H3. For the BI analyses, the best-fit models of nucleotide substitution for each partition were previously selected

**Table 3**

List of specimens analysed, GenBank references, specimen number, locations and museum voucher references. \* not generated from a single individual. IEBR – Institute of Ecology and Biological Resources, Hanoi, Vietnam; MNCS – Museo Nacional de Ciencias Naturales, Madrid, Spain; NCFM – Nanchang Freshwater Mollusk Collection, Nanchang University, Nanchang, Jiangxi Province, China; IEPN – Russian Museum of Biodiversity Hotspots; MCZ – Museum of Comparative Zoology, Harvard University, USA; UAUC – University of Alabama Unionid Collection, USA; BivAToL – Bivalve Tree of Life Project, USA.

Taxon	Specimen	COI	16S	18S	28S	H3	Location	Voucher
UNIONIDA								
MARGARITIFERIDAE								
GIBBOSULINAE								
<i>Gibbosula crassa</i>	1	MH293546	MH293536	MH293539	MH293542	MH293549	Bang River, Cao Bang, Vietnam	IEBR FM GC01
<i>Gibbosula crassa</i>	2	MH293547	MH293537	MH293540	MH293543	MH293550	Bang River, Cao Bang, Vietnam	IEBR FM GC03
<i>Gibbosula laosensis</i>	1	KU763224	KU763193	KU763255	KU763298	KU763342	Mun River, Thailand	
<i>Gibbosula laosensis</i>	2	KU763225	KU763194	KU763256	KU763299	KU763343	Luang Prabang, Laos	MNCS 15.07/12038 (N1687)
<i>Gibbosula rochehouartii</i>	1	MF072498	MF072505	MF072519	MF072512	MF072526	Poyang Lake, Yangtze, China	
<i>Gibbosula rochehouartii</i>	2	MF072502	MF072509	MF072523	MF072516	MF072530	Poyang Lake, Yangtze, China	
MARGARITIFERINAE								
<i>Cumberlandia monodonta</i>	1	AY579131	AY579089	AY579105	AY579121	AY579144	Missouri, USA	
<i>Cumberlandia monodonta</i>	2	MH293545	MH293535	MH293538	MH293541	MH293548	Meramec River, Missouri, USA	
<i>Margaritifera dahurica</i>	1	KJ161516	KJ943526	KT343730	KT343738	AY579133	Ilistaya River, Primorye, Russia	IEPN d0088/6
<i>Margaritifera dahurica</i> *	2	KJ161520	KJ943527	KJ943531	MH293544	MH293551	Ilistaya River, Primorye, Russia	IEPN d0089/2
<i>Margaritifera falcata</i>	1	AY579128	AY579085	AY579101	AY579117	AY579141	Idaho, USA	MCZ DNA100844
<i>Margaritifera falcata</i>	2	AY579127	AY579084	AY579100	AY579116	AY579140	North Umpqua River, Oregon, USA	MCZ DNA100699
<i>Margaritifera hembeli</i>	1	KU763218	KU763189	KU763250	KU763293	KU763336	Valentine Creek, Louisiana, USA	
<i>Margaritifera hembeli</i>	2	KU763219	KU763190	KU763251	KU763294	KU763337	Brown Creek, Louisiana, USA	
<i>Margaritifera laevis</i>		KU763222	KU763192	KU763253	KU763296	KU763340	Iwaizumi, Honshu, Japan	MNCS-FW1502-2
<i>Margaritifera margaritifera</i>	1	KU763227	KU763196	KU763258	KU763301	KU763345	Locust Creek, Pennsylvania, USA	
<i>Margaritifera margaritifera</i>	2	AF303342	AF303301	KU763274	KU763317	KU763360	Nore River, Ireland	MNCS FW1490-1
<i>Margaritifera marrianae</i>		KU763243	KU763214	KU763283	KU763326	KU763369	Hunter Creek, Alabama, USA	UAUC 1651
<i>Margaritifera middendorffi</i>	1	AY579124	AY579081	AY579092	AY579108	AY579134	Iturup, Kuril Islands, Russia	MCZ DNA100685
<i>Margaritifera middendorffi</i>	2	KJ161547	KJ943528	KT343726	KT343735	MH293552	Nachilova River, Kamchatka, Russia	IEPN d0099/6
<i>Pseudunio auricularius</i>	1	AY579125	AY579083	AY579097	AY579113	AY579137	Ebro River, Tarragona, Spain	MCZ DNA100674
<i>Pseudunio auricularius</i>	2	AF303309	AF303274	KU763247	KU763290	KU763333	Canal Imperial, Zaragoza, Spain	MNCS-FW1238-12
<i>Pseudunio homsensis</i>		KX550090	KX550092	KX550088	KX550086	MH293553	Karasu River, Turkey	
<i>Pseudunio maroccanus</i>	1	EU429678	EU429689	KU763281	KU763324	KU763367	Oum Er Rbia River, Morocco	MNCS N1254
<i>Pseudunio maroccanus</i>	2	EU429679	EU429691	KU763282	KU763325	KU763368	Laabid River, Morocco	MNCS N1264
UNIONIDAE								
<i>Lampsilis cardium</i>		KX713472	KX713226	KX713305	KX713394	KX713547	Illinois, USA	BivAToL-421
<i>Potomida littoralis</i>		KP217871	KP217981	KU763287	KU763330	KU763373	Cadiz, Spain	MNCS N706
<i>Unio pictorum</i>		KC429109	KC429266	KC429349	KC429447	KC429186	Thames River, UK	BivAToL-204
HYRIDAE								
<i>Hyridella australis</i>		KX713467	KX713224	KX713301	KX713389	KX713545	New South Wales, Australia	BivAToL-378
<i>Tripodion corrugatus</i>		KX713505	KX713262	KX713352	KX713438	KX713585	Peru	BivAToL-380
<i>Vesunio ambiguus</i>		KC429106	KC429263	KC429346	KC429444	KC429183	New South Wales, Australia	BivAToL-379
MULLERIIDAE								
<i>Anodontites elongata</i>		KX713444	KX713190	KX713268	KX713357	KX713512	Peru	BivAToL-323
<i>Lamproscapha ensiformis</i>		KX713471	KX713225	KX713304	KX713393	KX713546	Peru	BivAToL-382
ETHERIIDAE								
<i>Etheria elliptica</i>		KX713462	KX713219	KX713296	KX713384	KX713540	Zambia	BivAToL-401
IRIDINIDAE								
<i>Aspatharia pfeifferiana</i>		KC429107	KC429264	KC429347	KC429445	KC429184	Chambeshi River, Zambia	BivAToL-330
<i>Chambardia wahlbergi</i>		KX713448	KX713202	KX713277	KX713365	KX713520	Zambia	BivAToL-405
<i>Mutela hargeri</i>		KX713482	KX713237	KX713317	KX713405	KX713559	Zambia	BivAToL-401
TRIGONIDA								
TRIGONIIDAE								
<i>Neotrigonia lamarckii</i>		KC429105	KC429262	KC429345	KC429443	KC429182	North Stradbroke Island, Australia	BivAToL-97
<i>Neotrigonia margaritacea</i>		U56850	DQ280034	AF411690	AF411689	AY070155	Tasmania, Australia	

(Supplementary Table 2), under the Bayesian Information Criterion (BIC) using JModelTest 2.1.10 (Durriba et al., 2012). BI analyses were performed in MrBayes v3.2.6 (Ronquist et al., 2012) using the previously selected models. Analyses were initiated with program-generated trees and four Markov chains with default incremental heating. Two independent runs of  $20 \times 10^6$  generations were sampled at intervals of 1,000 generations producing a total of 20,000 trees. Burn-in was determined upon convergence of log likelihood and parameter values using Tracer 1.6 (Rambaut et al., 2014). For the ML phylogenetic analyses, sequences were analyzed in RaxML 8.0.0 (Stamatakis, 2014) with 1,000 bootstrap replicates, assuming a GTR + G + I model for each partition.

#### 2.4. Morphological and ecological assessments

To evaluate the systematics within Margaritiferidae and detect other potential margaritiferid species, detailed conchological and anatomical characters were evaluated on newly collected *G. crassa* specimens and on museum specimens of *Gibbosula*, *Lamprotula* and Margaritiferidae, including the type specimens of *Unio mansuyi* and *G. confragos*. Bibliographic data on the major ecological and physiological traits were also compiled for all margaritiferid species (Table 4). To characterize and compare glochidial size, the glochidial size index (Gln) was calculated following Lopes-Lima et al. (2017a).

**Table 4**Biological and ecological characters. (Gln) glochidial size index. Superscripts: <sup>U</sup>unknown; <sup>R</sup>rivers; <sup>L</sup>lakes.

	Host fish	Glochidia size (Gln)	Principal Habitats	Flow	Substrate	Water chemistry	References
<i>G. confragosa</i>	<sup>U</sup>	<sup>U</sup>	Rivers-floodplain <sup>L</sup>	<sup>U</sup>	<sup>U</sup>	<sup>U</sup>	He and Zhuang (2013)
<i>G. crassa</i>	<sup>U</sup>	<sup>U</sup>	Medium <sup>R</sup>	Moderate-strong	Boulder, cobble	Hard	Bogan and Do (2016)
<i>G. laosensis</i>	<sup>U</sup>	<sup>U</sup>	Headwater <sup>R</sup>	Moderate-strong	Sand, gravel boulder	Moderate-hard oligotrophic	Bolotov et al. (2014)
<i>G. rochechouartii</i>	<sup>U</sup>	<sup>U</sup>	Rivers-floodplain <sup>L</sup>	Slow-Moderate	Hard mud	Soft-moderate	Do (2011a)
<i>G. polysticta</i>	<sup>U</sup>	<sup>U</sup>	Rivers-floodplain <sup>L</sup>	Slow-Moderate	<sup>U</sup>	Oligotrophic	Do (2011b)
<i>C. monodonta</i>	Hiodontidae	0.004	Medium-large <sup>R</sup>	Moderate-strong	Under flat rocks rock crevices	Hard	S. McMurray pers. com. Sietman et al. (2017) Williams et al. (2008)
<i>M. dahurica</i>	Salmonidae	0.006	Headwater <sup>R</sup> -large <sup>R</sup>	Moderate-strong	Sand, gravel	Oligotrophic soft	Bolotov et al. (2015)
<i>M. falcata</i>	Salmonidae	0.006	Headwater <sup>R</sup> -large <sup>R</sup>	Moderate-strong	Sand, gravel	Oligotrophic soft	Nedeau et al. (2009)
<i>M. hembeli</i>	Esocidae	<sup>U</sup>	Headwater <sup>R</sup>	Moderate	Sand, gravel	Oligotrophic soft	Paul Johnson pers. com.
<i>M. laevis</i>	Salmonidae	0.004	Headwater <sup>R</sup> -large <sup>R</sup>	Moderate-strong	Sand, gravel	Oligotrophic soft	Bolotov et al. (2015)
<i>M. margaritifera</i>	Salmonidae	0.005	Headwater <sup>R</sup> -large <sup>R</sup>	Moderate-strong	Sand, gravel cobble	Oligotrophic soft	Lopes-Lima et al. (2017c)
<i>M. marrianae</i>	Esocidae	0.002	Headwater <sup>R</sup>	Slow-moderate	Sand, gravel	Oligotrophic soft	Paul Johnson pers. com.
<i>M. middendorffi</i>	Salmonidae	0.006	Headwater <sup>R</sup> -large <sup>R</sup>	Slow-moderate	Sand, gravel	Oligotrophic soft	Bolotov et al. (2015)
<i>P. auricularius</i>	Acipenseridae Blenniidae Gasterosteidae	0.018	Middle-lower Moderate-large <sup>R</sup>	Moderate-strong	Sand, gravel	Hard	Prié et al. (2010) Prié et al. (2018)
<i>P. homsensis</i>	<sup>U</sup>	<sup>U</sup>	Middle-lower Moderate-large <sup>R</sup>	Slow-moderate	Silt	Mesotrophic	Vikhrev et al. (2017)
<i>P. maroccanus</i>	<sup>U</sup>	<sup>U</sup>	Middle-lower Moderate-large <sup>R</sup>	Moderate-strong	Gravel, cobble	Hard	Sousa et al. (2016, 2018)

## 2.5. Divergence time estimates

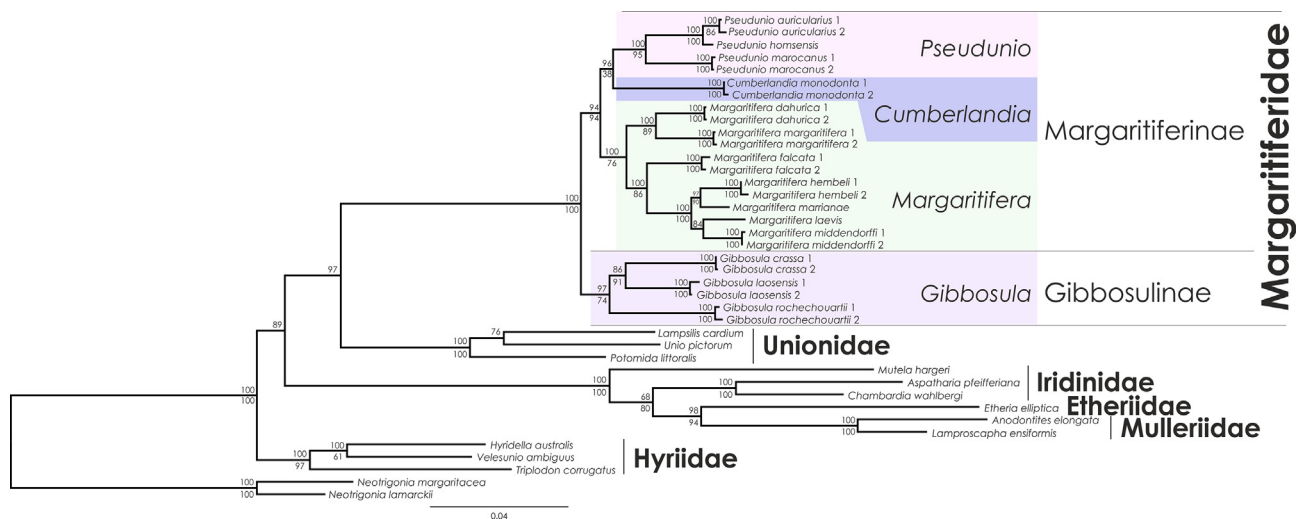
The acceptance of a global molecular clock to our multi-gene data set was estimated using the maximum likelihood test of MEGA6 (Tamura et al., 2013), which revealed that the null hypothesis of equal evolutionary rate throughout the tree was rejected ( $p < 0.001$ ). Thus, the time-calibrated haplotype-level Bayesian phylogeny was reconstructed in BEAST v. 1.8.4 based on multiple fossil calibration points using a lognormal relaxed clock algorithm with the Yule speciation process as the tree prior (Drummond et al., 2006, 2012; Drummond and Rambaut, 2007). Calculations were performed at the San Diego Supercomputer Center through the CIPRES Science Gateway (Miller et al., 2010). A fossil-calibrated ultrametric tree was obtained using BEAST v. 1.8.4. Similar settings were assigned to nine partitions (3 codons of COI + 16S rRNA + 18S rDNA + 28S rDNA + three codons of H3) as in the MrBayes analyses. The eight fossil calibrations were used for timing of the phylogeny (Supplementary Tables 3 and 4). Priors for out-group taxa were designated using a “Monophyly” option of BEAUti v. 1.8.4 (Drummond et al., 2012) as follows: (Trigoniidae, (Unionida)). Four replicate BEAST searches were conducted, each with 30 million generations. The trees were sampled every 1000th generation. The log files were checked visually with Tracer v. 1.6 for an assessment of the convergence of the MCMC chains and the effective sample size of parameters (Rambaut et al., 2014). The first 10% of trees were discarded as an appropriate burn-in. Almost all ESS values were recorded as  $> 1000$ , with a few values as  $> 250$ –800 and two values as  $> 100$ ; the subsequent distributions were similar to the prior distributions. The resulting tree files from four independent analyses were compiled with LogCombiner v. 1.8.4 (Drummond et al., 2012). The maximum clade credibility tree was obtained from 108,004 post-burn-in Bayesian trees using TreeAnnotator v. 1.8.4 (Drummond et al., 2012).

## 2.6. Ancestral geographic area reconstructions

Ancestral geographic area patterns were tested using three different approaches, i.e., Statistical Dispersal-Vicariance Analysis (S-DIVA), Dispersal-Extinction Cladogenesis (Lagrange configurator, DEC), and Statistical Dispersal-Extinction Cladogenesis (S-DEC) implemented in RASP v. 3.2 (Yu et al., 2015). The set of 108,004 fossil-calibrated binary trees that were combined from four runs of BEAST v. 1.8.4 (see above), was used for the ancestral area reconstruction. The user-specified, fossil-calibrated consensus tree, which was obtained based on this set of trees using TreeAnnotator v. 1.8.4 (see above), was used as a condensed tree. Outgroup sequences were removed from all datasets, using the appropriate option of RASP v. 3.2. Only a single sequence for each ingroup species was used for the analyses.

Six possible geographic areas of the in-group taxa were coded as follows: (A) Southeast Asia; (B) East Asia; (C) western North America; (D) eastern North America; (E) Mediterranean Region (South Europe, Middle East, and Morocco); and (F) Europe. Seven geographically unreliable distribution constrains were excluded from the input matrix as follows: Southeast Asia – western North America (AC), Southeast Asia – eastern North America (AD), Southeast Asia – Mediterranean Region (AE), Southeast Asia – Europe (AF), East Asia – eastern North America (BD), western North America – Mediterranean Region (CE), and western North America – Europe (CF). Geographic areas were assigned to the species as follows: Southeast Asia – *Gibbosula laosensis*, East Asia – *G. crassa*, *G. rochechouartii*, *Margaritifera dahurica*, *M. laevis*, and *M. middendorffi*, western North America – *M. falcata*, eastern North America – *Cumberlandia monodonta*, *Margaritifera marrianae*, and *M. hembeli*, and Mediterranean Region – *P. auricularius*, *P. homsensis*, and *Pseudunio maroccanus*. Taking into account the broad trans-Atlantic distribution of *Margaritifera margaritifera*, we assigned the ‘DEF’ range for this species.

The S-DIVA models were calculated with the following parameters: max areas = 2; allow reconstruction with max reconstructions = 100;



**Fig. 1.** Phylogenetic tree of the Paleoheterodonta obtained by Bayesian Inference (BI) and Maximum likelihood (ML) analyses of the combined (COI [3 codons] + 16S + 18S + 28S + H3 [3 codons]) dataset. Support values above the branches are posterior probabilities and bootstrap support below. Numbers after species names refer to specimen number (see Table 3).

max reconstructions for final tree = 1000; and allowing extinctions. The DEC and S-DEC analyses were run with default settings and max areas = 2. In addition to the evaluations obtained from each analysis separately, we used generalized results of all three modeling approaches, which were combined using an algorithm implemented in RASP v. 3.2.

## 2.7. Diversification rate analyses

The diversification rates were assessed based on the combined Bayesian phylogeny across the primary clades of the Margaritiferidae and the entire family. The set of 108,004 fossil-calibrated chronograms that were combined from four runs of BEAST v. 1.8.4 (see above) was used to construct semi-logarithmic lineage-through-time (LTT) plots in R-package ‘ape’ v. 4.0 (Paradis, 2012; Popescu et al., 2012) with the supplement of ‘paleotree’ v. 2.7 (Bapst, 2012). We did not include a simulation for missing taxa (Pybus and Harvey, 2000), because we assumed that our samples of the margaritiferid clades are nearly complete.

Two tests of a constant diversification rate for the endemic Indo-Chinese clades outlined above were calculated using ‘ape’ v. 4.0 based on the maximum clade credibility tree inferred from BEAST (Paradis, 2012; Popescu et al., 2012). First, the analysis of diversification with three survival models, i.e., a constant diversification model, a variable diversification rate through time (Weibull model), and diversification changes at a specified time point (Paradis, 1997). The delta parameter from the constant rate model of Paradis (1997) was used as mean diversification rates. Additionally, beta values of the Weibull model were tested where  $\beta > 1$  suggests declining and  $\beta < 1$  indicates an increasing rate of diversification. Second, the gamma statistic of Pybus and Harvey (2000) was applied. The null hypothesis of constant rate is rejected at the 5% level if a gamma statistic less than  $-1.645$ , which suggests a significantly decreasing rate of diversification through time (Pybus and Harvey, 2000).

## 3. Results

### 3.1. Mitogenome characteristics

The length of the newly sequenced female mitogenome haplotype of *G. crassa* (16,196 bp) is within the typical range of Unionida. It includes the 13 protein-coding genes, the gender-specific ORF described for all

Unionida mitogenomes with DUI system, 22 transfer RNA (tRNA) and 2 ribosomal RNA (rRNA) genes (Supplementary Fig. 1).

### 3.2. Phylogenetic analyses

The datasets included combinations of individual alignments (COI: 654 bp, 16S: 471 bp, 18S: 1778 bp, 28S: 309 bp, H3: 327 bp). No indels were observed and no stop codons were found after translating the sequences to amino acids in both COI and H3 datasets. All saturation tests showed significantly lower values of ISS than ISS.C (a critical value determined from computational simulation) indicating that the evaluated datasets (COI and H3) are not site saturated and are useful for phylogenetic comparisons. The resulting BI and ML trees of the concatenated (COI + 16S + 18S + 28S + H3) datasets generated the same topology within the ingroup, being the topology of the BI with 9 partitions presented (Fig. 1). With the exception of the Iridinidae, paraphyletic in all analyses, all Unionida families are represented by well-supported monophyletic clades, including the Margaritiferidae (Fig. 1; Table 5). Within the Margaritiferidae, four well supported clades can be found, identified here as *Gibbosula*, *Cumberlandia*, *Margaritifera*, and *Pseudunio* (Fig. 1; Table 5). In detail, a first division occurs between a *Gibbosula* clade (*G. rochechouartii* + *G. crassa* + *G. laosensis*) that is well supported in the BI analysis and a clade encompassing all remaining species (Fig. 1; Table 5). This latter clade is further divided into the *Cumberlandia* clade (*C. monodonta*) + the *Pseudunio* clade (*P. auricularius* + *P. homsensis* + *P. marcanus*) and the *Margaritifera* clade (*M. margaritifera*, *M. daturica*, *M. falcata*, *M. hembeli*, *M. laevis*, *M. marrianae*, and *M. middendorffi*) (Fig. 1; Table 5). The *Margaritifera* clade is further subdivided in the clade (*M. margaritifera* + *M. daturica*) sister to the “Pacific” clade (*M. falcata* + (*M. hembeli* + *M. laevis* + *M. marrianae* + *M. middendorffi*)) (Fig. 1; Table 5).

### 3.3. Morphological and ecological analyses

The literature review identified a total of 29 conchological, anatomical and physiological characters that are common to all analyzed Margaritiferid species and can therefore be used to diagnose the family (Table 2). Graf and Cummings (2006) listed five morphological synapomorphies for Margaritiferidae, characters: 7 - gills irregular scattered interlamellar connections; 8 - gills not fused with mantle posterior; 12 - pedal elevator muscle scars inconspicuous; 13 - anus located dorsal edge of posterior adductor muscle; and 27 - mantle attachment scars



**Table 5** Results of Repeatability Clade Analysis (RCA) of main clades corresponding to the preferred topology. High support values (BI posterior probability  $\geq 95\%$ , and ML bootstrap support  $\geq 70\%$ ) are highlighted in bold.

Clades	Analyses		Combined dataset					mtDNA					Nuclear				
	BI	ML	COI <sup>3</sup> + 16S + 18S + 28S + H3	COI + 16S + 18S + 28S + H3	COI <sup>3</sup> + H3 <sup>3</sup>	COI + 16S	COI <sup>3</sup> + 16S	COI + 16S	COI <sup>3</sup>	COI	16S	18S + 28S + H3 <sup>3</sup>	18S + 28S + H3	18S	28S	H3 <sup>3</sup>	H3
<i>Margaritifera</i>	100	100	99	99	100	100	100	100	99	58	-	-	-	-	-	-	
Pacific clade	76	100	84	84	90	93	90	58	78	24	-	-	37	-	-	-	
<i>Gibbosula</i>	86	97	85	99	95	89	62	61	83	-	100	100	98	-	-	-	
<i>Pseudunio</i>	74	100	64	64	65	61	52	61	78	78	98	99	60	85	-	-	
<i>Pseudunio</i> + <i>Cumberlandia</i>	96	96	93	93	100	100	96	75	64	64	100	100	40	57	-	-	
<i>Margaritifera</i> idae	38	38	47	47	78	84	79	42	68	68	91	91	93	-	-	-	
<i>Unionidae</i>	100	100	100	100	100	100	100	100	100	72	100	100	100	100	-	-	
<i>Etheriidae</i> + <i>Mulleriidae</i> + <i>Iridimidae</i>	100	100	100	100	95	100	94	94	74	74	100	100	100	100	-	-	
<i>Hyridae</i>	100	100	100	100	97	99	81	81	99	99	99	99	99	95	-	-	
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	100	100	100	100	55	93	76	98	97	97	100	100	84	100	100	100	
	97	97	98	98	76	75	70	72	63	63	93	93	-	-	82	91	

(Table 2). However, only three historically recognized characters, i.e. characters 7, 13 and 27, are synapomorphies of the Margaritiferidae since all other characters can be found in other members of the Unionida, outside the Margaritiferidae. In this study, we identified a new synapomorphy for the Margaritiferidae, i.e. papillae on the external surface of the excurrent aperture. In addition, two molecular characters are also synapomorphic, i.e. the F- and M- mitogenome gene orders (Lopes-Lima et al., 2017b).

Inspection of the conchological features revealed a few similarities across all species (Table 6). Mantle attachment scars were found consistently in all analyzed specimens and nacre color was generally white with the only exceptions being the purple nacre of *M. falcata* and *M. laevis*, and the peach color in the umbonal region of *G. laosensis* (Table 6). Interestingly, most of the inspected characters were distinct and consistent with the four clades retrieved with the phylogenetic analyses (i.e., *Gibbosula*, *Cumberlandia*, *Margaritifera*, and *Pseudunio*; Table 6). While thin shells are typical for *Cumberlandia*, thin to medium thick shells can be found in all species of *Margaritifera*. With the exception of *G. laosensis*, the remaining species belonging to *Pseudunio* and *Gibbosula* have ponderous, thick shells. All species within *Cumberlandia*, *Margaritifera* and *Pseudunio* have shallow and open umbo cavities (e.g., Fig. 2). Conversely, all species of *Gibbosula* have deep, compressed umbo cavities (e.g., Fig. 2), with the exception of *G. laosensis* (Table 6). Pseudocardinal teeth are also distinct among the clades (Fig. 2); while *Gibbosula* and *Pseudunio* species present large teeth (again with the exception of *G. laosensis*), *Margaritifera* presents peg-like smaller teeth, and those in *Cumberlandia* are reduced (Fig. 2). The lateral teeth are consistently well developed in most species across the clades, with a few exceptions (Table 6). However, the lateral teeth of species within *Pseudunio* and *Gibbosula* present vertical striations (except for *P. auricularius*), while this character is absent or visible only on the posterior end of laterals of *Cumberlandia* and *Margaritifera* species. Shell surface sculpture is also distinct across the genera (Table 6). Species within *Cumberlandia*, *Pseudunio* and *Margaritifera* are generally smooth, without any sculpture, the only exceptions being *M. hembeli* and *M. marrianae*, which present plications on the posterior slope and onto the posterior disk. A distinct pattern can be seen in *Gibbosula*, where all species, except *G. laosensis*, are strongly sculptured with pustules, plications or both (Table 6). Shell shape is also distinct among the four clades: species within *Gibbosula* present a typically convex ventral margin and a variable shell shape; *Cumberlandia* have a concave ventral margin and elongated shape; *Margaritifera* shells are elongated and typically straight to slightly concave ventral margin; and finally *Pseudunio* shells are elongated-oval with a straight to concave ventral margin (Table 6). The umbo in most of the examined shells was eroded and therefore hindered a proper analysis of its sculpture. Nevertheless, concentric bars in the umbo were present in all species, where this feature was visible (Table 6). All of the soft body anatomical traits were similar in all analyzed species (Table 7).

The ecological and other biological characters analyzed here also corroborate the existence of four genera (Table 4). The host fishes of *Margaritifera* species belong exclusively to the Salmonidae and the closely related Esocidae, while the hosts for *Pseudunio* and *Cumberlandia* do not belong to these fish families (Table 4). *Cumberlandia* uses two species of Hiodontidae, while members of three unrelated families of fish are found to be suitable for *P. auricularius* (Table 4). As for the other two species of *Pseudunio*, no hosts have yet been identified but no salmonid species occur sympatrically within their current known distribution (Table 4). The fish hosts for *Gibbosula* species are all unknown, although at least for the Southeast Asian taxa (*G. laosensis* and *G. crassa*) do certainly not include Salmonidae, since this family does not occur in this area (Table 4). The glochidia size of *P. auricularius* is much larger than those of *Margaritifera* and *Cumberlandia*. Since the glochidia of the other two *Pseudunio* and all *Gibbosula* species are undescribed, its utility for systematics still needs to be confirmed (Table 4). The habitat preferences are also distinct among the genera. While *Margaritifera* species

**Table 6**  
Analysed conchological characters of Margaritiferidae species. Superscripts: <sup>1</sup>W-shaped pustules on umbo and onto disk; <sup>2</sup>plications on posterior slope, posterior disk; <sup>3</sup>plications on posterior slope, pustules on umbo and disk.

	Shell thickness		Mantle attachment scars	Umbo pocket	Pseudocardinal teeth	Lateral teeth	Lateral teeth sculpture	Umbo sculpture	Nacre colour	Ventral margin	Shell shape	Surface sculpture
	Thick	Thin										
<i>G. confragosa</i>	Thick	Thin	Present	Deep Open	Large	Well developed	Reduced	unknown	white	slight convex	oval	yes <sup>1</sup>
<i>G. crassa</i>	Thick	Thin	Present	Deep Compressed Open	Large Peg like	Well developed Reduced	Yes	unknown	white	slight convex	rectangular	yes <sup>2</sup>
<i>G. laosensis</i>	Medium	Thick	Present	Deep Open	Large Peg like	Well developed	Yes	unknown	White peach umbo area white	slight concave	elongate	no
<i>G. polysticta</i>	Thick	Thin	Present	Deep Compressed	Large Peg like	Well developed	Yes	unknown	white	convex	oval	yes <sup>2</sup>
<i>G. rochechouartii</i>	Thick	Thin	Present	Deep Compressed	Large Peg like	well developed	Yes	unknown	white	straight convex	rectangular	yes <sup>3</sup>
<i>C. monodonta</i>	Thin	Thick	Present	Shallow Open	reduced Peg like	reduced	No	Concentric bars	white	concave	elongate	no
<i>M. dahurica</i>	Medium	Thin	Present	Shallow Open	reduced Peg like	reduced	No	unknown	white	straight	elongate	no
<i>M. falcata</i>	Thin-medium	Medium	Present	Shallow Open	reduced Peg like	Well developed	No	unknown	white or purple	straight slight concave	elongate	no
<i>M. hembéli</i>	Medium	Thin	Present	Shallow Open	reduced Peg like	Well developed	Posterior end	unknown	white	straight slight concave	elongate	yes <sup>2</sup>
<i>M. laevis</i>	Medium	Thin	Few	Shallow Open	reduced Peg like	Reduced	Posterior end	unknown	white or purple	straight slight concave	elongate	no
<i>M. margaritifera</i>	Thin-medium	Medium	Present	Shallow Open	reduced Peg like	Well developed	No	Concentric bars	white	straight slight concave	elongate	no
<i>M. marrianae</i>	Thin-medium	Medium	Present	Shallow Open	reduced Peg like	Well developed	Posterior end	Concentric almost double looped <sup>1</sup>	white	straight	elongate	yes <sup>2</sup>
<i>M. middendorffi</i>	Medium	Thin	Present	Shallow Open	reduced Peg like	Well developed	Posterior end	unknown	white	straight	elongate	no
<i>P. auricularius</i>	Thick	Thin	Present	Shallow Open	Large Peg like	Well developed	No	concentric bars	white	concave	elongate oval	no
<i>P. homsensis</i>	Thick	Thin	Few	Shallow Open	Large Peg like	Well developed	Yes	unknown	white	straight concave	elongate oval	no
<i>P. maroccanus</i>	Thick	Thin	Present	Shallow Open	Large Peg like	Well developed	Yes	concentric bars	white	straight concave	elongate oval	no

prefer oligotrophic soft-water rivers and are more prevalent in headwaters, *Pseudunio* generally inhabits the middle to lower sections of moderate to hard-water mesotrophic rivers. *Cumberlandia* seems to occur in habitats similar to those of *Pseudunio* (Table 4). However, contrary to all of the other genera it is mostly found in a very particular microhabitat, i.e. under large flat rocks or in rock crevices (Table 4). *Gibbosula* seems to be much more plastic in its habitat preferences (Table 4) although the ecological features of most species need to be more thoroughly studied.

### 3.4. Origin and ancient radiations of the Margaritiferidae

The combined results of the biogeographic modeling (S-DIVA, DEC and S-DEC approaches) based on the fossil-calibrated chronogram obtained from the relaxed molecular clock analyses returned a robust ancestral area reconstruction for the primary clades of the Margaritiferidae (Figs. 3 and 4, Supplementary Fig. 2, and Table 8). The model suggests that the Margaritiferidae Most Recent Common Ancestor (MRCA) was widespread across the eastern part of Laurasia (probability 55.0%). The S-DIVA, DEC, and S-DEC models support the same scenario (probability 53.3–58.3%). The origin of the crown group of the family was placed in the Jurassic (mean age 172 Ma, 95% HPD 168–178 Ma). Based on the combined biogeographic model, the Gibbosulinae MRCA most likely originated in East Asia (probability 78.6%), with a subsequent vicariance event separating the Southeast Asian species *G. laosensis* (probability 79.9%). The origin of the crown group of the subfamily is placed in the mid-Cretaceous (mean age ~ 103 Ma, 95% HPD 86–131 Ma).

The Margaritiferinae MRCA most likely evolved in the East Laurasia (East Asia + Mediterranean Region, probability 62.0%), with the crown group of the subfamily originating in the Late Jurassic (mean age ~ 151 Ma, 95% HPD 132–170 Ma). Among Margaritiferinae clades, the crown group of the *Cumberlandia* + *Pseudunio* clade most likely originated in the Early Cretaceous (mean age ~ 135 Ma, 95% HPD 129–146 Ma) within the Mediterranean region, with subsequent dispersal to eastern North America followed by a vicariance event (probability 45.0%). In contrast, S-DIVA model suggests a rather primary broad range of the MRCA across the Mediterranean Region and eastern North America followed by vicariance (probability 100%). The crown group of *Pseudunio* originated in the Mediterranean Region (probability 99.9%) in the Eocene (mean age 47 Ma, 95% HPD 35–66 Ma).

The crown group of *Margaritifera* is of Late Cretaceous origin (mean age 86 Ma, 95% HPD 51–131 Ma) and most likely evolved in East Asia (probability 52.4%). The sister species pair of *M. dahurica* and *M. margaritifera* diverged in the mid-Eocene (mean age 42 Ma, 95% HPD 34–57 Ma) via a dispersal event forming a continuous trans-Eurasian range of their MRCA followed by a vicariance event (probability 70.4%). The origin of the ‘Pacific’ clade, i.e., *M. falcata*, *M. laevis*, *M. middendorffi*, *M. hembeli*, and *M. marrianae*, is placed near the Paleocene – Eocene boundary (mean age 57 Ma, 95% HPD 46–73 Ma). The diversification of this group was largely associated with several dispersal and vicariance events via the Beringian land bridge (probability 49.2–86.0%).

### 3.5. Diversification rates

The lineage-through-time modeling suggests extremely slow diversification rates in the Margaritiferidae (Fig. 5). The constant-rate test suggests that all clades diversified under the pure-birth (constant) model (Supplementary Table 5). Paradis’ test of diversification with three survival models returned a declining diversification rate in *Gibbosula* and *Margaritifera*, but not in *Pseudunio* + *Cumberlandia* and the Margaritiferidae as a whole (Supplementary Table 5).

## 4. Discussion

### 4.1. Definition of the Margaritiferidae

Since the first definition of the Margaritiferidae by Ortmann, its supposed diagnostic characters have varied considerably (Table 2). Graf and Cummings (2006), based on a molecular (COI + 28S) and morphological phylogeny, revised margaritiferid synapomorphies noting that there was no previous consensus on characters diagnosing the family Margaritiferidae. These authors retained only five morphological synapomorphies, two conchological (characters 12 and 27, Table 2) and three anatomical (characters 7, 8, and 13, Table 2) characters. All other analyzed characters were considered plesiomorphic (Graf and Cummings, 2006). The main synapomorphies of the family were again re-evaluated by Araujo et al. (2017) (Table 2). They rejected Graf and Cummings (2006) character 12, and considered character 27 as the only conchological synapomorphy for the Margaritiferidae. These authors retained anatomical characters 7, 8, and 13, but were not able to fully evaluate the anal position in all taxa (see Table 2). Other characters previously used to characterize Margaritiferidae were found in other genera of the Unionidae (Table 2). Finally, a recent mitogenomics study provided the F- and M- type gene-orders of the Margaritiferidae as two additional synapomorphic diagnostic characters (Lopes-Lima et al., 2017b).

In the present study, 29 analyzed characters were common to all margaritiferid species, and therefore can be used to diagnose the family (Table 2). However, only six, i.e. characters 7, 13, and 27 (Table 2), the papillae on the external surface of the excurrent aperture, plus both mitogenome orders are synapomorphies of the Margaritiferidae. All of the other characters can be found on other members of the Unionida and *Neotrigonia*, outside the Margaritiferidae.

### 4.2. Expansion of Margaritiferidae

Based on morphological and molecular evidence, the family Margaritiferidae is here expanded to 16 species and separated into two subfamilies (i.e. Margaritiferinae and Gibbosulinae) and four genera (i.e. *Pseudunio*, *Cumberlandia*, *Margaritifera*, and *Gibbosula*) (Fig. 1; Table 9; Supplementary Table 6).

Until recently, two different species of *Gibbosula* used to be recognized. Firstly, the type species *G. crassa* was described by Wood (1815) from a specimen collected in an unknown location in China. Since then, only a few specimens of *G. crassa* or its synonym *Unio*

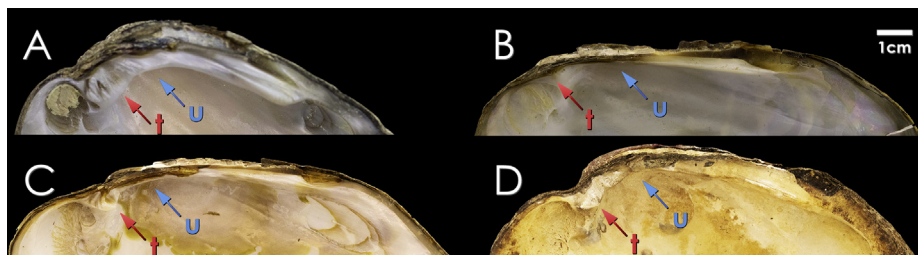


Fig. 2. Hinge plate and umbo cavity of Margaritiferidae. A - *Gibbosula crassa* (NCSM 102194.2), B - *Cumberlandia monodonta* (NCSM 55359.18), C - *Margaritifera margaritifera*, (NCSM 5771.1) D - *Pseudunio auricularius* (NCSM 44514.2). t - pseudocardinal teeth, u - umbo cavity.

**Table 7**  
Anatomical characters. \*Not analysed for anatomy.

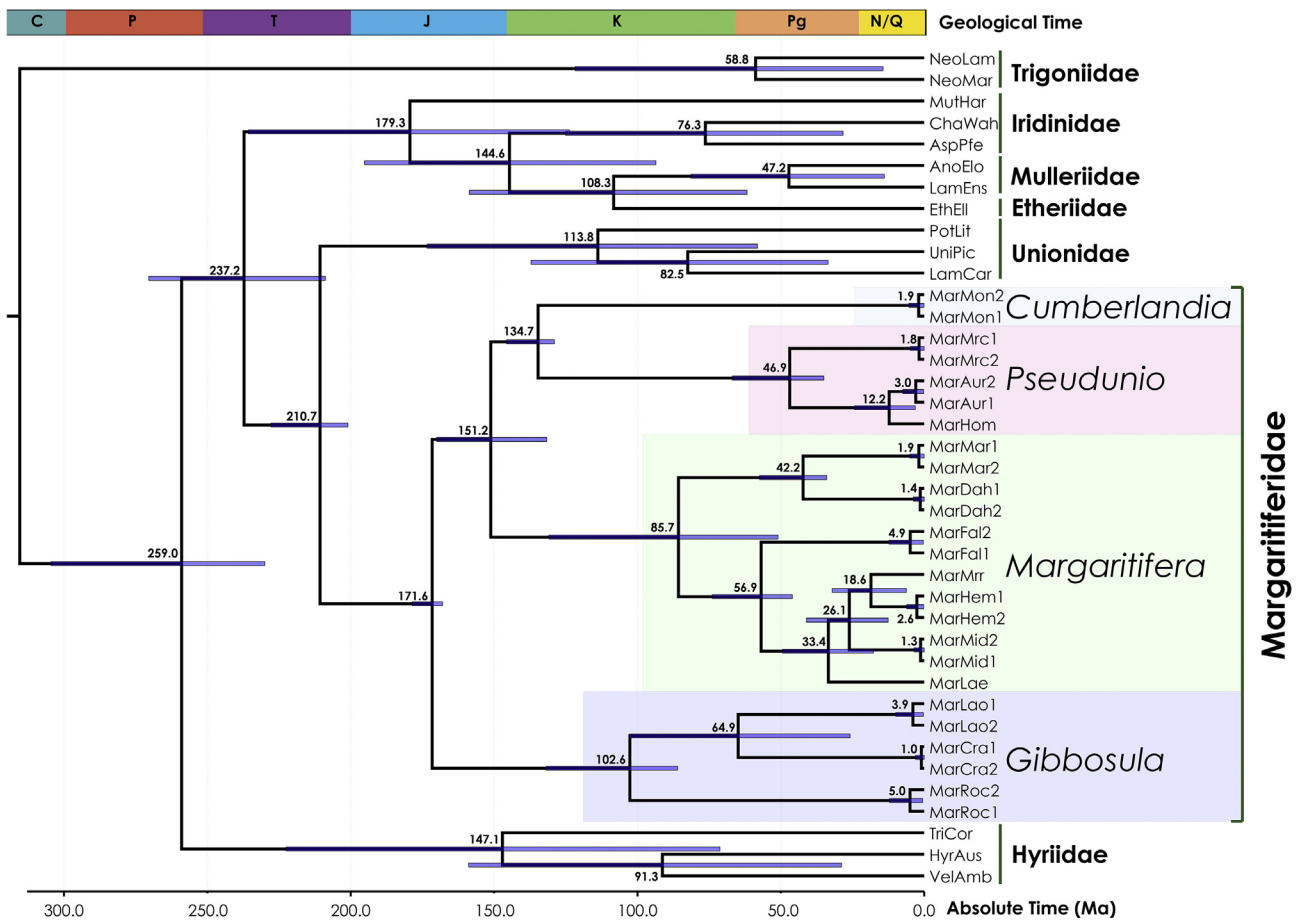
	Incurrent aperture	Excurrent aperture	Papillae exc. aperture	Anal position	Gill attachment	Gill structure	Labial Palp	Foot muscle pigmented	Diaphragm
<i>G. confragosa</i> *	-	-	-	-	-	-	-	-	-
<i>G. crassa</i>	Arborescent	Grenulated	Yes	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>G. laosensis</i>	Arborescent	Grenulated	-	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>G. polysticta</i> *	-	-	-	-	-	-	-	-	-
<i>G. rochechouartii</i>	Arborescent	Grenulated	-	posterior dorsal	Unknown	Interrupted	Unknown	Yes	Ridge
<i>C. monodonta</i>	Arborescent	Grenulated	Yes	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>M. daturica</i>	Arborescent	Grenulated	Yes	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>M. falcata</i>	Arborescent	Grenulated	Yes	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>M. hembeli</i>	Arborescent	Grenulated	Yes	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>M. laevis</i>	Arborescent	Grenulated	-	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>M. margaritifera</i>	Arborescent	Grenulated	No	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>M. marrianae</i>	Arborescent	Grenulated	Yes	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>M. middendorffi</i>	Arborescent	Grenulated	Yes	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>P. auricularius</i>	Arborescent	Grenulated	-	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>P. homsenis</i>	Arborescent	Grenulated	-	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge
<i>P. marocanus</i>	Arborescent	Grenulated	Yes	Posterior dorsal	Anterior	Interrupted	Falcate	Yes	Ridge

*mansuyi* have been collected, almost a hundred years ago, in the Bang River, Pearl/Zhu River basin, either in China or Vietnam. During recent surveys, the species was re-discovered but seems to be quite rare and restricted to the middle stretches of Bang River in Cao Bang Province, Vietnam. The second previously recognized species within *Gibbosula* is *G. confragosa*, described by Frierson from a single specimen, collected in an uncertain location in north China. Although Prozorova et al. (2005) stated that this species was present in the Yangtze and other Eastern Chinese basins, there is no current evidence of its occurrence in the Yangtze basin. In fact, since *G. confragosa* original description, only one specimen has been collected and described, i.e. a specimen from Lake Baiyangdian, Hai River basin, Hebei province, northern China, previously incorrectly labelled as *U. microstictus* (He and Zhuang, 2013). Beside the shell surface sculpture differences, the disjunct distribution of *G. confragosa* suggests a distinct specific rank.

The newly found specimens and shells of *G. crassa* from Vietnam, here analyzed in detail, feature the characteristics diagnostic and synapomorphies of the Margaritiferidae (Tables 6 and 7). Additionally, the F-type whole mitogenome sequence of one of the specimens collected revealed the typical gene order of the Margaritiferidae (Supplementary Fig. 1), which is unique to this family (Lopes-Lima et al., 2017b). The phylogenetic analyses also confirm the inclusion of *G. crassa* in the Margaritiferidae family, forming a well-supported clade (BI only) with *G. laosensis* and *G. rochechouartii*. The shells of *G. confragosa* and *G. polysticta* present mantle attachment scars exclusive to the Margaritiferidae and were therefore included in the Margaritiferidae (Fig. 1; Table 6) and assigned to *Gibbosula* due to similarities in shell characteristics with the type species, *G. crassa* (Table 6). An additional *Gibbosula* species was recently described, *Gibbosula nanningensis* (Qian et al., 2015). No specimens of this species were available for evaluation, but based on the description, i.e. the absence of mantle attachment scars and its distinct morphology, we reject its assignment to *Gibbosula* and therefore to the Margaritiferidae. A detailed systematic description of the species within *Gibbosula* is presented in Supplementary Appendix 1.

Most of the earlier works on the systematics of margaritiferid genera have failed to retrieve monophyletic clades based on morphological characters alone (Huff et al., 2004). More recently, authors showed that previous generic assignments were inconsistent with the molecular phylogenetic patterns (Huff et al., 2004; Bolotov et al., 2016; Araujo et al., 2017). Whilst all these studies recognized *Margaritifera* as the single genus within the Margaritiferidae, the rationale for this generic assignment is not always clear. In fact, Bolotov et al. (2016) suggested that the clades found should be assigned to distinct subgenera but maintained *Margaritifera* as a monotypic genus due to the morphological similarity and moderate level of genetic divergence among the clades.

In the present study, four well supported clades (mainly in the BI analyses) were consistently retrieved using the most comprehensive Margaritiferidae data set analyzed to date (Fig. 1, Table 5). The divergence of these clades, corresponding to the subgenera identified by Bolotov et al. (2016), is older (from late Jurassic to early Cretaceous) than previously believed due to the inclusion of new species and improvements in the fossil calibration (see details below). The present study further revealed a set of consistent morphological, biological and ecological features characteristic to each of the clades. Based on these results, each clade was assigned to a separate generic rank (Fig. 1). The genus *Gibbosula* includes the species *G. crassa*, *G. confragosa*, *G. laosensis*, *G. polysticta*, and *G. rochechouartii* (Fig. 1; Supplementary Table 6). The morphological and ecological features of *Gibbosula* are consistently more distinct from the other three genera (Tables 6 and 7). This agrees with the molecular phylogeny developed here, which presents two main clades, one with all *Gibbosula* species and another including (*Margaritifera* + (*Cumberlandia* + *Pseudunio*)) (Fig. 1; Table 5). Due to their old divergence (late Jurassic, see below) and clear morphological differences, a subfamily rank was assigned to each of these



**Fig. 3.** Fossil-calibrated ultrametric chronogram of the Margaritiferidae calculated under a lognormal relaxed clock model and a Yule process speciation implemented in BEAST 1.8.4 and obtained for the complete data set of mitochondrial and nuclear sequences (nine partitions: three codons of COI + 16S rRNA + 18S rDNA + 28S rDNA + three codons of H3). Bars indicate 95% confidence intervals of the estimated divergence times between lineages (Ma). Black numbers near nodes are mean ages (Ma). Stratigraphic chart according to the International Commission on Stratigraphy, 2015.

two clades, i.e. Margaritiferinae and Gibbosulinae Bogan, Bolotov, Froufe, Lopes-Lima, new subfamily. Distribution of the two Margaritiferidae subfamilies is mutually exclusive, with the Gibbosulinae being restricted to East and Southeast Asia, while the Margaritiferinae are widespread throughout the rest of the Holarctic (Fig. 6).

#### 4.3. Systematics

**Margaritiferidae** Henderson, 1929 (Ortmann, 1910)

**Type genus:** *Margaritifera* Schumacher, 1816

**Type species:** *Mya margaritifera* Linnaeus, 1758

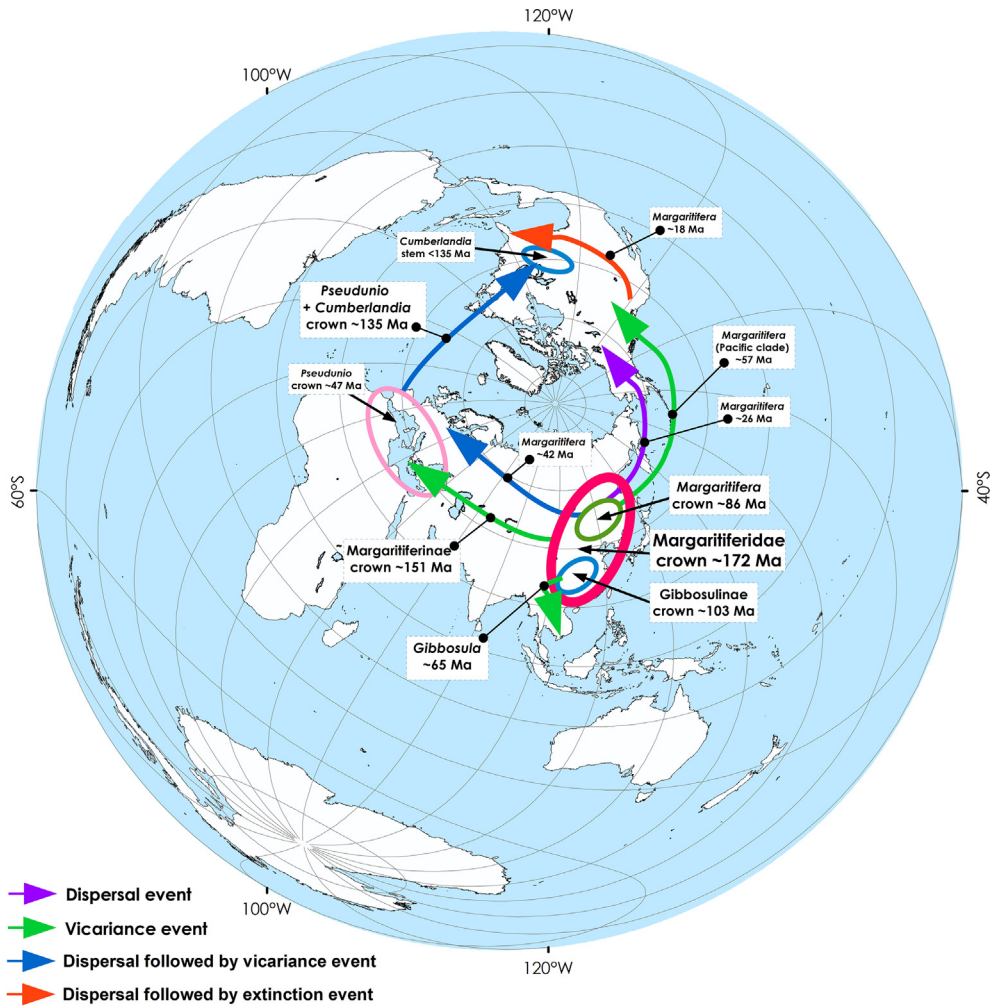
**Type Locality:** “Habitat in totius orbis arctici cataractis” [Arctic habitat in the entire world cataracts] (Linnaeus, 1758).

**Comments:** This family was split from the Unionidae and four more species were moved from the Unionidae, refining the definition of the family and the variation in shell shape, anatomy and geographic distribution.

**Diagnosis:** Shell shape varies from elongate to rectangular or oval, shell thickness varies from thin to very thick. Posterior ridge of shell varies from low and rounded to well developed and posterior slope with or without plications, maximum shell length about 200 mm. Umbo sculpture presents angular un-joined chevron-like hooks but Zieritz et al. (2015) have referred to this sculpture as double looped. Periostracum color varies from a dark green to typically black. Lateral teeth vary from vestigial to well-defined with vertical sculpture on all or the posterior portion of the teeth. Pseudocardinal teeth vary from peg like in both valves to thick and massive. Umbo pocket varies from shallow

and open to deep and compressed (Fig. 2). Lateral mantle attachment scars are present in varying numbers inside of the pallial line. Nacre varies from white to purple. Mantle free around edges of animal. Apertures open without any mantle fusions to separate the incurrent, excurrent or supra-anal apertures. Branchial and supra-branchial areas not separated posteriorly by gills, but by a diaphragm comprised by a ridge of mantle tissue. Incurrent aperture with arborescent papillae and in at least one species has simple papillae on the external side of incurrent aperture mantle surface typically along the length of the aperture. Excurrent aperture smooth or crenulated, lacking papillae, external side of excurrent aperture mantle surface typically has small papillae along the length of the aperture. Gills attached to the visceral mass only anteriorly. Labial palps falcate in outline. Interlamellar gill connections are “irregularly scattered or forming irregular oblique row, or incomplete septa which run obliquely to the direction of the gill filaments” (Heard and Guckert, 1970). Gills lack water tubes. Marsupium occupies all four gills. Muscular section of the food pigmented either dark red or black. Anus is located on the posterior dorsal margin of the posterior adductor muscle. This family is a short term brooder or tachyitic. Most species are dioecious with only a few listed as hermaphroditic or having hermaphroditic populations. Fish hosts, when known, are Salmonidae, Esocidae, Acipenseridae, Blenniidae, Gasterosteidae, and Hiodontidae, with each margaritiferid genus being restricted to a single or few host fish families. Female and male mitochondrial genome orders are unique for Margaritiferidae and different from Unionidae.

**Distribution:** The family is found in North America north of



**Fig. 4.** Simplified scheme of origin and expansion routes inferred across clades of the Margaritiferidae. The black numbers show the mean age of putative expansion events obtained from the multi-locus fossil-calibrated phylogenetic model (see Fig. 3 for details). Circles indicate the putative places of origin of the family and several clades. The map was created using ESRI ArcGIS 10 software ([www.esri.com/arcgis](http://www.esri.com/arcgis)); the topographic base of the map was created with ESRI Data and Maps.

Mexico, Western and Northern Europe, western North Africa in Morocco, western Middle-East in Syria, Turkey and Lebanon, Southeast Asia and north to eastern Russia and Japan (Fig. 6).

**Subfamily Margaritiferinae Henderson, 1929**

**Type genus:** *Margaritifera* Schumacher, 1816

**Type species:** *Mya margaritifera* Linnaeus, 1758

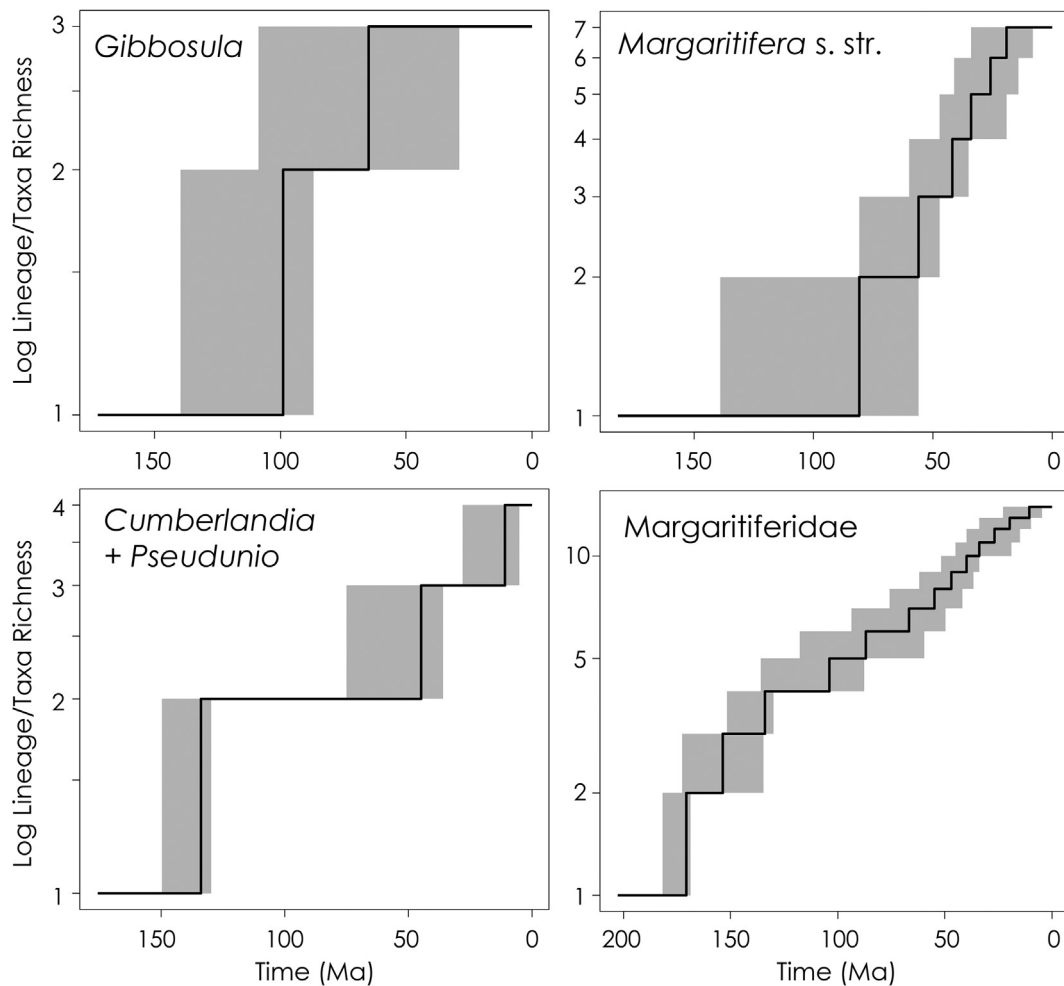
**Type Locality** “Habitat in totius orbis arctici cataractis” [Arctic habitat in the entire world cataracts] (Linnaeus, 1758).

**Comments:** This subfamily contains three genera, *Margaritifera*, *Cumberlandia* and *Pseudunio*. Species of *Cumberlandia* and *Margaritifera* have thin to medium thick, elongated shells, while *Pseudunio* has thick shells and well-developed teeth. All have a shallow open umbo cavity.

**Table 8**

The most probable ancestral areas of the primary clades within Margaritiferidae inferred from three different statistical modeling approaches. High support values (probability ≥ 70%) are highlighted in bold. \*Mediterranean + Eastern North America.

Clades	Ancestral areas	Biogeographic events	Probability of ancestral areas (%)			
			S-DIVA	DEC	S-DEC	Combined results
Margaritiferidae	E. Asia + Mediterranean	Dispersal	58.3	53.3	53.4	55.0
Gibbosulinae ( <i>Gibbosula</i> )	E. Asia	Dispersal	<b>100.0</b>	67.6	68.2	<b>78.6</b>
<i>G. laosensis</i> – <i>G. crassa</i>	E. Asia + SE. Asia	Vicariance	<b>100.0</b>	<b>71.2</b>	68.6	<b>79.9</b>
Margaritiferinae ( <i>Margaritifera</i> + <i>Pseudunio</i> + <i>Cumberlandia</i> )	E. Asia + Mediterranean	Vicariance	41.7	<b>73.4</b>	<b>71.0</b>	62.0
<i>Margaritifera</i>	E. Asia	Dispersal	65.0	49.1	43.1	52.4
<i>M. dahurica</i> – <i>M. margaritifera</i>	E. Asia + Europe	Dispersal + Vicariance	50.0	<b>81.4</b>	<b>79.9</b>	<b>70.4</b>
<i>M. falcata</i> – <i>M. laevis</i> (Pacific clade)	E. Asia + W. North America	Vicariance	<b>100.0</b>	<b>81.4</b>	<b>76.7</b>	<b>86.0</b>
<i>M. laevis</i> – <i>M. middendorffi</i>	E. Asia	Dispersal	<b>97.3</b>	63.2	67.3	49.2
<i>M. middendorffi</i> – <i>M. hembeli</i>	E. Asia + W. North America	Dispersal + Vicariance	33.3	66.0	63.8	54.4
<i>M. hembeli</i> – <i>M. marrianae</i>	W. North America + E. North America	Dispersal + Extinction	33.3	40.5	41.9	38.2
<i>Pseudunio</i> + <i>Cumberlandia</i>	Mediterranean	Dispersal + Vicariance	<b>100.0</b> *	64.6	<b>70.5</b>	45.0
<i>Pseudunio</i>	Mediterranean	Intra-area radiation	<b>100.0</b>	<b>100.0</b>	<b>99.7</b>	<b>99.9</b>
<i>P. auricularius</i> – <i>P. homsensis</i>	Mediterranean	Intra-area radiation	<b>100.0</b>	<b>100.0</b>	<b>100.0</b>	<b>100.0</b>



**Fig. 5.** Semilogarithmic lineage-through-time (LTT) median plots of chronograms estimated from 108,004 post-burn-in Bayesian trees for the primary Margaritiferidae clades, including *Gibbosula*, *Cumberlandia* + *Pseudunio*, *Margaritifera*, and the entire family. The gray filling indicates 95% confidence intervals.

**Table 9**

Margaritiferidae systematics and taxonomy.

**Margaritiferidae** Henderson, 1929

*Gibbosulinae* Bogan, Bolotov, Froufe, Lopes-Lima, *nom. nov.*

*Gibbosula* Simpson, 1900,

*Gibbosula confragosa* Frierson, 1928

*Gibbosula crassa* (Wood, 1815)

*Gibbosula laosensis* (Lea, 1863), *comb. nov.*

*Gibbosula polysticta* (Heude, 1877), *comb. nov.*

*Gibbosula rochechouartii* (Heude, 1875), *comb. nov.*

**Margaritiferinae** Henderson, 1929

*Cumberlandia* Ortmann, 1912

*Cumberlandia monodonta* (Say, 1829)

*Margaritifera* Schumacher, 1816

*Margaritifera dahurica* (Middendorff, 1850)

*Margaritifera falcata* (Gould, 1850)

*Margaritifera hembeli* (Conrad, 1838)

*Margaritifera laevis* (Haas, 1910)

*Margaritifera margaritifera* (Linnaeus, 1758)

*Margaritifera marrianae* Johnson, 1983

*Margaritifera middendorffi* (Rosen, 1926)

*Pseudunio* Haas, 1910

*Pseudunio auricularius* (Spengler, 1793)

*Pseudunio homsensis* (Lea, 1864)

*Pseudunio maroccanus* (Pallary, 1928)

The three genera use different fish families as hosts.

**Diagnosis:** Shell shape elongate, with a concave or straight ventral margin. Shell thin to moderately thick or thick, posterior ridge rounded. Shell surface smooth or with plications on the posterior slope and the

posterior edge of the shell disk. Umbo sculpture is listed as concentric bars but usually eroded. Umbo pocket shallow and open (Fig. 2). Nacre color usually white but may be purple. Lateral teeth usually well-developed but may be reduced; some species have vertical sculpture. Pseudocardinal teeth are peg-like to large (Fig. 2). Fish hosts when known are Salmonidae, Esocidae, Acipenseridae, Blenniidae, Gasterosteidae and Hiodontidae, with host fish families being mutually exclusive to each margaritiferine genus.

**Distribution:** This subfamily is Holarctic in distribution including North America, Europe, Morocco, Turkey, Syria and Lebanon, China, Japan and eastern Russia (Fig. 6).

*Cumberlandia* Ortmann, 1912

**Type species:** *Unio monodonta* Say, 1829

**Type locality:** “at the falls of the Ohio, on the rocky flats which are exposed in a low state of the water” (Say, 1829).

**Type specimen:** The type specimen of *Unio monodonta* appears to be lost (Watters et al., 2009).

**Comments:** This large, arcuate shell is distinctive in shape, being very thin shelled and living in fast water usually under large flat rocks. It has been recognized as different from the typical *Margaritifera* and based on the gill structure, Heard and Guckert (1971) erected a subfamily for this genus.

**Diagnosis:** Shell shape elongate usually with a convex ventral margin, shell is thin, shell surface is smooth except for growth arrest line, posterior ridge rounded. Lateral teeth reduced to a slight rounded ridge. Pseudocardinal teeth are reduced (Fig. 2). Umbo cavity open and shallow (Fig. 2). Interlamellar gill connections were described as



Fig. 6. Distribution map of the Margaritiferidae.

“scattered and in interrupted rows, but developed as continuous septa which run obliquely forward” (Heard and Guckert, 1970). Fish hosts are Hiodontidae.

**Distribution:** “*Cumberlandia monodonta* occurs in the Mississippi Basin from southern Minnesota and Wisconsin south to the Ouachita River drainage in south-central Arkansas, and in the Ohio River drainage from Ohio and West Virginia downstream to the mouth of the Ohio River, including some tributaries” such as the Tennessee and Cumberland River drainages (Williams et al., 2008) (Fig. 6).

***Margaritifera* Schumacher, 1816**

**Type species** *Mya margaritifera* Linnaeus, 1758

**Type locality:** “Habitat in totius orbis arctici cataractis” [Arctic habitat in the entire world cataracts]. (Linnaeus, 1758).

**Type specimens:** There exists a specimen in the Linnean Society of London, Box No. LSL 22, Dance label image Ref. G-M 00101251. Dance was uncertain this was a Linnean specimen, so the listing by Graf and Cumming (2018) may be invalid. There are two additional lots in the Linnean Collection, Uppsala University, Museum of Evolution, Zoology Section (Uppsala University, 1999) which are potentially part of the syntype series (UUZM, 2018).

**Comments:** *Margaritifera* is the most widespread genus within the family with a Pacific, Atlantic and central Eurasian distribution. Since Bolotov et al. (2016), the Japanese endemic *M. togakushiensis* (Kondo and Kobayashi, 2005) has been considered a synonym of *M. mid-dendorffi* based on morphology and phylogenetic data.

**Diagnosis:** Shell shape elongate, usually with concave ventral margin. Shell is thin to moderately thick. Posterior ridge rounded. Shell surface smooth except for growth arrest lines. Lateral teeth are distinct and peg-like. Pseudocardinal teeth vary from well-developed to reduced (Fig. 2). Umbo cavity shallow and open (Fig. 2). Nacre color typically white but purple in *M. falcata* and also in some *M. laevis* individuals. Host fish are species of the Salmonidae or Esocidae for two species restricted to the Gulf Coast of the United States- (Table 4).

**Distribution:** The genus *Margaritifera* is widespread across North America, Western Europe, China, Japan and eastern Russia (Fig. 6).

***Pseudunio* Haas, 1910**

**Type species:** *Unio sinuata* Lamarck, 1819 = *Unio auricularius* Spengler, 1793

**Type locality:** “Habite dans le Rhin, la Loire, et les autres grandes rivières du continent européen tempéré et austral” [Lives in the Rhine, the Loire and other great rivers of continental Europe] (Lamarck,

1819).

**Type specimen:** the Mollusk Collection, Muséum d’histoire naturelle, Genève contains one valid syntype of *Unio sinuata* Lamarck, 1819 MHNG-MOLL-50572 and 3 possible syntypes MHNG-MOLL-50573. Lamarck had only three specimens in total so at least one of these specimens is not a valid type. Dr. Tardy noted the specimens in lot 50573 measured 104 to 117 mm while Lamarck listed a range of size from 140 to 145 mm (Tardy, Pers. Comm.). The type of *Unio auricularius* was first listed and figured by Lister (1685) and is pre-Linnean. Spengler (1793) validated this species. There is a lectotype in lot ZMUC Biv-315 (Knudsen et al., 2003). [Zoological Museum, University of Copenhagen, Copenhagen, Denmark].

**Comments:** Placement of the three species here assigned to *Pseudunio* have often been assigned to *Margaritifera*. However, in the phylogeny presented herein, they form a separate clade from *Margaritifera*, using a different suite of host fish families.

**Diagnosis:** Shell shape elongate oval. Shells thick. Posterior ridge rounded. Umbo sculpture is concentric bars. Posterior slope smooth. Shell surface is smooth. Lateral teeth are well developed and most have vertical striations. Pseudocardinal teeth are large and well developed (Fig. 2). Umbo cavity open and shallow (Fig. 2). Fish hosts include species of the Acipenseridae, Blenniidae and Gasterosteidae (Table 4).

**Distribution:** Species assigned to *Pseudunio* presently occur in rivers in northern Morocco, the Iberian Peninsula, France, southern Turkey, Syria, Lebanon, and formerly part of England, Italy, Germany and the Netherlands (Fig. 6).

**Subfamily Gibbosulinae** Bogan, Bolotov, Froufe and Lopes-Lima, new subfamily

**Type genus:** *Gibbosula* Simpson, 1900

**Type species:** *Mya crassa* Wood, 1815

**Type locality:** unknown (Wood, 1815); but listed as China, freshwater (Wood, 1825)

**Comments:** All the taxa included in this subfamily clade except for *G. laosensis* were historically included in the Unionidae. The only previous reference recognizing that *Gibbosula* belonged in the Margaritiferidae was by Morrison (1975). Transferring these four taxa from the Unionidae to the Margaritiferidae has changed our understanding of the range in morphological characteristics (including shell shape and anatomy) within this family.

**Diagnosis:** Shell shape ranges from elongate to rectangular or oval. Shell moderately thick to thick. Posterior ridge rounded to rather sharp.



Shell surface is smooth with growth arrest rings or with the posterior slope marked with heavy plications and the disk of the shell covered with pustules or w-shaped nodules. Umbo sculpture is unknown. Lateral teeth well developed with vertical sculpture. Pseudocardinal teeth well developed and large (Fig. 2). Umbo pocket deep and compressed (Fig. 2) and one species with the pocket shallow and open. Nacre color is white to some with peach color. Fish hosts for this subfamily are unknown (Table 6).

**Distribution:** Species assigned to *Gibbosula* occur or used to occur in the upper Mekong River basin in Thailand, Laos, Vietnam, the Bang River in the Pearl River basin of Vietnam, the middle Sittaung River basin in Myanmar, the Yangtze River basin of southern China and one species from North China (Fig. 6).

***Gibbosula* Simpson, 1900**

Type species: *Mya crassa* Wood, 1815

Type locality: unknown (Wood, 1815); but listed as China, freshwater (Wood, 1825:12)

**Type specimens:** *Mya crassa* types are unknown; *Unio* (*Quadrula*) *mansuyi* Dautzenberg & Fischer, 1908, a junior synonym, lectotype MNHN-MP-0136 here designated.

**Comments:** *Gibbosula* now contains five species, is restricted to Southeast Asia and northeast China. *Margaritanopsis laosensis* is included in *Gibbosula*, but conchologically resembles *Margaritifera* and *Cumberlandia* with a thin, elongate smooth shell rather than the thick rectangular or oval sculptured shells of the other species assigned to this genus. As *Gibbosula nanningensis* Qian, Fang and He, 2015, does not conform to the diagnosis of *Gibbosula* and has simple papillae and not arborescent papillae in the incumbent aperture, it is here transferred to the genus *Lamprotula*, Unionidae.

**Diagnosis:** Shell shape varies from rectangular, oval to elongate in *G. laosensis*. Ventral margin varies from concave in *G. laosensis* to rounded or convex. Shell thickness ranges from medium-thick in *G. laosensis* to thick. Posterior ridge varies from rounded especially in *G. laosensis* to rather sharp. Umbo sculpture is unknown. Posterior slope has plications but is smooth in *G. laosensis*. Shell surface is smooth, with plications or covered with pustules of various shapes. Lateral teeth are typically well developed except for the reduced teeth in *G. laosensis* and have vertical striations. Pseudocardinal teeth are usually large and well developed (Fig. 2), except in *G. laosensis* where they are peg-like. Umbo cavity deep and compressed (Fig. 2) or open and shallow as in *G. laosensis*. Nacre color is typically white. Fish hosts are unknown (Table 4).

**Distribution:** Species assigned to *Gibbosula* occur in rivers of northern Thailand, Laos, central Myanmar, western Vietnam, northern Vietnam in the headwaters of Pearl River system, tributaries of the Yangtze River basin in southern China, and north China (Fig. 6).

#### 4.4. Origin and diversification of the Margaritiferidae

In this study, we provide an updated fossil-calibrated phylogeny of the Margaritiferidae, which includes almost all known members of the family, with exception of *G. confragosa* and *G. polysticta*. These new results suggest that East Asia was the most likely place of origin of the Margaritiferidae. Although the statistical biogeographic models assume that the crown group of the family was widely distributed across the East Laurasia (East Asia + Mediterranean), the fossil evidence shows an East Asian origin for both the stem and the crown group (e.g., Chen, 1984; Jingshan et al., 1993; Ma, 1994, 1996; Jiang et al., 2005; Pan and Sha, 2009; Fang et al., 2009; Yao et al., 2011), i.e., the region of the Yangtze Plate and the adjoining complex of small terranes that formed the present Tibetan Plateau (Van Damme et al., 2015). Additionally, †*Shifangella margaritiferiformis* Liu & Luo, 1981 from the Late Triassic deposits of China (Fang et al., 2009) is here proposed as a fossil member of the crown group of Margaritiferidae + Unionidae, most likely representing a separate ancestral family (Supplementary Tables 3 and 4). This agrees with Graf et al. (2015) and Skawina and Dzik (2011), who suggested that pre-Jurassic freshwater bivalves may represent the stem-

groups of modern unionoid clades. Bolotov et al. (2017a) showed that the Unionidae most likely originated in East and Southeast Asia, which is consistent with the hypothesis of an Asian origin for both families.

With respect to combined results of our fossil-calibrated and biogeographic modeling, we suggest that the Margaritiferidae family originated in East Asia (Figs. 3 and 4) in the mid-Jurassic, most likely simultaneously with the Unionidae (Bolotov et al., 2017a). We advance that †*Palaeomargaritifera guangyuanensis* Ma, 1984 **comb. res.** from the Middle Jurassic deposits of Sichuan is the earliest known fossil member of the family (Supplementary Tables 3 and 4). This dating is not consistent with the three earlier fossil-calibrated models (Bolotov et al., 2016; Araujo et al., 2017; Huang et al., 2017). Bolotov et al. (2016) placed the origin of Margaritiferidae in the mid-Cretaceous but did not use any fossil calibrations for the deep nodes, which led to a possible underestimation of the family age. In contrast, Araujo et al. (2017) suggested that the family originated in the Late Triassic based on the age of †*Shifangella*, which is actually the most probable MRCA of Margaritiferidae and Unionidae (Supplementary Tables 3 and 4). Huang et al. (2017) assigned †*Shifangella* as a stem calibration for the Margaritiferidae and placed the origin of the family crown group in the Late Cretaceous that is close to the dating of Bolotov et al. (2016).

The divergence between Gibbosulinae and Margaritiferinae in the Late Jurassic represented the earliest split within the Margaritiferidae. The Gibbosulinae, a local clade of East Asian origin, diversified during the Late Cretaceous possibly via connections between the paleo-river systems of East and Southeast Asia. We suggest that †*Gibbosula tibetica* (Gu, 1976) **comb. nov.** from the Late Cretaceous deposits of the Tibetan Plateau could be considered the earliest known fossil member of the Gibbosulinae (Supplementary Tables 3 and 4). Whilst Bolotov et al. (2016) hypothesized that *G. laosensis* clustered with *C. monodonta*, this was not confirmed in our phylogeny. This discrepancy can be explained by the absence of other members of the Gibbosulinae in the reconstruction by Bolotov et al. (2016). The external resemblance between *G. laosensis* and *C. monodonta* that was a subject of long-term discussion (Walker, 1910; Smith, 2001; Bolotov et al., 2016) is surely a result of morphological convergence. Interestingly, both clades (Gibbosulinae and *Pseudunio* + *Cumberlandia*) include species with narrow, elongated shells (*G. laosensis* and *C. monodonta*) as well as broad, rounded shells (*G. crassa*, *G. rochechouartii*, *P. homsenensis*).

The Margaritiferinae MRCA had a continuous range from East Asia to the Mediterranean Region in the Late Jurassic, which was most likely facilitated by host fish dispersal within a continuous paleo-river system or along the Tethys coastal line (Hou and Li, 2017). The earliest history of this clade is well documented via fossil records from Jurassic deposits of North Africa and Europe (Delvene et al., 2013, 2016; Van Damme et al., 2015). †“*Margaritifera*” *crosthwaitei* (Newton, 1909) from the Late Jurassic deposits of Egypt and †*Asturianaia soudanensis* (Van Damme & Bogan, 2015) **comb. nov.** from the Middle to Late Jurassic deposits of Niger are the earliest fossil members from North Africa that could be assigned to this clade (Van Damme et al., 2015). Fossils identified as “*Margaritifera*” cf. *valdensis* (Mantell, 1844) are known from the Late Jurassic deposits of Spain (Delvene et al., 2013, 2016). There are three additional Late Jurassic margaritiferid species that were recently described from Spain: †*Asturianaia colunghensis* Delvene, Munt, Piñuela & García-Ramos, 2016, †*A. lastrensis* Delvene, Munt, Piñuela & García-Ramos, 2016 and †“*Margaritifera*” *lagriega* Delvene, Munt, Piñuela & García-Ramos, 2016 (Delvene et al., 2016).

The MRCA of *Pseudunio* + *Cumberlandia* clade most likely originated in the Mediterranean Region and dispersed to eastern North America with subsequent vicariance event in the Early Cretaceous. †*Paraheudeana idubedae* (Palacios & Sánchez, 1885) from the Early Cretaceous deposits of Spain appears to be the earliest known member of the crown group of this clade (Supplementary Tables 3 and 4). The evolutionary history of *Pseudunio* was associated with the intra-Mediterranean radiation from the mid-Eocene to mid-Miocene. Our results support the assumption of Bolotov et al. (2016) that the split between *P.*

*auricularius* and *P. maroccanus* was well before the Messinian Salinity Crisis (MSC). Additionally, the new model indicates that the split between *P. auricularius* and *P. homsensis* most likely preceded this paleogeographic event. In contrast, the divergence between *Unio* species in Morocco and Iberia was coincident with the MSC (Froufe et al., 2016). The earliest fossils resembling the extant *Cumberlandia* are known from the Early Cretaceous deposits in North Africa: †*C. rhazensis* (Mongin, 1968) **comb. nov.** and †*C. saharica* (Mongin, 1968) **comb. nov.** (Van Damme et al., 2015).

*Margaritifera* is the most widespread and diverse group of recent margaritiferids. This clade most likely originated in East Asia in the Late Cretaceous. The earliest fossils that may belong to this clade are known from the mid-Cretaceous deposits of Mongolia: †*Margaritifera elongata* (Martinson, 1982) **comb. nov.**, †*M. sainshandensis* (Martinson, 1982) **comb. nov.** and †*M. glabra* (Kolesnikov, 1956) **comb. nov.** (Supplementary Table 3). However, the first two species together with nine additional fossil taxa from Mongolia were considered synonyms of †*Unio longus* (Zhu, 1976) from China (Sha et al., 2006). A detailed discussion of the fossil taxa taxonomy is beyond the scope of the present investigation but it should be mentioned that Sha et al. (2006) provided their revision without studies of the type series of the synonymized species. Our reconstruction of the diversification patterns within this clade is largely congruent with the multiple trans-Beringian exchange model developed by Bolotov et al. (2015, 2016) and is supported by numerous fossil records (Supplementary Table 3). In fact, an expanded sampling of species from the ‘Pacific’ clade (*M. falcata*, *M. laevis*, *M. middendorffi*, *M. hembeli*, and *M. marrianae*) indicates the possibility of an extinction event that closes the gap between East Asian *M. middendorffi* and its relatives from southeastern North America, i.e., *M. hembeli* and *M. marrianae*. Previously, Bolotov et al. (2016) suggested that an additional *Margaritifera* species could be within this gap in accordance with the hypothesis of Taylor (1988) regarding vicariate forms of Margaritiferidae on both sides of the Pacific. However, Taylor’s unnamed taxon is actually a morphological form of *M. falcata*, which differs by nacre color (white with salmon spots) but is not genetically different from the typical violet-nacre form (our unpubl. data).

The new fossil-calibrated model also supports the hypothesis that the Mekong and Yangtze unionoid faunas must have developed as independent radiations during the entire Cenozoic (Schneider et al., 2013; Bolotov et al., 2017a,b) because *G. laosensis* (Mekong River basin) and *G. crassa* (Pearl River basin) split ~65 Ma ago, and the *G. laosensis* + *G. crassa* subclade diverged from *G. rochechouartii* (Yangtze) ~103 Ma ago. The two largest paleo-Mekong radiations in the Unionidae most likely originated in the Early Cenozoic (mean age = 51–55 Ma) or even pre-Cenozoic (mean age = 65–71 Ma) (Bolotov et al., 2017a,b). These results are in accordance with the concept of long-lived (ancient) rivers, suggesting that several large rivers on Earth may have existed for long-term periods comparable with geological epochs (Bolotov et al., 2017a).

The present results highlight that the placement of several Jurassic and Early Cretaceous margaritiferid species within the genus *Margaritifera* (e.g., Delvene et al., 2013, 2016; Van Damme et al., 2015) needs to be revised because these taxa most likely represent ancestral fossil lineages that are not directly associated with the crown group of the latter genus despite their morphological similarity. The description of two fossil species from the same deposit on the basis of small conchological differences, a common procedure in systematic paleontology (e.g., Delvene et al., 2016), most likely leads to overestimation of the actual diversity of fossil taxa, e.g. Margaritiferidae, because the sympatric occurrence of several closely related species is an unusual phenomenon. The co-occurrence of *M. laevis* and *M. middendorffi* in several rivers of Japan, South Kuriles and Sakhalin Island (Bolotov et al., 2015, 2016; Araujo et al., 2017) is the only example of such a secondary sympatry known to date, whereas distribution ranges of the other species reflect a drainage-dependent allopatric speciation model without clear secondary contact zones. This evolutionary pattern

suggests a limited number of ancestral fossil lineages not only by the single confirmed extinction event but also by the slow substitution and diversification rates within the family. Modeling results suggest delayed diversification rates in the Margaritiferidae (Fig. 5 and Supplementary Table 5) that are consistent with findings for the Indo-Chinese Unionidae, which also reveal slow diversification rates (Bolotov et al., 2017a). Indeed, the rates in margaritiferids are ~2.5 times slower compared with the Unionidae (Bolotov et al., 2016). These results may be associated with slower rates of molecular evolution in the Margaritiferidae, which support the hypothesis of a possible link between delayed diversification and slow molecular evolution in freshwater mussels (Bolotov et al., 2017a), although this enigmatic pattern is in need of further investigation.

## 5. Conclusions

The current study supports the increase of extant margaritiferid species to 16 and suggests their division into two subfamilies and four genera. Since a better understanding of phylogenetic diversity is central for determining conservation priorities (Lopes-Lima et al., 2017c, 2018), the results reported here may be important in the definition of future management strategies devoted to conservation of margaritiferid species. The inclusion of *G. crassa*, *G. polysticta*, *G. rochechouartii*, and *G. confragosa* in the Margaritiferidae, confirms the family as the most threatened among unionoids (IUCN, 2018). In fact, the first three mentioned species have a threatened status (IUCN, 2018), while *G. confragosa* has never been evaluated (IUCN, 2018). All four “new” margaritiferids seem to have small distribution ranges and are affected by multiple impacts (IUCN, 2018). Further studies on the Margaritiferidae should include basic ecological and physiological research, collecting data on distribution, abundance, habitat preferences, host-fish identification and reproductive cycles, as well as a phylogenomics approach to complement the current phylogenetic evaluation. Finally, a complete revision of numerous fossil margaritiferid taxa is necessary for the future development of reliable phylogenetic, phylogenomic and biogeographic reconstructions.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ympmv.2018.04.041>.

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