Mesozoic Mitogenome Rearrangements and Freshwater Mussel (Bivalvia: Unionoidea) Macroevolution

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Abstract

Using a new fossil-calibrated mitogenome-based approach, we identified macroevolutionary shifts in mitochondrial gene order among the freshwater mussels (Unionoidea). We show that the early Mesozoic divergence of the two Unionoidea clades, Margaritiferidae and Unionidae, was accompanied by a synchronous split in the gene arrangement in the female mitogenome (i.e. gene orders MF1 and UF1). Our results suggest that this macroevolutionary jump was completed within a relatively short time interval (95% HPD 201-226 Ma) that coincided with the Triassic–Jurassic mass extinction. Both gene orders have persisted within these clades for ~200 Ma. The monophyly of the so-called “problematic” Gonideinae taxa was supported by all the inferred phylogenies in this study using, for the first time, the M- and F-type mitogenomes either singly or combined. Within Gonideinae, two additional splits in the gene order (UF1 to UF2, UF2 to UF3) occurred in the Mesozoic and have persisted for ~150 and ~100 Ma, respectively. Finally, the mitogenomic results suggest ancient connections between freshwater basins of East Asia and Europe near the Cretaceous–Paleogene boundary, probably via a continuous paleo-river system or along the Tethys coastal line, which are well supported by at least three independent but almost synchronous divergence events.

Keywords:

Mitogenome rearrangements, macroevolutionary jumps, Triassic–Jurassic mass extinction

Introduction

The tempo, timing and mode of evolution have attracted considerable debate among evolutionary biologists. Here we use a new approach using mitogenome rearrangements to investigate changes at the geological time scale in the speciose and imperilled freshwater mussels.

In many taxonomic groups, the gene arrangement within mitogenomes is highly conserved, e.g. many vertebrate groups share the same gene order (Pereira 2000). Other faunal groups, such as the Bivalvia, exhibit a number of different mitochondrial gene arrangements (e.g., Yuan et al. 2012), which are the result of different mechanisms such as tandem duplication followed by gene loss (Boore 2000). Although local homoplastic arrangements have been identified in some invertebrate groups (e.g. Flook and Rowel 1995; Dowton and Austin 1999), complete gene orders generally remain unique and represent signatures with diagnostic value (Basso et al. 2017), providing a powerful signal for inferring ancient evolutionary relationships (Boore 2000).

Among freshwater mussels of the order Unionida, which spans about 900 species and represents the major bivalve radiation in the freshwater environment (Lopes-Lima et al. 2017a, 2018a), five mitogenome rearrangements have been described so far (Lopes-Lima et al. 2017b). Within the superfamily Unionoidea (Margaritiferidae + Unionidae), the mitochondria are furthermore unusual in that two highly divergent mtDNA molecules exist in males (Female or F- and Male or M-type) as a result of Doubly Uniparental Inheritance (DUI) (Zouros et al. 1994; Breton et al.

2009). This is in contrast to the vast majority of animal taxa, which inherit their mtDNA exclusively through the maternal lineage and thus exhibit only F-type mtDNA. In Unionoidea males, M-type mtDNA is restricted to the gonadal tissue inherited from the paternal lineage, and F-type mtDNA is present in all somatic tissues transmitted from the maternal lineage and also in female gonadal tissue (Breton et al. 2009; Froufe et al. 2016; Fonseca et al. 2016; Lopes-Lima et al. 2017b).

In recent decades, complete mitochondrial genome sequences have been published for a wide range of taxa, enabling reconstruction of shallow and deep phylogenies in both vertebrates and invertebrates (e.g. Jacobsen et al. 2014; Liu et. al 2016). However, the number of available mitogenomes for Unionida is low, particularly for M-type genomes (Froufe et al. 2016; Fonseca et al. 2016; Lopes-Lima et al. 2017b; Huang et al. 2019). A further shortcoming is that published mitogenomes are restricted to only a few higher Unionida taxa, with no mitogenomes being available for several families and subfamilies. In fact, of the six recognized Unionida families (Lopes-Lima et al. 2014), published mitogenomes are essentially restricted to the Unionoidea (Unionidae + Margaritiferidae) with a distribution predominantly within the Northern Hemisphere. While some studies have questioned the monophyly of the Unionoidea (e.g. Combosch et al. 2017; Whelan et al. 2011) the most comprehensive recent studies, using either full mitogenomes (Huang et al. 2019; Wu et al. 2019) or hundreds of nuclear loci (Pfeiffer et al. 2019) support its monophyletic status. Moreover, mitogenome-based Unionida phylogenies reconstructed to date have been

144 based on either F- or M-type mitogenomes (Froufe et al. 2016; Fonseca et
145 al. 2016; Lopes-Lima et al. 2017b). Although in these studies the highly
146 divergent F- and M-type mitogenomes recovered identical phylogenies,
147 concatenated phylogenetic analyses of M- and F-type datasets would be
148 expected to recover a more robust phylogeny.
149 The Unionidae is the most species-rich Unionida family, comprising 620
150 species in several subfamilies and distributed widely (Lopes-Lima et al.
151 2017a). However, phylogenetic relationships within and between Unionidae
152 subfamilies are still contentious and different phylogenies have been
153 resolved with different analysed markers (e.g., Lopes-Lima et al. 2017a;
154 Bolotov et al. 2017a).
155 One of the least studied Unionidae subfamilies, the Gonideinae, has a
156 scattered distribution in the Northern Hemisphere (Lopes-Lima et al.
157 2017a). Species in this subfamily have suffered major declines, and half of
158 the assessed Gonideinae species are currently listed as Near Threatened
159 or Threatened (IUCN 2019). Moreover, 70% of recognized Gonideinae
160 species have either never been assessed or are listed as Data Deficient by
161 the IUCN Red List (IUCN 2019), indicating an urgent need for research into
162 this family's diversity, distribution and ecology.
163 Another outcome of the general lack of data on Gonideinae is their
164 unresolved phylogeny. In fact, monophyly of this sub-family is disputed.
165 The first molecular study to include the so-called "problematic" Gonideinae
166 taxa (Graf 2002) only examined the type species, i.e. *Gonidea angulata*
167 (Lea 1838). Subsequent studies included several additional Gonideinae

taxa but the clade Gonideinae was never recovered as monophyletic (Graf and Cummings 2006; Whelan et al. 2011; Pfeiffer and Graf 2013). More recently, multi-marker and mitogenomic approaches have consistently recovered Gonideinae as monophyletic (Huang et al. 2013; Pfeiffer and Graf 2015; Fonseca et al. 2016; Froufe et al. 2016; Lopes-Lima et al. 2017a, b). Bolotov et al. (2017a, b) subsequently elevated one of the four Gonideinae tribes established by Lopes-Lima et al. (2017a), i.e. Pseudodontini, to the subfamily level (i.e. Pseudodontinae).

A good understanding of the evolutionary biogeography of the Gonideinae can be fundamental for reconstructing patterns of connections of freshwater systems through space and time on a global scale. Our knowledge in this respect is still far from complete. The first biogeographic scenarios developed using Unionida data (e.g. Starobogatov 1970; Banarescu 1991) proved highly inaccurate, as they were mostly descriptive and based solely on the (dis-)similarity between unionid faunas. Furthermore, these scenarios were generated at a time when unionid taxonomy was poorly resolved and included numerous paraphyletic higher-order taxa as well as nominal taxa, determined by shell shape rather than reliable indicators of true biological species (e.g. Bolotov et al. 2017a; Konopleva et al. 2017). Modern paleontology-based models seem to be much more reliable. Based on the fossil record from Vietnam, Schneider et al. (2013) developed the hypothesis of an independent development of Unionida faunas in the Yangtze and Mekong basins, at least during the entire Cenozoic. In addition, Van Damme et al. (2015) showed that the African Early

Cretaceous Unionida are representatives of Asian/Eurasian taxa with the lack of Gondwanan elements, while the African Jurassic assemblages are distinctly related to those in Eurasia.

Recently, a first statistical biogeographic model for the Unionidae at the global level indicated that the Unionidae most likely originated in Southeast and East Asia in the Jurassic, with the earliest expansions into North America and Africa (since the Albian), following the colonization of Europe and India (Bolotov et al. 2017a). However, the Jurassic fossil record of western North America (for a review see Watters 2001) and Africa (Van Damme et al. 2015) indicate that these continents were colonized before the Cretaceous. Additionally, two species-rich monophyletic mussel radiations with an early Cenozoic or even pre-Cenozoic origin were discovered within the paleo-Mekong catchment (Bolotov et al. 2017a, b). These findings revealed that the largest river systems (e.g. Mekong, Yangtze and Mississippi) may represent ancient evolutionary hotspots of freshwater mussels (Scholz and Glaubrecht 2004; Wesselingh 2007).

On the basis of the most comprehensive dataset of mitogenomes sampled to date, including eight newly sequenced mitogenomes, this paper aims to improve our understanding of the higher-order phylogeny and classification of Unionidae by: 1) testing the monophyly of the poorly known Gonideinae subfamily using both full F- and M- mitogenomes and, for the first time, mitogenomes concatenated; 2) estimating macroevolutionary patterns in freshwater mussels of the Unionidae using, for the first time, a fossil-calibrated mitogenomic approach; 3) estimating the timing of major

divergence events and comparing them to those of mitogenome rearrangements; and 4) developing an updated integrative approach to the systematics of Unionidae, on the basis of the mitogenomic results. This will allow the reconstruction of the potential origin and ancient radiations of the Unionidae and detect the most probable ancestral areas.

Methods

Sampling, DNA extractions, sequencing, assembly and annotation

One male specimen of *Chamberlainia hainesiana*, *Microcondylaea bonellii*, *Pilsbryoconcha exilis* and *Monodontina vondembuschiana* were dissected for sampling of gonadal (to recover M-type mtDNA) and mantle (to recover F-type mtDNA) tissues. DNA extractions followed Froufe et al. (2016). The complete M- and F-type mitogenome sequencing and assemblage followed Gan et al. (2014), while annotations were performed using MITOS (Bernt et al. 2013). The final limits of tRNA genes were rechecked with ARWEN (Laslett and Canbäck 2008). All F- and M- mitogenomes have been deposited in GenBank database under the accession numbers XXXXXXX (submitted), respectively and were visualized using GenomeVx (Conant and Wolf 2008).

DNA (NUC) and amino acid (AA) sequences of all mtDNA protein-coding genes (PCG), except ATP8 and the gender-specific open reading frames (M-ORF, H-ORF and F-ORF; Breton et al. 2011), were used in the phylogenetic analyses. The sequences of each gene were aligned using

MAFFT software (version 7.304, Katoh and Standley 2013) and trimmed with GUIDANCE2 (Sela et al. 2015; see Froufe et al. (2016) for the parameters used).

The gene alignments were then concatenated, resulting in two alignments with the following length: 13449 aligned nucleotide positions and 3870 aligned amino acids positions + 1889 aligned nucleotide positions from the rRNA genes. The optimal partitioning scheme for each alignment was selected using PartitionFinder v. 2.1.1 software (Lanfear et al. 2016) under the greedy algorithm with proportional branch lengths across partitions. The best substitution models of DNA and protein evolution for each partition were selected under the BIC ranking method (Schwarz 1978). The codon positions of the protein-coding genes and each rRNA were defined as the initial data blocks for the partitioning schemes search.

An additional dataset was also created, concatenating both F- and M-type gene alignments, with the following length: 26780 aligned nucleotide positions and 7661 aligned amino acid positions + 3797 aligned nucleotide positions from the rRNA genes. This alignment included 45 Unionida species plus *Mytilus galloprovincialis* as an outgroup (Table 1) using the same partitioning method and model selection as described above.

Phylogenetic analyses

All phylogenetic analyses were performed using both Maximum Likelihood (ML) and Bayesian Inference (BI) methods. ML analyses were performed using RAxML (v. 8.0.0, Stamatakis 2014) with 100 rapid bootstrap

replicates and 20 ML searches. The BI was applied using MrBayes v. 3.2.7a (Ronquist et al. 2012) with two independent runs (10^7 generations with a sampling frequency of 1 tree for every 100 generations), each with four chains (3 hot and 1 cold). All runs reached convergence (average standard deviation of split frequencies below 0.01). The posterior distribution of trees was summarized in a 50% majority rule consensus tree (burn-in of 25%).

Divergence time estimates

The time-calibrated mitogenomic phylogeny was reconstructed in BEAST v. 1.8.4 based on two reliable fossil calibrations (Supplementary Table 1) 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). The sample of M-type mitogenomes was used as outgroup. Similar settings to each gene partition as in the MrBayes analyses were specified but using a simplified evolutionary model (HKY; see Bolotov et al. 2017a for details). Five replicate BEAST searches were conducted, each with 5×10^7 generations and a tree sampling every 5,000th generation. The log files were checked visually with Tracer v. 1.7 for an assessment of the convergence of the MCMC chains and the effective sample size of parameters (Rambaut et al. 2018). The chains in one run did not reach the convergence and were excluded, the other runs were compiled with

LogCombiner v. 1.8.4 (Drummond et al. 2012) using an appropriate burn-in depending on the start of convergence of MCMC chains in each run. Most of ESS values were recorded as >300, with a few ESS values >100. The maximum clade credibility tree was obtained from the post-burn-in trees using TreeAnnotator v. 1.8.4 (Drummond et al. 2012).

Ancestral gene order and ancestral area reconstructions

TreeREx (Bernt et al. 2008) was used for inferring the most parsimonious putative ancestral gene orders and gene rearrangements along the obtained Unionidae F-haplotype phylogenetic sub-tree with the default settings (http://pacosy.informatik.uni-leipzig.de/185-0-TreeREx.html). Ancestral area reconstruction models were calculated for the Unionidae 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) following Bolotov et al. (2017a). Margaritiferidae were not used in this analysis due to the limited number of available mitogenomes. Four possible distribution areas of the in-group taxa were coded as follows: (A) Southeast Asia, (B) East Asia, (C) North America, and (D) Europe. From the input matrix, two geographically unreliable constrains (AC and AD) were excluded.

Results

Mitogenome characteristics and gene arrangements

All the eight sequenced haplotypes include the 13 protein-coding genes (PCGs) typically found in metazoan mitochondrial genomes, the sex-specific ORF described for all Unionida mitogenomes with DUI system (Breton et al. 2009, 2011) and 22 transfer RNA (tRNA) and two ribosomal RNA (rRNA) genes (Fig. 1). As expected, the length of the four newly sequenced M-type mitogenomes is larger than the corresponding F-type (Breton et al. 2009), ranging from 16,267 bp in *P. exilis* to 17,465 bp in *C. hainesiana*, while the F-type ranged from 16,020 bp in *M. bonellii* to 16,746 bp in *C. hainesiana* (Table 2). The A+T content, and GC and AT skews are similar in all sequenced species in both F and M mtDNA types, averaging around 60%, 37 (+) and -0.23 (+), respectively (Table 2).

The gene arrangements of *Microcondylaea bonellii, P. exilis* and *Monodontina vondembuschiana* are identical to all Gonideinae mitogenomes available on GenBank (2018), named UF2 (Lopes-Lima et al. 2017b). However, *C. hainesiana* has a new and distinct gene arrangement, here named UF3 (Fig. 2).

Phylogenetic analyses

All the phylogenies inferred in this study that are based on M and F mitogenomes alone (i.e. not combined) support the monophyly of Gonideinae (Fig. 3). Moreover, the four tribes Chamberlainiini, Gonideini, Lamprotulini and Pilsbryoconchini, are also monophyletic in both M- and F-type trees (Fig. 3). The same results were obtained when using for the first

time the M and F mitogenomes combined, despite the lower number of species (Fig. 4). The only unsupported result on the topology is seen in the relationship among the tribes Gonideini, Pilsbryoconchini and Lamprotulini in the ML AA data set (Fig. 4).

Ancestral gene order and ancestral area reconstructions

The TreeREx analysis indicated that the evolution of gene orders in the Unionidae F-type mtDNA is characterized by two independent events of tandem duplication and random loss (TDRL), with every ancestral gene order showing the highest consistency scores. The analysis suggests that the ancestral gene order for Unionidae F mitogenome is UF1, which is also found in the contemporary species of the subfamilies Ambleminae and Unioninae (Fig. 5). The fossil-calibrated mitogenomic analysis placed the split between the UF1 and MF1 gene orders in the Late Triassic (mean age = 208 Ma, 95% high posterior density (HPD) 201-226 Ma) (Fig. 6A).

The ancestral gene order of the Gonideinae species represented in our study is UF2, which results from a TDRL event of an mtDNA segment involving nad3, trnH, trnA, trnS2, trnS1, trnE, nad2, and trnM (Fig.2 Box A). In UF2, the genes trnH, trnS1, nad2 and trnM pertain to the original segment, while the remaining genes – nad3, trnA, trnS2, and trnE – are present in the duplicated segment (Fig.2 Box A). The fossil-calibrated model developed suggests that the UF1 and UF2 gene orders split near the Jurassic – Cretaceous boundary (mean age = 149 Ma, 95% HPD 138-162 Ma) (Fig. 6A).

Finally, the UF3 gene order also arises after a TDRL event within Gonideinae (Fig.2 Box B). It involved an mtDNA segment containing twelve genes: trnQ, trnC, trnI, trnV, trnL2, nad1, trnG, nad6, nad4, nad4l, atp8 and trnD. In UF3, the genes trnC, trnI, trnV, trnG, nad6, atp8 and trnD are retained in the original segment, whilst genes trnQ, trnL2, nad1, nad4 and nad4l were not lost in the duplicated one (Fig.2 Box B). The fossil-calibrated model placed the split between the UF2 and UF3 gene orders in the Cretaceous near the Albian – Cenomanian boundary (mean age = 102 Ma, 95% HPD 77-124 Ma) (Fig. 6A).

The combined ancestral area reconstruction model suggests that the Most Recent Common Ancestor (MRCA) of the crown group of the Ambleminae + (Gonideinae + Unioninae) clade used to be widely distributed across the supercontinent of Laurasia (probability 100%) (Fig. 7). The earliest split was between the Laurentian (Ambleminae) and Eurasian (Gonideinae + Unioninae) taxa. This vicariance event is placed in the Late Jurassic (mean age = 159 Ma, 95% HPD 155-170 Ma). Early diversification of the Gonideinae + Unioninae clade is placed within East Asia (probability 100%; Fig. 7). The origin of the MRCA of this large clade (mean age = 149 Ma, 95% HPD 138-162 Ma) and subsequent splitting into two subclades (mean ages of crown groups = 137 and 106 Ma and 95% HPD 123-152 and 90-124 Ma for Gonideinae and Unioninae, respectively) most likely resulted from an intra-area radiation (probability 100% in each case) during the early Cretaceous. The Yangtze and Mekong unionid faunas have likely been

separated since the Albian (mean ages = 95-102 Ma, 95% HPD 77-124 Ma) (Fig. 7).

Discussion

Phylogenetic patterns

The new mitogenomic results presented here place the Pilsbryoconchina subtribe (previously under the Pseudodontinae as described by Bolotov et al. 2017a) as a subclade within the monophyletic Gonideinae in both the M- and F-type phylogenies. Our results are thus in agreement with the phylogeny recovered by Lopes-Lima et al. (2017a), which is also supported by morphological data. However, the recovered results contradict that of Bolotov et al. (2017a, b), which suggested elevation of the Pseudodontini to the subfamily level.

Our results further indicate that the number of recognized subfamilies within the Unionidae is most likely lower than has been suggested by recent phylogenetic studies (Lopes-Lima et al. 2017; Bolotov et al. 2017a, b). The mitogenomic results fully support three large subfamily-level clades: Ambleminae, Gonideinae and Unioninae. It is important to note that our analyses did not include members of the Parreysiinae and Rectidentinae. Nor did it include sequences of *Modellnaia siamensis*, the only species of the monotypic Modellnaiinae, which is characterized by a number of morphological and anatomical autapomorphies suggesting its separation within the Unionidae as a “phylogenetic relic” (Brandt 1974; Heard and

Hanning 1978). Future studies including full mitogenomes of several taxa from Parreysiinae, Rectidentinae and Modellnaiinae are needed to fully resolve the higher-level phylogeny of the global Unionidae.

Our results highlight that resolving the systematics of a large, species-rich clade such as the Unionidae is a complex task. Previous taxonomic schemes for the Unionidae included only two levels of family-group names, i.e., subfamilies and tribes (reviews: Lopes-Lima et al. 2017a; Bolotov et al. 2017a, b). However, our whole mitogenome analyses reveal that despite the limited number of taxa included, the Unionidae classification scheme could be better explained by including three levels of family-group names (i.e. subfamilies, tribes and subtribes) to accurately reflect the presence of several levels of highly divergent clades within this family (Fig. 6A). Subfamilies represent the largest clades that are fully supported by the mitogenomic approach (Fig. 7); some of which may be characterized by unique morphological synapomorphies, although several subfamilies have been supported by molecular data only (e.g., Lopes-Lima et al. 2017a).

The most recent nuclear-based Unionoida phylogeny (using hundreds of nuclear protein-coding loci; Pfeiffer et al. 2019) shows strong similarity to our own findings in regard to the relationships of both families and subfamilies. Moreover, mitogenome data currently available suggest that the Unionidae comprise seven (Lopes-Lima et al. 2017a) or eight (Bolotov et al. 2017a) subfamily clades. Of these, the Gonideinae (encompassing Pseudodontinae), Unioninae (encompassing the Anodontinae) and Ambleminae were well supported in the mitogenomic results obtained

herein, whilst the validity and placement of the Parreysiinae, Rectidentinae and Modellnaiinae clades are yet to be confirmed by mitogenomic analyses.

The largest monophyletic clades, within each subfamily, exhibiting significant morphological synapomorphies and fully supported by the present mitogenomic results, are herein considered as tribes. Therefore, using these criteria, the Gonideinae comprise two tribes, i.e. Gonideini (trapezoidal to rectangular shells with none or only vestigial hinge teeth and tetragenous brooding type) and Chamberlainiini (round oval shells, with a well-developed hinge structure and ectobranchous brooding type).

The subtribes represent smaller but distant clades within the tribes, comprising several genera or even a single highly divergent genus that usually does not reveal any unique synapomorphies but can be distinguished on the basis of molecular characters. Based on data available to date, including the present results, the Gonideini comprise at least five subtribes, i.e. Chamberlainiina, Gonideina, Lamprotulina, Pilsbryoconchina and Pseudodontina (Lopes-Lima et al. 2017a; Bolotov et al. 2017a, b).

Macroevolutionary patterns of the Unionidae

The new mitogenomic analysis presented herein supports the hypothesis of an ancient Mesozoic origin and diversification of the Unionoidea (Taylor 1988; Ma 1996; Van Damme et al. 2015; Bolotov et al. 2016; Araujo et al. 2017; Bolotov et al. 2017a, b). The new results indicate that the Late Triassic split between the Margaritiferidae and Unionidae coincided

approximately with the Triassic–Jurassic extinction that was one of the largest mass extinction events in the Phanerozoic (Watters 2001; Hesselbo et al. 2002; Bogan and Weaver 2012; Percival et al. 2017; Smithwick and Stubbs 2018). The divergence event between the two families was associated with TDRL event leading to formation of the two stable mitochondrial gene orders, i.e., MF1 and UF1, which have persisted without changes for ~200 Ma. However, there were at least two additional Mesozoic splits in the mitochondrial gene order (i.e., UF1 vs. UF2 and UF2 vs. UF3) within the Unionidae, with UF2 and UF3 being restricted to a single subfamily, the Gonideinae. The first split coincided with the origin of this subfamily but the UF3 is a third, new and distinct gene arrangement derived from UF2 present in a single species, *Chamberlainia hainesiana*. These two mitochondrial gene orders have also persisted for long-term periods of ~150 and ~100 Ma for UF2 and UF3, respectively.

At least two splits in the mitochondrial gene order were associated with the origin of the MRCAs of large and diverse clades of family (Unionidae vs. Margaritiferidae) or subfamily (Unioninae vs. Gonideinae) levels. With respect to this evidence, these TDRL events could be considered progressive evolutionary innovations because they lead to formation of stable gene orders that have persisted within widely distributed and diverse clades for ~150-200 Ma. As for the mitogenome gene order, our ancestral state analyses suggest UF1 (in the Unionidae) as the ancestral gene order, which is maintained in the subfamilies Ambleminae and Unioninae *sensu lato* (Fig. 6). Additionally, it indicates that the evolution of F-type mtDNA

gene orders is characterized by two independent events of TDRL (Moritz et al. 1987; Boore 2000). One resulted in the evolution of UF2, present in the Gonideinae, and the other in UF3, within Gonideinae but restricted to *Chamberlainia hainesiana*. In contrast, all sequenced M-type Unionidae mitogenomes to date present the same gene order, i.e. UM1 (Lopes-Lima et al. 2017b) (Fig.2). Possibly this could be explained by the higher natural selection pressure and/or due to the tight control of the DUI system on the paternal mitochondrial inheritance. In summary, our results reveal that each TDRL event was followed by the stable long-term persistence of a mitochondrial gene order through evolving lineages (or even a single lineage, although the *Chamberlainia* clade may actually be under-sampled) and corresponds to the first reliable mitogenomic evidence supporting the evolutionary stasis in molecular traits of freshwater bivalves. However, this should be further explored using an expanded data set of mitochondrial genomes that may facilitate the understanding of how evolutionary rates have shifted across multiple genetic loci and how that corresponds to ecologically relevant traits.

Diversification and Biogeography

Combining our new fossil-calibrated mitogenomic analyseswith robust ancestral area reconstruction provides new insights into early diversification patterns and biogeography of the Unionidae. According to our results, the Ambleminae + (Gonideinae + Unioninae) clade originated in the late Jurassic, with their MRCA distributed across Laurentia and Eurasia of the

504 supercontinent of Laurasia. The split between the Ambleminae and
505 Gonideinae + Unioninae clades was likely associated with a vicariance
506 event driven by plate tectonics, i.e., the formation of the early Jurassic
507 Transcontinental Laurasian Seaway (Bjerrum et al. 2001). The Ambleminae
508 is an entirely Laurentian subfamily, which diversified primarily through
509 radiation within the Mississippi drainage basin from the Early Cretaceous
510 (Bolotov et al. 2017a). In this context, a peculiar Unionidae fauna from the
511 Late Jurassic of western North America (Watters 2001) appears to be
512 ancestral lineages and stem groups of the Ambleminae + (Gonideinae +
513 Unioninae) clade. The Gonideinae and the Unioninae (Unionini, Anodontini,
514 Lanceolariini, and Lepidodesmini) (Fig. 6) originated in East Asia, most
515 likely via intra-area radiation within the paleo-Yangtze River system during
516 the Cretaceous (Fang et al. 2009; Wang et al. 2018). The Southeast Asian
517 Gonideinae taxa (Mekong basin) were separated via several vicariance
518 events in the Albian - Cenomanian, which may indicate the drainage
519 rearrangement of paleo-river systems of the Indochina Peninsula and
520 surrounding terrains during this period (Wang et al. 2018). The
521 mitogenomic results suggest ancient connections between freshwater
522 basins of East Asia and Europe near the Cretaceous – Paleogene
523 boundary, probably via a continuous paleo-river system or along the Tethys
524 coastal line (Hou and Li 2017), and this is also depicted in the
525 Margaritiferinae subfamily within Margaritiferidae (Lopes-Lima et al. 2018b).
526 This pattern is well supported by at least three independent but almost
527 synchronous divergence events: *Potomida* vs. *Lamprotula* and

Pronodularia, *Microcondylaea* vs. *Solenaia*, and *Unio* vs. *Nodularia* and its relatives. During the same period, faunal exchange via the Beringian Land Bridge with subsequent vicariance events may also have started. The question of the origin of the family-clade, i.e. Unionidae, remains unanswered due to the lack of available mitogenomes of Parreysiinae and Rectidentinae, although combined COI, 28S and 16S data indicated that this family most likely originated within East or Southeast Asia (Bolotov et al. 2017a).

The new results presented herein support the hypothesis that several of the largest river basins on Earth may represent so-called ancient (long-lived) rivers, the Unionida faunas of which have existed throughout long-term periods comparable with geological epochs (Bolotov et al. 2017a; Lopes-Lima et al. 2018b). The mitogenomic results suggest that the MRCA of the entire Gonideinae + Unioninae clade may have originated within the paleo-Yangtze drainage basin. This indicates that the modern Yangtze may be a system of at least Late Jurassic origin and a stable refugium for very ancient, relic lineages that have existed for approximately 150 Ma. The unionid fauna of the paleo-Mississippi system seems to be of Early Cretaceous origin (mean age of the crown group in our model) that has diversified for at least 120 Ma. The paleo-Mekong fauna appears to be younger as it likely separated from the paleo-Yangtze fauna in the Albian - Cenomanian, and its two largest monophyletic unionid radiations may have had a Late Cretaceous or Paleocene origin (Bolotov et al. 2017a, b). These results agree with the dating of divergence between two primary clades of

the Southeast Asian cave spitting spiders that were separated at ~55 Ma by the paleo-Mekong River, which served as a biogeographic barrier (Luo and Li 2017).

Systematics

Based on the morphological evidence, we propose the putative MRCA of the Unionidae and Margaritiferidae as a new fossil family-level taxon in the Unionoidea.

Superfamily Unionoidea Rafinesque, 1820

Family †Shifangellidae Bolotov, Bogan, Lopes-Lima & Froufe **fam. nov.**

Type genus: †*Shifangella* Liu & Luo in Liu (1981)

Diagnosis: The Margaritiferidae and Unionidae are the most conchologically similar families to the †Shifangellidae. However, †Shifangellidae can be distinguished from the Margaritiferidae by having a weakly developed, narrow hinge plate (vs. typically well-developed and rather thick) and a shallow, smooth anterior adductor scar (vs. deep with arborescent-like striations), and from the Unionidae by an elongated *Margaritifera*-like shell with strongly concave ventral margin (vs. typically straight, rounded or slightly concave).

Distribution: Late Triassic, southwestern China (Sichuan).

Biology: This ancestral family likely had parasitic glochidial larvae similar to its descendants (ancestral state reconstruction, probability 100%).

Comments: Synonymy of the genus †*Palaeomargaritifera* Ma, 1984 (Middle Jurassic, China) with †*Shifangella* suggested by Fang et al. (2009) most likely erroneous because †*Palaeomargaritifera* has a well-developed, thick hinge plate, strong pseudocardinal teeth and deep anterior adductor scar with arborescent-like striations supporting its original placement within the Margaritiferidae. The genus †*Dianoconcha* Guo, 1988 (Middle Jurassic, China), another synonym of †*Shifangella* proposed by Fang et al. (2009), differs by a subtrapezoid, elongate-elliptical or rhomboid shell. This feature together with a narrow hinge plate and an observable but shallow anterior adductor scar suggest that it most likely belongs to the Unionidae. With respect to their age and diagnostic features mentioned above, †*Palaeomargaritifera* and †*Dianoconcha* appear to be the MRCAs of the crown groups of the Margaritiferidae and Unionidae, respectively. The family-level placement of several unionoid genera described from the Early Jurassic of China (e.g., †*Pseudomargaritifera* Ma, 1996 and †*Solenoides* Ma, 1996) is unclear and is in need of further revision; some of them might actually be members of the †Shifangellidae.

Conclusions

All the phylogenies inferred in this study using, for the first time, both the M and F mitogenomes individually and combined support the monophyly of the so called “problematic” Gonideinae taxa. Moreover, the new mitogenomic results place the Pseudodontinae, as previously described by

Bolotov et al. (2017a), as a subclade within the monophyletic Gonideinae in both M- and F-type phylogenies. Additionally, the present work supports the hypothesis of an ancient Mesozoic origin and diversification of the Unionoidea and reveals that each TDRL event was followed by the stable, long-term persistence of a mitochondrial gene order through evolving lineages and corresponds to the first reliable mitogenomic evidence supporting the evolutionary stasis in molecular traits of freshwater mussels. Finally, we propose a new systematics framework with three infrafamilial levels (i.e. subfamilies, tribes, and subtribes) that better explains the evolutionary patterns within the Unionidae. Future application of the phylogenetic mitogenome-based approach outlined here to Parreysiinae, Rectidentinae and Modellnaiinae will be an important step to further resolve current taxonomic classification uncertainties within the Unionidae. Moreover, this study demonstrates the considerable potential for using comparative genomic techniques for unravelling patterns in the tempo, timing and mode of evolutionary processes.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data archiving

Sequence data have been submitted to GenBank accession numbers: XXX-XXX (submitted).

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Figure legends:

Fig. 1 Gene maps of the F- and M-type mitochondrial genomes of *Chamberlainia hainesiana*, *Microcondylaea bonellii*, *Pilsbryoconcha exilis* and *Monodontina vondembuschiana*. Genes positioned inside the circle are encoded on the heavy strand, and genes outside the circle are encoded on the light strand. Color codes: Small and large ribosomal RNAs (red), transfer RNAs (purple); FORF, F-specific open reading frame (yellow) and MORF, M-specific open reading frame (yellow); PCGs genes (green).

Fig. 2 Diagrams of the four distinct gene orders known in Unionidae to date. In the F-type, three gene orders are depicted: UF1, UF2 and UF3. In the male M-type lineage, the only Unionidae gene arrangement is shown: M-type 1 (UM1). Blue boxes highlight gene rearrangement region from UF1 to UF2 (Box A) and from UF2 to UF3 (Box B). Small and large ribosomal RNAs and transfer RNAs are depicted by one letter of the amino acid code; Arrow colour codes, follow Fig 1.

977

978 **Fig. 3** Phylogenetic (BI-NUC) tree of Unionida estimated from 14
979 concatenated individual mtDNA gene sequences (12 protein-coding and 2
980 rRNA genes). Values for branch support are represented in the following
981 order: (1) Bayesian posterior probabilities (PP) for BI-NUC tree, (2)
982 Bayesian PP for BI-AA tree, (3) ML bootstrap support (BS) values for ML-
983 NUC and (4) ML BS values for ML-AA tree. Maximum support values (PP =
984 1, BS = 100) are represented by asterisks. Gonideinae subfamily and tribes
985 are highlighted. For details see text. Codes in Table 1.

986

987 **Fig. 4** Phylogenetic (BI-NUC) tree of Unionida estimated from 28
988 concatenated individual mtDNA gene sequences (24 protein-coding and 4
989 rRNA genes) of the first combined Female+Male concatenated data set.
990 Maximum branch support values (BI-NUC/BI-AA PP = 1; ML-NUC/ML-AA
991 BS = 100) are represented by asterisks, while # represents the only non-
992 supported branch by ML-AA tree. Gonideinae subfamily and tribes are
993 highlighted. Codes in Table 1.

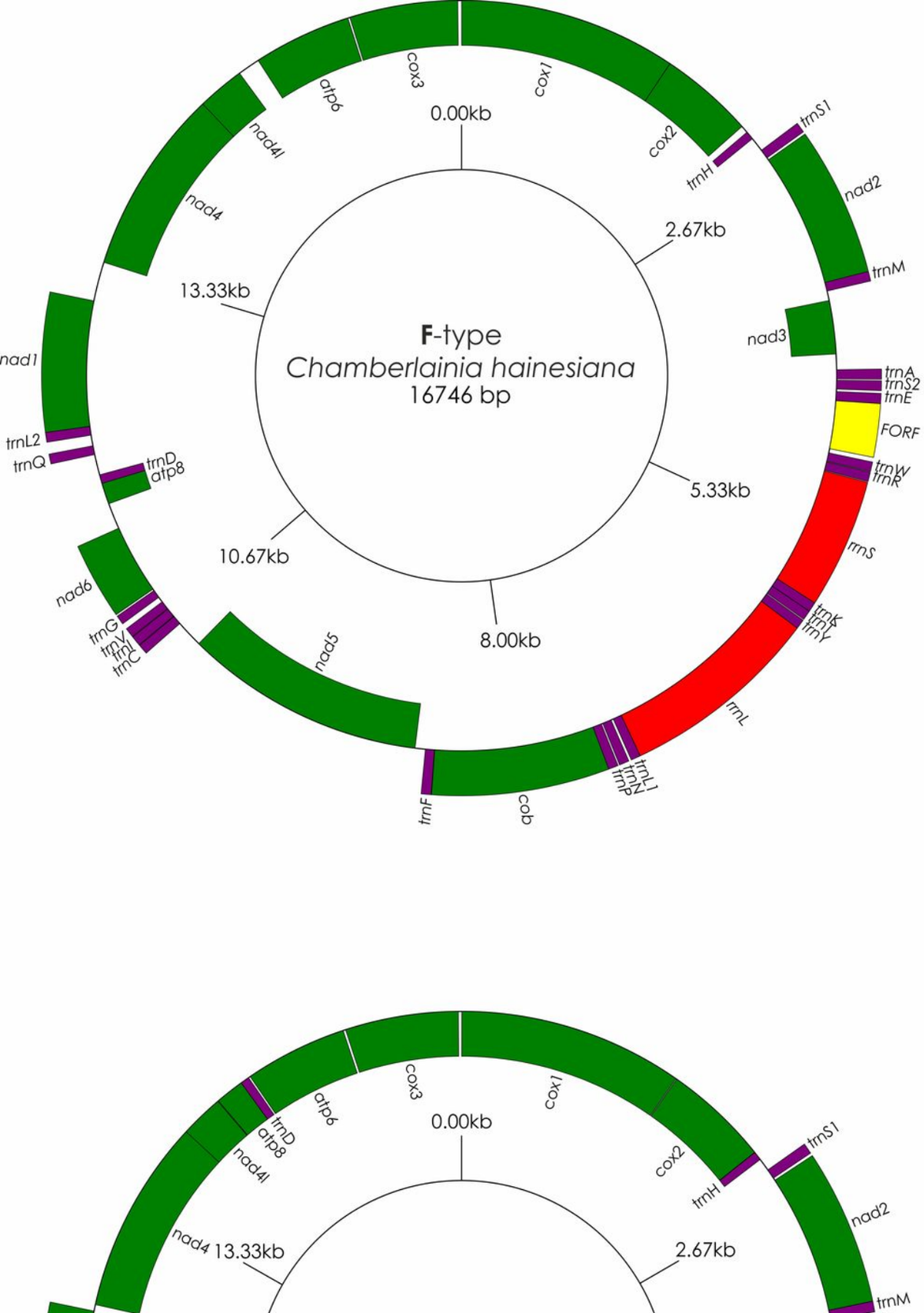
994

995 **Fig. 5** Unionidae F-haplotype phylogenetic sub-tree (BI-NUC) used to infer
996 the most parsimonious putative ancestral gene orders and gene
997 rearrangements, mapped as MF1, UF1, UF2 and UF3 (see text for details).
998 Margaritiferidae and all subfamily nodes were collapsed for visual
999 purposes.

1000

Fig. 6 Time-calibrated mitogenomic phylogeny, an example of three-level classification scheme (subfamilies, tribes and subtribes) and evolution of the mitochondrial gene order in the Unionoidea. Fossil-calibrated ultrametric chronogram of the Unionoidea calculated under a lognormal relaxed clock model and a Yule process speciation implemented in BEAST and obtained for the complete mitogenome data set. The outgroup sample is not shown. Bars indicate 95% confidence intervals of the estimated divergence times between lineages (Ma). Black numbers near nodes are mean ages (Ma). Color labels indicate the mitochondrial gene order (MF1, UF1, UF2, and UF3). Red asterisks indicate fossil calibrations (Supplementary Table 1). Stratigraphic chart according to the International Commission on Stratigraphy, 2015.

Fig. 7 Historical biogeography of the Unionidae. This combined scenario has been inferred from three different statistical modeling approaches (S-DIVA, DEC and S-DEC) based on the time-calibrated mitogenomic phylogeny (Fig. 6A). Pie charts near nodes indicate probabilities of certain ancestral areas. Color circles on the tip nodes indicate the range of each species. Color labels indicate the mitochondrial gene order (UF1, UF2, and UF3).


F-type
Chamberlainia hainesiana
16746 bp
0.00kb
2.67kb
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8.00kb
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cox1
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cox3
atp6
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trnM
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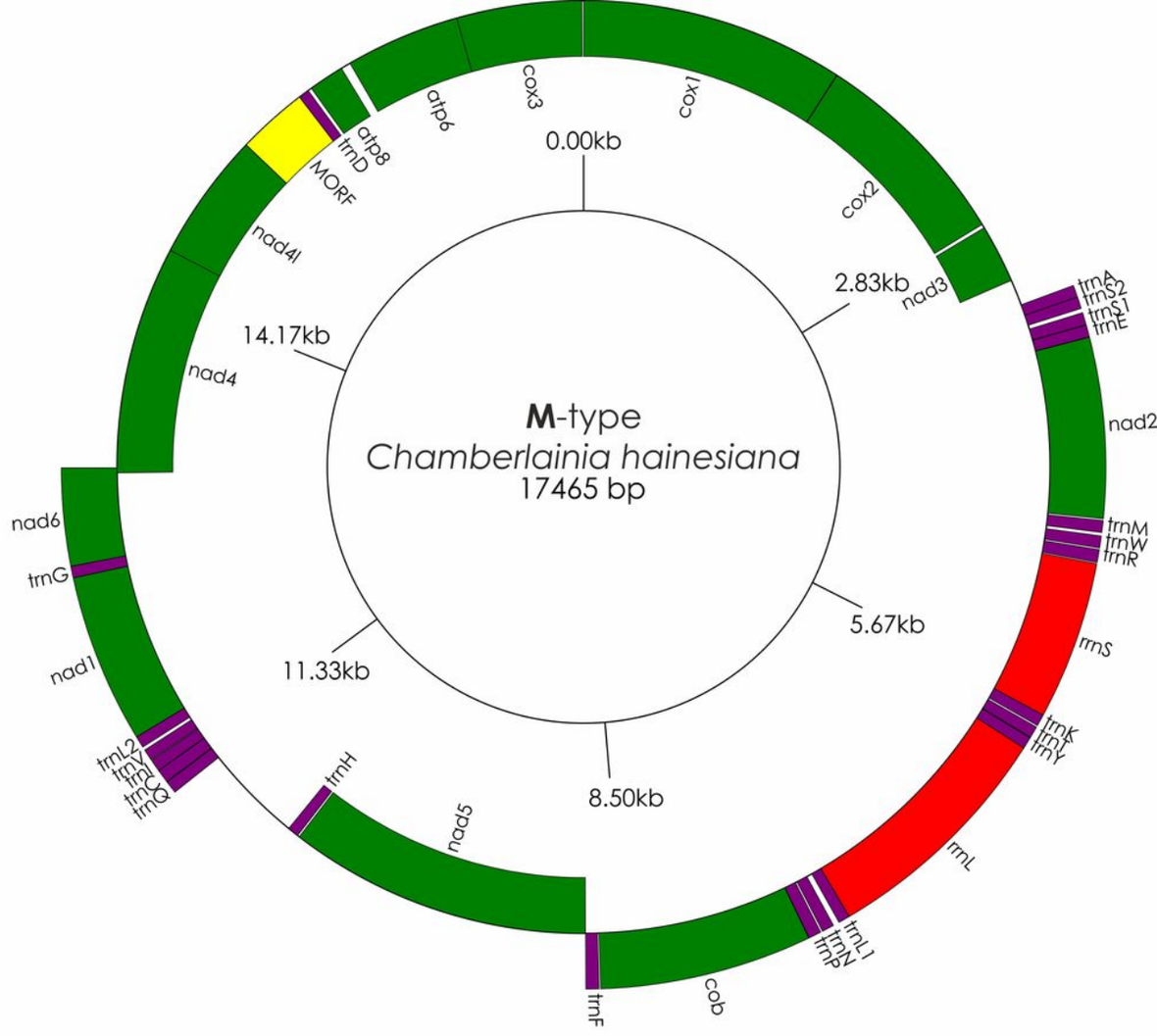
trnL2
trnQ
trnD
atp8
trnG
trnV
trnI
trnC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trnC
trnQ
trnH
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trnF
trnL1
trnN
trnP
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trnK
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trnS1
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trnR

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trnG
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trnC
trnQ
nad5
cob
trnF
trnL1
trnN
trnP
rnL
trnK
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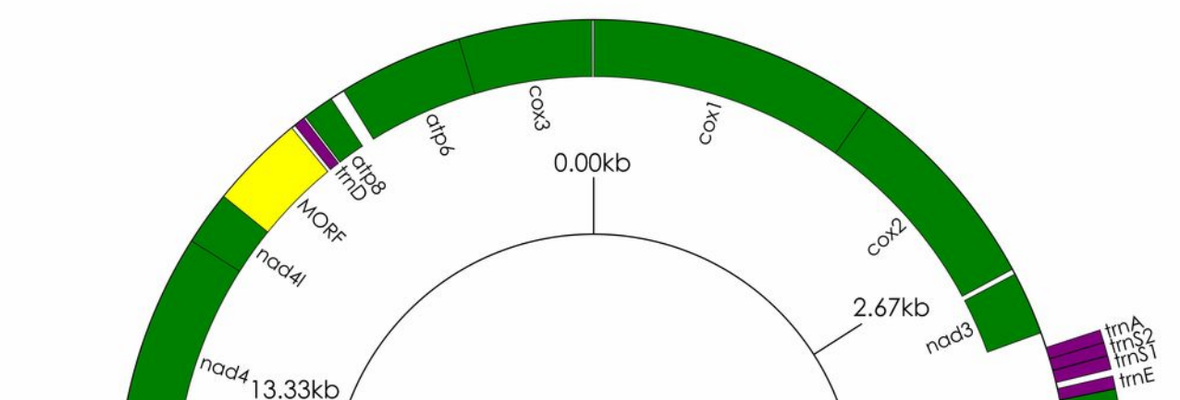
trnS1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nad6
trnG
nad1
trnL2
trnY
trnL
trnC
trnQ
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trnP
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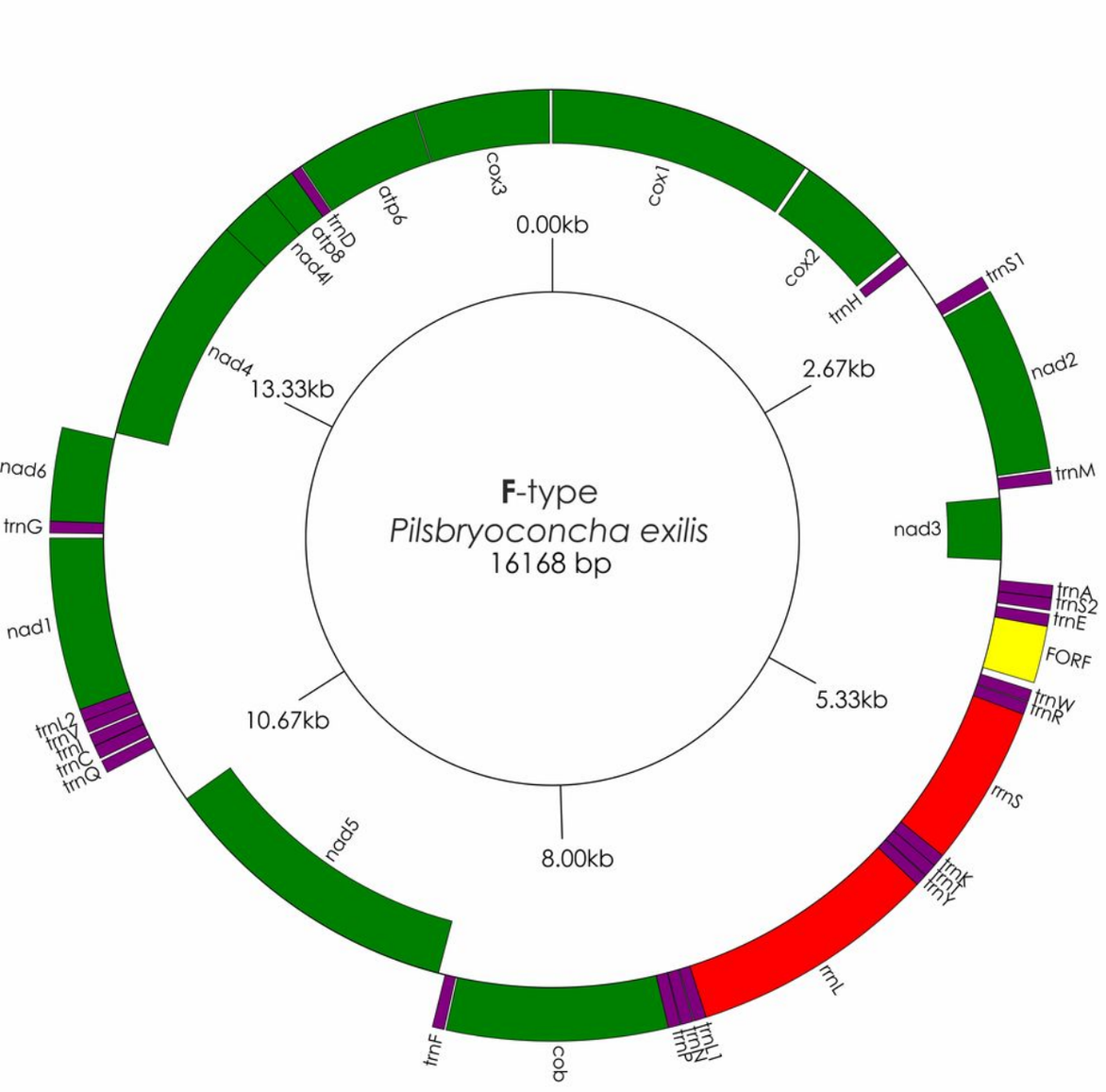
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trnN
trnP
rnL
trnK
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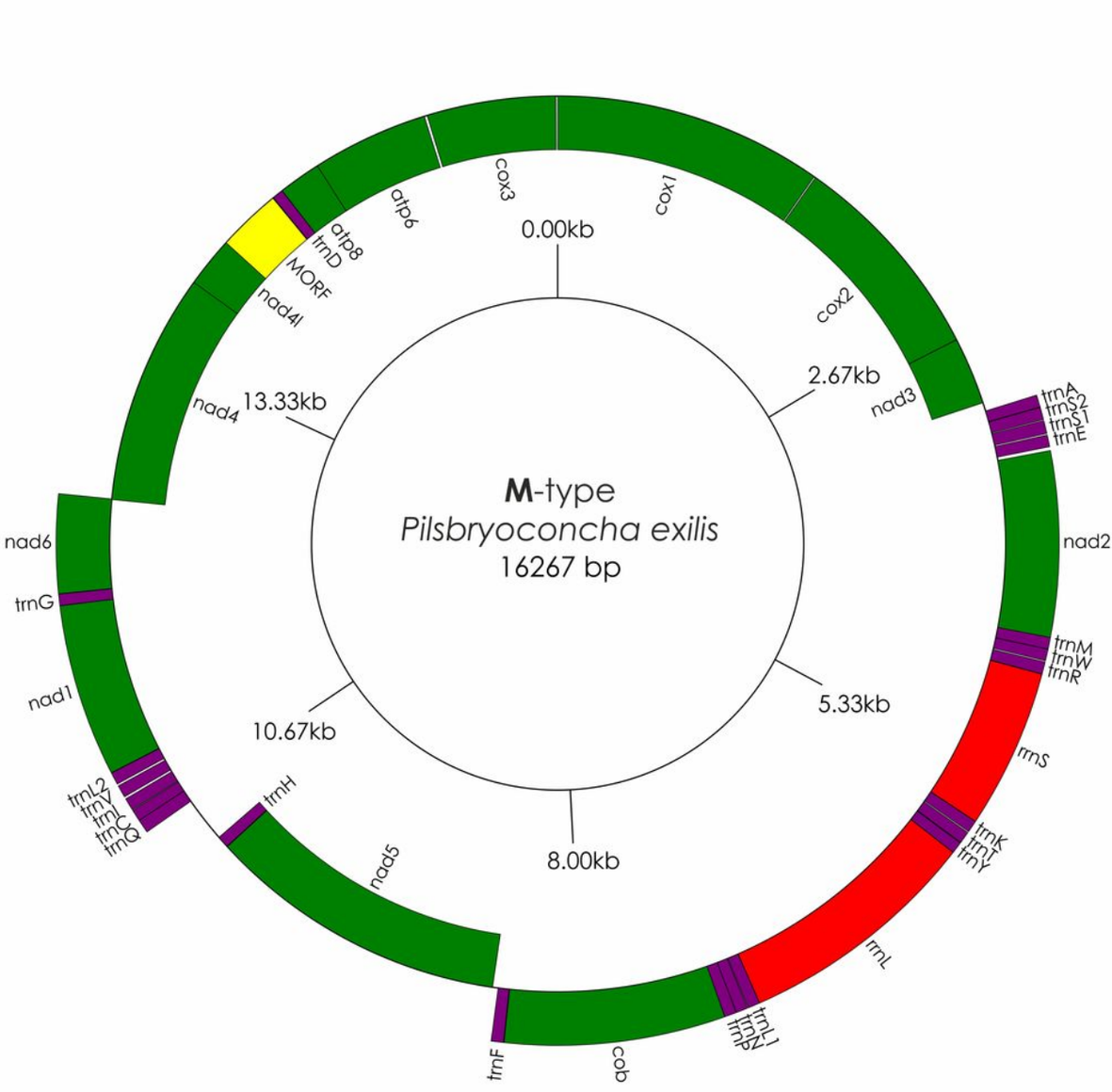
trnS1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trnY
trnL
trnC
trnQ
trnH
nad5
cob
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trnL1
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trnR

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cob
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trnL1
trnN
trnP
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trnK
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rnS
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trnW
trnR
trnS1

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MORF
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nad6
trnG
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trnY
trnL
trnC
trnQ
trnH
nad5
cob
trnF
trnL1
trnN
trnP
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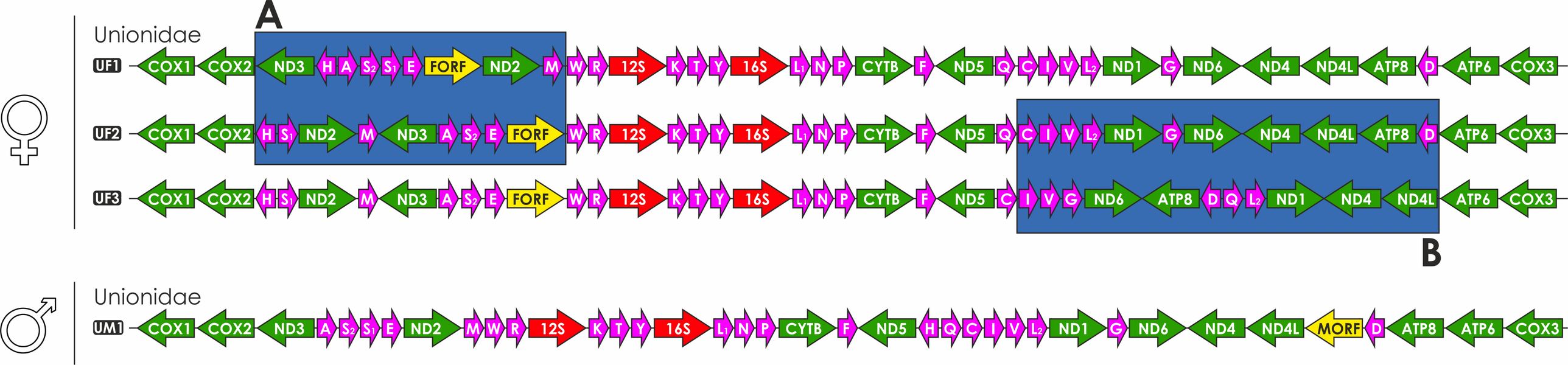
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UF2
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UF3
COX1 COX2 H S1 ND2 M ND3 A S2 E FORF W R 12S K T Y 16S L1 N P CYTB F ND5 C I V G ND6 ATP8 D Q L2 ND1 ND4 ND4L ATP6 COX3
B
Unionidae
UM1
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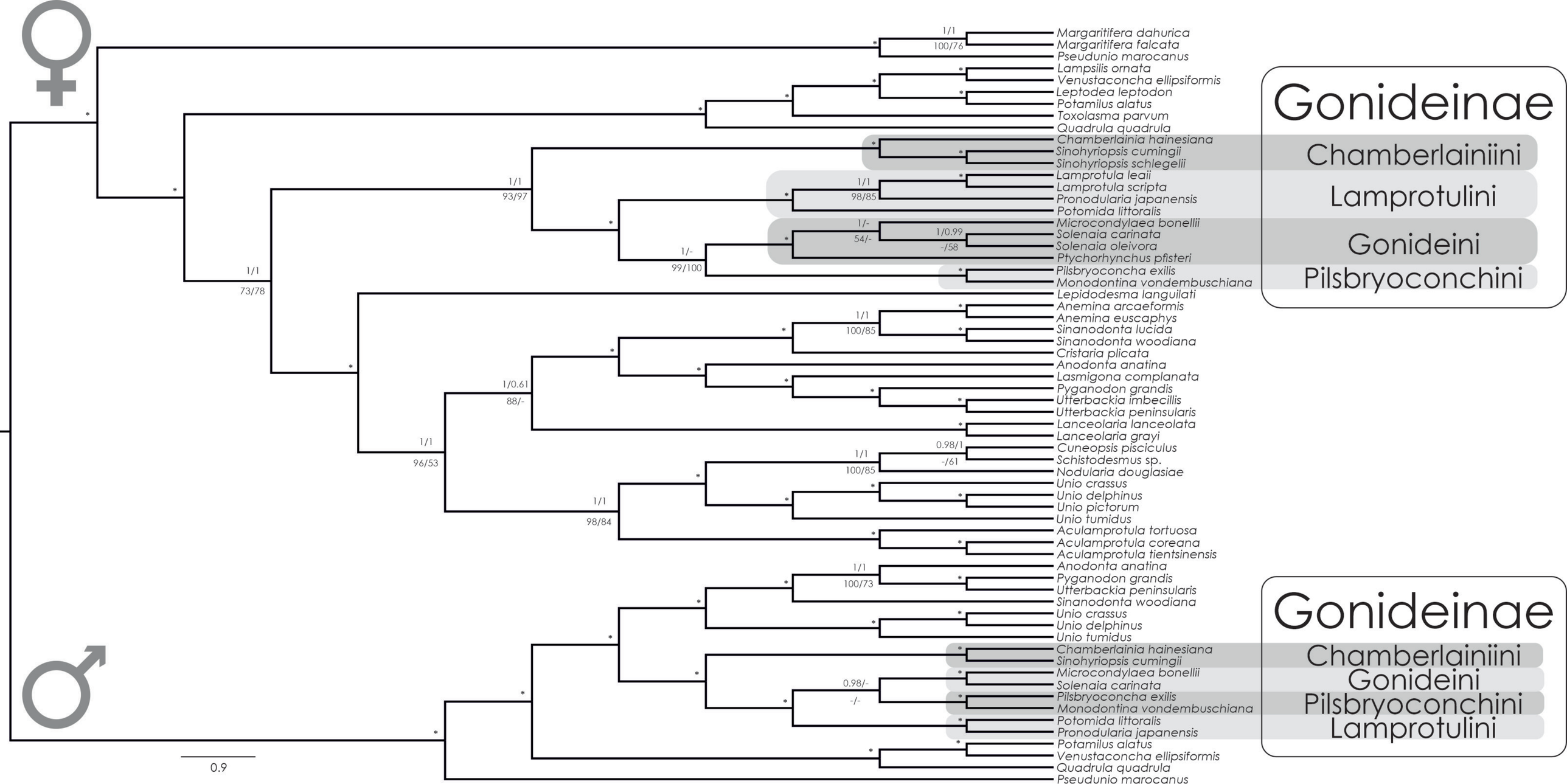
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Margaritifera falcata
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Lampsilis ornata
Venustaconcha ellipsiformis
Leptodea leptodon
Potamilus alatus
Toxolasma parvum
Quadrula quadrula
Chamberlainia hainesiana
Sinohyriopsis cumingii
Sinohyriopsis schlegelii
Lamprotula leaii
Lamprotula scripta
Pronodularia japanensis
Potomida littoralis
Microcondylaea bonellii
Solenaia carinata
Solenaia oleivora
Ptychorhynchus pfisteri
Pilsbryoconcha exilis
Monodontina vondembuschiana
Lepidodesma languilati
Anemina arcaeformis
Anemina euscaphys
Sinanodonta lucida
Sinanodonta woodiana
Cristaria plicata
Anodonta anatina
Lasmigona complanata
Pyganodon grandis
Utterbackia imbecillis
Utterbackia peninsularis
Lanceolaria lanceolata
Lanceolaria grayi
Cuneopsis pisciculus
Schistodesmus sp.
Nodularia douglasiae
Unio crassus
Unio delphinus
Unio pictorum
Unio tumidus
Aculamprotula tortuosa
Aculamprotula coreana
Aculamprotula tientsinensis
Anodonta anatina
Pyganodon grandis
Utterbackia peninsularis
Sinanodonta woodiana
Unio crassus
Unio delphinus
Unio tumidus
Chamberlainia hainesiana
Sinohyriopsis cumingii
Microcondylaea bonellii
Solenaia carinata
Pilsbryoconcha exilis
Monodontina vondembuschiana
Potomida littoralis
Pronodularia japanensis
Potamilus alatus
Venustaconcha ellipsiformis
Quadrula quadrula
Pseudunio marocanus
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93/97
1/1
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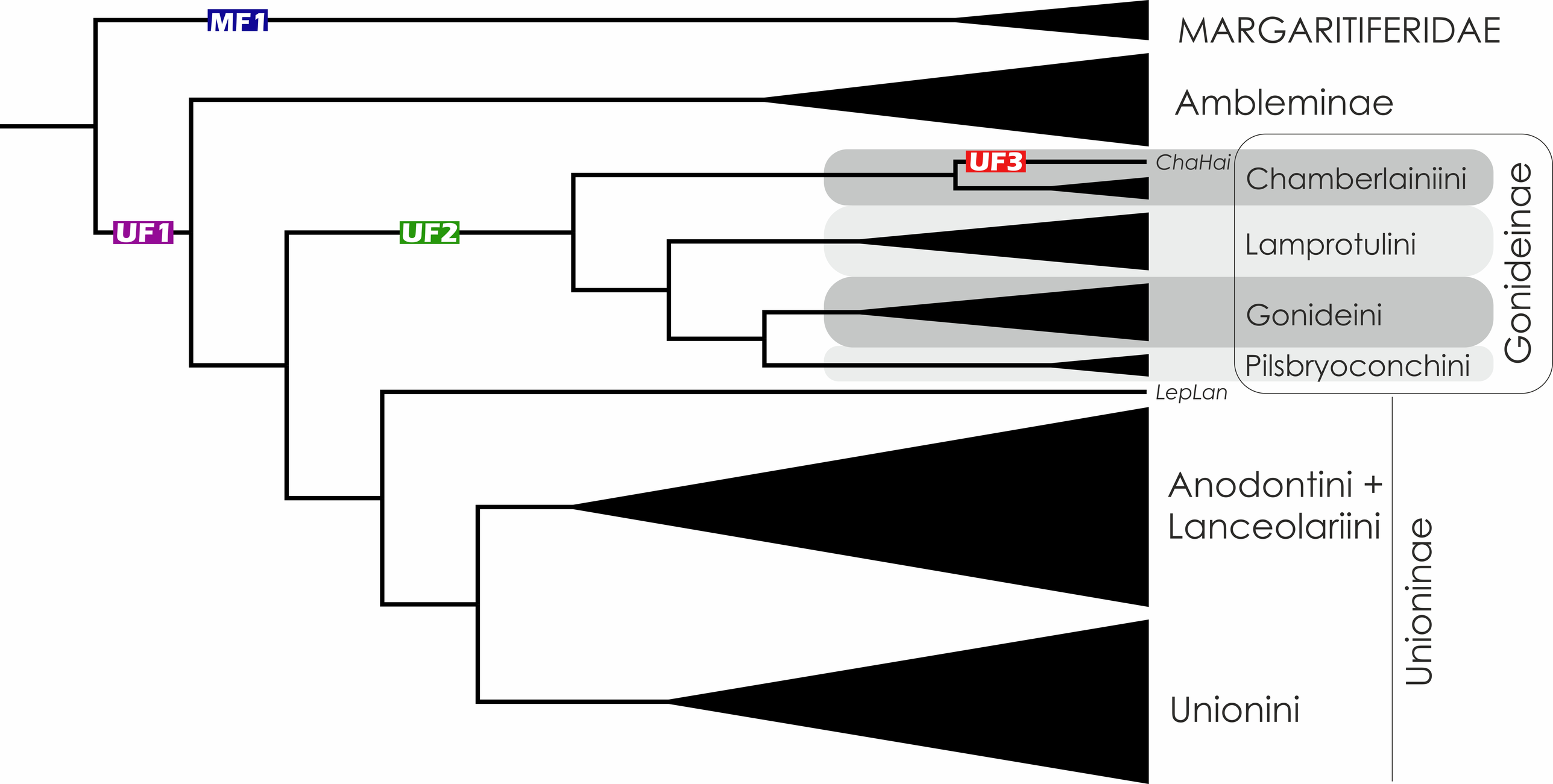
-/-
0.9
Gonideinae
Chamberlainiini
Lamprotulini
Gonideini
Pilsbryoconchini
Gonideinae
Chamberlainiini
Gonideini
Pilsbryoconchini
Lamprotulini

Pseudunio marocanus
Potamilus alatus
Venustaconcha ellipsiformis
Quadrula quadrula
Chamberlainia hainesiana
Sinohyriopsis cumingii
Microcondylaea bonellii
Solenaia carinata
Pilsbryoconcha exilis
Monodontina vondembuschiana
Potomida littoralis
Pronodularia japanensis
Unio crassus
Unio delphinus
Unio tumidus
Sinanodonta woodiana
Anodonta anatina
Pyganodon grandis
Utterbackia peninsularis
Mytilus galloprovincialis

Gonideinae
Chamberlainiini
Gonideini
Pilsbryoconchini
Lamprotulini

5.0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MARGARITIFERIDAE
Ambleminae
UF3
ChaHai
Chamberlainiini
Lamprotulini
Gonideinae
UF1
UF2
Gonideini
Pilsbryoconchini
LepLan
Anodontini +
Lanceolariini
Unioninae
Unionini

T
J
K
Pg
N/Q
Geological Time

Aculamprotula tientsinensis
Aculamprotula coreana
Aculamprotula tortuosa
Aculamprotulina
Cuneopsis pisciculus
Schistodemus sp.
Nodularia douglasiae
Nodulariina
Unio crassus
Unio pictorum
Unio delphinus
Unio tumidus
Unionina
Unionini
Lanceolaria grayii
Lanceolaria lanceolata
Lanceolariina
Lanceolariini
Utterbackia imbecillis
Utterbackia peninsularis
Pyganodon grandis
Lasmigona complanata
Anodonta anatina
Anodontina
Sinanodonta woodiana
Sinanodonta lucida
Anemina euscaphys
Anemina arcaeformis
Cristaria plicata
Cristariina
Anodontini
Lepidodesma languilati
Lepidodesmina
Lepidodesmini
Chamberlainia hainesiana
Sinohyriopsis schlegelii
Sinohyriopsis cumingii
Chamberlainiina
Chamberlainiini
Potomida littoralis
Lamprotula scripta
Lamprotula leaii
Pronodularia japanensis
Lamprotulina
Ptychorhynchus pfisteri
Solenaia carinata
Solenaia oleivora
Microcondylaea bonellii
Gonideina
Pilsbryoconcha exilis
Monodontina vondembuschiana
Pilsbryoconchina
Gonideini
Gonideinae
Potamilus alatus
Leptodea leptodon
Lampsilis ornata
Venustaconcha ellipsiformis
Toxolasma parvum
Quadrula quadrula
Ambleminae
Pseudunio marocanus
Margaritifera dahurica
Margaritifera falcata
Margaritiferidae
Unioninae

24.0
47.0
85.7
53.7
59.0
72.0
36.9
9.9
57.9
95.4
11.8
106.3
89.9
22.5
39.5
51.0
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4.9
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22.9
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43.8
61.5
106.6
67.4
52.4
59.8
95.0
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28.0
50.9
30.7
77.0
117.5
55.8
37.0
159.3
208.2

UF1
UF2
UF3
MF1
†*Hadrodon jurassicus*
†Shifangellidae

250
200
150
100
50
0
Absolute Time

Aculamprotula tientsinensis
Aculamprotula coreana
Aculamprotula tortuosa
Aculamprotulina

Cuneopsis pisciculus
Schistodesmus sp.
Nodularia douglasiae
Nodulariina

Unio crassus
Unio pictorum
Unio delphinus
Unio tumidus
Unionina

Lanceolaria grayii
Lanceolaria lanceolata
Lanceolariina

Utterbackia imbecillis
Utterbackia peninsularis
Pyganodon grandis
Lasmigona complanata
Anodonta anatina
Anodontina

Sinanodonta woodiana
Sinanodonta lucida
Anemina euscaphys
Anemina arcaeformis
Cristaria plicata
Cristariina

Lepidodesma languilati
Lepidodesmina

Chamberlainia hainesiana
Sinohyriopsis schlegelii
Sinohyriopsis cumingii
Chamberlainiina

Potomida littoralis
Lamprotula scripta
Lamprotula leaii
Pronodularia japanensis
Lamprotulina

Ptychorhynchus pfisteri
Solenaia carinata
Solenaia oleivora
Microcondylaea bonellii
Gonideina

Pilsbryoconcha exilis
Monodontina vondembuschiana
Pilsbryoconchina

Potamilus alatus
Leptodea leptodon
Lampsilis ornata
Venustaconcha ellipsiformis
Toxolasma parvum
Quadrula quadrula
Ambleminae

Unioninae

Gonideinae

UF1
UF1
UF3
UF2
UF1

Biogeographic events:
Dispersal
Vicariance

Ancestral areas:
East Asia
North America
North America + East Asia
North America + Europe
Southeast Asia
Europe
Europe + East Asia
East Asia + Southeast Asia

Table 1. List of specimens analysed (based on Lopes-Lima et al. 2017), GenBank references, and country. *original identification.

TAXON	CODE	F-TYPE GenBank	M-TYPE GenBank	COUNTRY
UNIONIDA				
UNIONIDAE				
AMBLEMINAE				
Lampsilis ornata	LamOrn	NC_005335	-	USA
Leptodea leptodon	LepLeo	NC_028522	-	China (Introduced)
Potamilus alatus	PotAla	KU559011	KU559010	China (Introduced)
Quadrula quadrula	QuaQua	NC_013658	FJ809751	USA
Toxolasma parvum	TaxPar	NC_015483	-	USA
Venustaconcha ellipsiformis	VenEll	FJ809753	NC_013659	USA
GONIDEINAE				
CHAMBERLAINIINI				
Chamberlainia hainesiana	ChaHai	Submitted	Submitted	Thailand
Sinohyriopsis cumingii	SinCum	NC_011763	KC150028	China
Sinohyriopsis schlegelii	SinSch	NC_015110	-	China (Introduced)
GONIDEINI				
Microcondylaea bonellii	MicBon	Submitted	Submitted	Italy
Ptychorhynchus pfisteri	PtyPfi	KY067440	-	China
Solenaia carinata	SolCar	NC_023250	KC848655	China
Solenaia oleivora	SolOle	NC_022701	-	China
LAMPROTULINI				
Lamprotula leai	LamLea	NC_023346	-	China
Lamprotula scripta	LamScr	NC_030258	-	China
Potomida littoralis	PotLit	NC_030073	KT247375	Portugal
Pronodularia japanensis	ProJap	AB055625	AB055624	Japan
PILSBRYOCONCHINI				
Pilsbryoconcha exilis	PilExi	Submitted	Submitted	Malaysia
Monodontina vondembuschiana	PseVon	Submitted	Submitted	Malaysia
UNIONINAE				
Aculamprotula tientsinensis	AcuTie	NC_029210	-	China
Aculamprotula coreana	AcuCor	NC_026035	-	South Korea
Aculamprotula tortuosa	AcuTor	NC_021404	-	China
Anemina arcaeformis	AneArc	NC_026674	-	China
Anemina euscaphys	AneEus	NC_026792	-	China
Anodonta anatina	AnoAna	NC_022803	KF030962	Poland
Cristaria plicata	CriPli	NC_012716	-	China
Cuneopsis pisciculus	CunPis	NC_026306	-	China
*'Lamprotula gottschei'**	LamGot	NC_023806	-	China
Lanceolaria grayana	LanGra	NC_026686	-	China
Lanceolaria lanceolata	ArcLan	NC_023955	-	China
Lasmigona compressa	LasCom	NC_015481	-	USA
Lepidodesma languilati	LepLan	NC_029491	-	China
Nodularia douglasiae	NodDou	NC_026111	-	China
Pyganodon grandis	PygGra	NC_013661	FJ809755	USA

Sinanodonta lucida	SinLuc	NC_026673	-	China
Sinanodonta woodiana	SinWoo	HQ283348	KM434235	China
Unio crassus	UniCra	KY290447	KY290450	Poland
Unio delphinus	UniDel	KT326917	KT326918	Portugal
Unio pictorum	UniPic	NC_015310	-	Poland
Unio tumidus	UniTum	KY021076	KY021073	Poland
Utterbackia imbecillis	UttImb	NC_015479	-	USA
Utterbackia peninsularis	UttPen	HM856636	NC_015477	USA
MARGARITIFERIDAE				
Margaritifera dahurica	MarDah	NC_023942	-	China
Margaritifera falcata	MarFal	NC_015476	-	USA
Pseudunio marocanus	PseMrc	KY131953	KY131954	Morocco
MYTILIDA				
Mytilus galloprovincialis	MytGal	AY497292	AY363687	Greece

Table 2. Main structural features of the female (above) and male (below) transmitted mitochondrial genomes of Gonideinae species. Newly sequenced species are presented in bold.

	Chamberlainiini			*Gonideini*				*Lamprotulini*				*Pilsbryoconchini*	
♀	***C. hainesiana***	*S. cumingii*	*S. schlegelii*	***M. bonellii***	*P. pfisteri*	*S. carinata*	*S. oleivora*	*L. leai*	*L. scripta*	*P. littoralis*	*P. japanensis*	***P. exilis***	***M. vondembuschiana***
Tot. size (pb)	**16,746**	15,954	15,939	**16,020**	16,040	16,716	16,392	16,530	16,250	15,789	16,826	**16,168**	**16,028**
A+T %	**58.10**	60.24	60.30	**62.00**	60.77	60.89	59.93	60.28	58.95	58.23	57.20	**60.72**	**58.97**
GC (+) skew	**0.37**	0.36	0.35	**0.35**	0.36	0.39	0.37	0.37	0.36	0.36	0.36	**0.37**	**0.38**
AT (+) skew	**-0.29**	-0.23	-0.23	**-0.20**	-0.22	-0.22	-0.22	-0.21	-0.23	-0.23	-0.21	**-0.22**	**-0.24**
♂	***C. hainesiana***	*S. cumingii*		***M. bonellii***		*S. carinata*				*P. littoralis*	*P. japanensis*	***P. exilis***	***M. vondembuschiana***
Tot. size (pb)	**17,465**	17,100		**16,737**		17,102				16,451	16,967	**16,267**	**16,364**
A+T %	**62.35**	59.71		**59.79**		61.01				58.93	57.12	**61.90**	**59.55**
GC (+) skew	**0.43**	0.41		**0.35**		0.38				0.34	0.36	**0.35**	**0.37**
AT (+) skew	**-0.24**	-0.27		**-0.26**		-0.27				-0.24	-0.25	**-0.25**	**-0.26**