Analysis of mitochondrial genomes resolves the phylogenetic position of Chinese freshwater mussels (Bivalvia, Unionidae)

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Abstract

The Yangtze River basin is one of the most species-rich regions for freshwater mussels on Earth, but is gravely threatened by anthropogenic activities. However, conservation planning and management of mussel species has been hindered by a number of taxonomic uncertainties. In order to clarify the taxonomic status and phylogenetic position of these species, mitochondrial genomes of four species (Acuticosta chinensis, Schistodesmus lampreyanus, Cuneopsis heudei and Cuneopsis capitatus) were generated and analyzed along with data from 43 other mitogenomes. The complete F-type mitogenomes of A. chinensis, S. lampreyanus, C. heudei, and C. capitatus are 15652 bp, 15855 bp, 15892 bp, and 15844 bp, respectively, and all four F-type mitogenomes have the same pattern of gene arrangement. ML and BI trees based on the mitogenome dataset are completely congruent, and indicate that the included Unionidae belong to three subfamilies with high bootstrap and posterior probabilities, i.e., Unioniniae (Acutamprotula, Cuneopsis, Nodularia, and Schistodesmus), Anodontinae (Cristaria, Arconaia, Acuticosta, Lanceolaria, Anemina, and Sinoanodonta), and Gonideinae (Ptychorhynchus, Solenaia, Lamprotula, and Sinohyriopsis). Results also indicate that A. chinensis has affinities with Arconaia lanceolata and Lanceolaria grayii and is a member of the subfamily Anodontiniae.
Keywords
China, classification, freshwater, F-type mitogenome, mussel

Introduction

The freshwater mussel family Unionidae is the most species-rich family within the order Unionida, including more than 620 species representing 142 genera (Graf and Cummings 2007; Bogan 2008). The Unionidae is widely distributed, and its members are found on all continents, with the exception for Antarctica (Graf and Cummings 2007; Bogan 2008; Lopes-Lima et al. 2017a). Unfortunately, freshwater mussels are one of the most threatened animal groups in the world, due to habitat destruction, commercial exploitation, and water pollution (Lydeard et al. 2004; Vaughn et al. 2010; Lopes-Lima et al. 2014; Wu et al. 2017a).

Well-supported phylogenetic hypotheses for the Unionidae are crucial for understanding the evolutionary history and biogeography of its genera (e.g., Roe 2013; Graf et al. 2015), for formulating reliable classifications (e.g., Campbell et al. 2005), and for developing conservation priorities (Lopes-Lima et al. 2017b, 2018). Advances in developing improved phylogenetic hypotheses for the Unionidae have occurred in the past several decades (Davis 1984; Lydeard et al. 1996; Nagel and Badino 2001; Hoeh et al. 2001, 2002; Giribet and Wheeler 2002; Graf 2002; Campbell et al. 2005; Zanatta and Murphy 2006; Graf and Cummings 2007; Campbell and Lydeard 2012a, b; Froufe et al. 2014; Prié and Puillandre 2014; Graf et al. 2015; Pfeiffer and Graf 2015). Most of these studies have focused on North American, Australian, and European taxa, although more recently, African (Whelan et al. 2011; Graf 2013; Elderkin et al. 2016) and Asian (Huang et al. 2002; Zhou et al. 2007; Huang et al. 2013; Bolotov et al. 2017a, b) taxa have been included, and a global phylogenetic framework of the Unionidae has recently been established (Bolotov et al. 2017a; Lopes-Lima et al. 2017a).

Despite these advances, the incorporation of Asian taxa into unionid phylogenetic hypotheses, particularly those from China has lagged.

The middle and lower reaches of the Yangtze River are a diversity hotspot for unionids in East Asia (Graf and Cummings 2007; He and Zhuang 2013; Zieritz et al. 2017), and this region may harbor as many as 15 unionid genera (Wu et al. 2000; Shu et al. 2009; Wu et al. 2017a). As with North American freshwater mussels, much of the early descriptive work on Chinese taxa occurred during the latter part of the 19th Century (Heude 1875, 1877a, b, 1878, 1879, 1880a, b, 1881, 1883, 1885). Pierre Marie Heude was a Jesuit priest who collected freshwater and terrestrial mollusks in China. During a ten-year period between 1882 and 1902, Heude described close to 600 species including 140 freshwater mussel species (Johnson 1973). However, the validity and classification of many of these species were called into question by Simpson (1900, 1914) and Haas (1969). Simpson (1900, 1914) presented a modified classification based on anatomical information such as marsupium size and shape, larval type and umbo sculpture in addition to conchological characters. Simpson condensed the number of Chinese freshwater mussels down to 85 species in 14 genera and placed them into two subfamilies, the Unioniniae and the Hyriiniae. Haas (1969) further re-
vised the classification of the Unionidae and reduced the number of Chinese unionids to 56 species and subspecies in 20 genera, and placed them into four subfamilies: Unioninae, Quadrulinae, Anodontinae and Lampsilinae. After 1949, Chinese malacologists (e.g., Lin 1962; Tchang et al. 1965a, b; Liu et al. 1964, 1979, 1980, 1982; Wu et al. 2000) conducted a substantial amount of work on the classification of the Unionidae, and placed Chinese species into either the Unioninae or Anodontinae, based on the presence or absence of hinge teeth. In the 1990s, malacologists began to refocus their attention on the soft anatomy and changes to the classification, based on the shape of the glochidia and type of marsupium were made (Wei and Fu 1994; Wu et al.1999a, b; Shu et al. 2012). Despite these advances, the higher-level taxonomy of Chinese unionids was not updated, and only the subfamilies Unioninae and Anodontinae remained in the revised system.

At the beginning of this century, Chinese researchers investigated the molecular systematics of the Unionidae and made great progress revising the earlier classifications (Huang et al. 2002; Wang et al. 2013; Ouyang et al. 2011, 2015; Huang et al. 2013, 2015, 2018; Song et al. 2016; Zhou et al. 2007, 2016a, b; Wu et al. 2016, 2017b). However, there continued to be many discrepancies regarding the classification of genera (Table 1). Most recently, Lopes-Lima et al. (2017a) constructed a phylogenetic framework for the worldwide Unionidae; however, it only contained 17 Chinese freshwater mussel species. Wu et al. (2018b) generated a phylogeny based on portions of the mitochondrial COI and ND1 genes that included 34 Chinese unionids. While the resultant trees from these studies resolved a number of relationships, branch support values at certain nodes were low, and the placements of some genera (Sinohyriopsis and Lepidodesma) were not clarified.

The purpose of this study was to clarify the taxonomic status and phylogenetic position of Chinese Unionidae using the DNA sequences of mitochondrial genomes to infer phylogenetic relationships. Phylogenetic hypotheses based on the analysis of mitochondrial genomes of unionids are becoming more common (Walker et al. 2006; Huang et al. 2013, 2018; Burzyński et al. 2017). In the Unionoida, Mytiloida, and Veneroida, an unusual mode of mitochondrial DNA transmission termed Doubly Uniparental Inheritance (DUI) occurs, in which two distinct, tissue-specific and gender-associated mitogenomes (i.e., F-type and M-type) (Breton et al. 2007) are present. For the remainder of this paper, all references to mitogenomes refer to the F-type mitogenome.

In this study, we sequenced and described the complete mitogenomes of four Chinese unionids: Acuticosta chinensis (Lea, 1868), Schistodesmus lampreyanus (Baird & Adams, 1867), Cuneopsis heudei (Heude, 1874), and Cuneopsis capitatus (Heude, 1874), with the aim of combining these new genome sequences with existing mitochondrial genomes to develop a phylogenetic framework for the Chinese Unionidae. In addition, we were particularly interested in determining the taxonomic position of the genus Acuticosta. This genus was erected by Simpson (1900) and Acuticosta chinensis (Lea, 1868) was used as the type species. The genus Acuticosta has been placed in a number of unionid subfamilies including the Hyriinae (Simpson, 1900), Unioninae (Liu 1979), Acuticostinae (Prozorova et al. 2005), and Unioninae (Huang et al. 2002, Graf and Cummings 2007; Zhou et al. 2007, Ouyang et al. 2011, 2015), and most recently, the Anodontinae (Wu et al. 2018b).
**Table 1.** Chinese freshwater mussels (Unionidae) systematic taxonomy history. Shaded genera indicate classification disputes.

| Genus               | Liu et al. 1979 | Huang et al. 2002 | Zhou et al. 2007 | Ouyang et al. 2011 | Huang et al. 2013 | Ouyang et al. 2015 | Wu et al. 2018b | This study |
|---------------------|-----------------|-------------------|-------------------|-------------------|-------------------|-------------------|-----------------|-----------|
| Aculamprotula       | Wu et al., 1999 | –                 | –                 | –                 | –                 | –                 | –                | Unioninae |
| Sinanodonta         |                  |                   | Anodontinae       | Anodontinae       | Anodontinae       | –                 | Anodontinae     | Anodontinae |
| Modell, 1944        |                  |                   |                   |                   |                   |                   |                   | Anodontinae |
| Cristaria           | Schumacher, 1817 | Anodontinae       | Anodontinae       | Anodontinae       | –                 | Anodontinae       | Anodontinae     | Anodontinae |
| Conopeps           | Simpson, 1900    | Unioninae         | Unioninae         | Unioninae         | Unioninae         | Unioninae         | Unioninae       | Unioninae |
| Schistodensmus      | Simpson, 1900    | Unioninae         | Unioninae         | Unioninae         | –                 | Unioninae         | Unioninae       | Unioninae |
| Nodularia           | Conrad, 1853     | Unioninae         | Unioninae         | Unioninae         | Unioninae         | Unioninae         | Unioninae       | Unioninae |
| Anemina      | Haas, 1969       | Anodontinae       | Anodontinae       | –                 | –                 | Anodontinae       | Anodontinae     | Anodontinae |
| Aricicosta           | Simpson, 1900    | Unioninae         | Unioninae         | Unioninae         | –                 | –                 | Unioninae       | Anodontinae |
| Arconasia           | Conrad, 1865     | Unioninae         | Unioninae         | Unioninae         | –                 | –                 | Unioninae       | Anodontinae |
| Lamprotula       | Simpson, 1900    | Unioninae         | Ambleminae        | Ambleminae        | Ambleminae        | –                 | Ambleminae      | Gonideinae |
| Lanceolatia         | Conrad, 1853     | Unioninae         | Unioninae         | Unioninae         | –                 | Unioninae         | Anodontinae     | Anodontinae |
| Lepidodesma         | Simpson, 1896    | Anodontinae       | –                 | –                 | –                 | –                 | Unioninae       | Incertae sedis |
| Psychorhynchus      | Simpson, 1900    | –                 | Ambleminae        | –                 | –                 | –                 | –                | Gonideinae |
| Solenaria           | Conrad, 1869     | Anodontinae       | Ambleminae        | Ambleminae        | Gonideinae        | Ambleminae        | Gonideinae      | Gonideinae |
| Sinobryopisis       | Starobogatov, 1970 | Unioninae          | Ambleminae        | Ambleminae        | Ambleminae        | Ambleminae        | –                | Incertae sedis |

**Materials and methods**

**Taxon sampling, mitochondrial genome sequencing, and assembly**

Samples of four species were collected from Poyang Lake (28°47.84’N; 116°2.03’E) in Jiangxi Province, China (Figure 1), and specimens were preserved and vouchers deposited in the Biological Museum of Nanchang University. Information for primers used for PCR amplification of F-type mitogenomes can be found in Table 2. Complete mitogenomes were sequenced and annotated according to our previous study (Wu et al. 2016).

**Dataset construction**

We downloaded all published unionid mitogenomes from GenBank (as of March 2018), and combined them with the four mitogenomes generated in this study for a total of 41 unionid mitogenomes (22 Chinese taxa). In addition, we included additional genomes, also downloaded from GenBank, from the Margaritiferidae (four species), Iridinidae (one species), and Hyriidae (one species) as out-groups for the phylogenetic analysis (Table 3).
Figure 1. Shells of the unionids species in this study. A Acuticosta chinensis (Lea, 1868) B Schistodesmus lampreyanus (Baird & Adams, 1867) C Cuneopsis heudei (Heude, 1874) D Cuneopsis capitatus (Heude, 1874). Scale bar: 4 cm. Photographs R-W Wu.

Alignments, partitioning strategies, and phylogenetic analyses

Nucleotide sequences of 12 mitochondrial protein-coding genes (we excluded atp8) and 2 rRNA genes were concatenated for construction of the phylogenetic trees. Nucleotide sequences of protein coding genes (PCG) were translated to amino acid sequences using MEGA 5.0 (Tamura et al. 2011), and genes were aligned based on the amino acid sequence (PNGs), or nucleotide sequence (rRNA) using the MUSCLE program (Edgar 2004) with default settings. Alignments of sequences were manually checked and areas of ambiguous alignment were excluded. Finally, 12 PCGs and the 2 rRNA genes were concatenated (11862 bp) using SequenceMatrix (Vaidya et al. 2011). The dataset was then partitioned according to codon position of each PCG and each rRNA gene for phylogenetic analysis. Prior to phylogenetic analysis, a partition homogeneity test was carried out in PAUP* version 4.0b10 (Swofford 2003) to determine rate heterogeneity among genes and codon positions. The partition homogeneity test indicated there was no significant difference in signals (P > 0.05).

PartitionFinder v1.1.1 (Lanfear et al. 2012) was used to select optimal substitution models for the 2 rRNA genes and each codon position of the 12 PCG. Bayesian analyses were undertaken in MrBayes Version 2.01 (Ronquist et al. 2012), four chains were run simultaneously for 1 million generations, and trees were sampled every 1000
Table 2. Primers used for PCR amplification of female *Acuticosta chinensis*, *Schistodesmus lampreyanus*, *Cuneopsis heudei*, and *Cuneopsis capitatus* mitochondrial genomes.

| Fragment                  | Primer name | Primer sequence (5’ to 3’)                          | Length  |
|---------------------------|-------------|------------------------------------------------------|---------|
| COI (universal primer)    | LCO1490     | GGTCAACAAATCATAAGATATTGG                             | ~700 bp |
|                           | HCO2198     | TAAACCTCAGGGTGACCAAAAATCA                            |         |
| 16S (universal primer)    | 16SarL      | CGCCTGGTTTATCAAAACAT                                 | ~500 bp |
|                           | 16SbrH      | CCGGTCTGAACTCAGTACGTCGAT                           |         |
| ND1 (universal primer)    | Leu-urRF    | TGGCAGAAAAGTGCACTAGATAAAGCC                        | ~1000 bp|
|                           | LoGlyR      | CCTGCTTTGAAGCCAGTACT                                  |         |
| COI→ND1 (*A. chinensis*)  | ZGCNH       | TTGGGACTGGCTGGGAC                                   | ~500 bp |
|                           | ZGCNR       | CGAAGCCTGCAAGATGCTATA                                | ~4500 bp|
| ND1→16S (*A. chinensis*)  | ZGN1H       | CGAAGCCTGCAAGATGCTATA                                | ~5200 bp|
|                           | ZGN1R       | TACCCGAAGTCTGGTTTGC                                 |         |
| 16S→COI (*A. chinensis*)  | ZG1CH       | CTAGTGTCCTCCAGTACT                                   | ~3200 bp|
|                           | ZG1CR       | AGACAAGGGAGGTATAACCG                                |         |
| COI→ND1 (*S. lampreyanus*)| SXCNH      | CTGTTGGCAAGCTGATATC                                 | ~2100 bp|
|                           | SXCNR       | ATAGCTTGGCACATG                                    |         |
| ND1→16S (*S. lampreyanus*)| SXN1H       | GAGATGTTGGCTACG                                    | ~4500 bp|
|                           | SXN1R       | CGATGTTGGCTACG                                     |         |
| 16S→COI (*S. lampreyanus*)| SX1CH       | TTCTACTGCTTCGGCCAGT                                  | ~3600 bp|
|                           | SX1CR       | GCAGCAGATGGACAGAC                                   |         |
| COI→ND1 (*C. heudei*)     | YTCNH       | TCTTGGCTGATGCTGCAAATAATGCA                          | ~6200 bp|
|                           | YTCNR       | TCCCCCTTCTTTTATGCAATGCA                             |         |
| ND1→16S (*C. heudei*)     | YTN1H       | TGTCTCTGGGAGGATTACT                                 | ~1300 bp|
|                           | YTN1R       | ACATAAGTGGCAACGGCTATT                               |         |
| 16S→COI (*C. heudei*)     | YT1CH       | TTACTGGTTTCAAGATGCG                                 | ~5600 bp|
|                           | YT1CR       | AATAACACCAGGAAGATCGT                                |         |
| COI→ND1 (*C. capitatus*)  | JSCNH       | GTGTGCTGAGCTATATCTCTTT                              | ~5300 bp|
|                           | JSNR        | TTGGTACCTTTGCGAGG                                   |         |
| ND1→16S (*C. capitatus*)  | JSN1H       | GTATTGTGGAGTGGATGAC                                 | ~4700 bp|
|                           | JSN1R       | GAATGCCAGACTGAGAATA                                   |         |
| 16S→COI (*C. capitatus*)  | JS1CH       | TATCTGCTGGAGTGGTCCAATC                              | ~5000 bp|
|                           | JS1CR       | CAATAATCTTCCAGGTTGAC                                 |         |

generations, with a burn-in of 25%. Stationarity was considered to be reached when the average standard deviation of split frequencies was less than 0.01.

The gene and codon site-based partitioned ML analysis was performed in RAxML implemented in raxmlGUI v.1.3 (Stamatakis 2014), using the GTRGAMMAI model of nucleotide substitution with the search strategy set for rapid bootstrapping. ModelFinder (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017) implemented


| Taxon | GenBank accession number | Reference          |
|-------|--------------------------|--------------------|
| UNIONIDAE |                        |                    |
| Ambleminae |                        |                    |
| Quadrula quadrula (Rafinesque, 1820) | FJ809750          | Breton et al. 2009 |
| Venustaconcha ellipisformis (Conrad, 1836) | FJ809753          | Breton et al. 2009 |
| Potamilus alatus (Say, 1817) | KU559011          | Wen et al. 2017    |
| Leptodea leptodon (Rafinesque, 1820) | NC_028522         | Feng et al. 2016   |
| Toxolasma parvum (Barnes, 1823) | HM856639          | Breton et al. 2011 |
| Lampis ornata (Conrad, 1835) | NC_005335         | Serb and Lydeard 2003 |
| Gonideinae |                        |                    |
| Pronodularia japonensis (Lea, 1859) | AB055625          | Unpublished        |
| Lamprotula leaii (Griffith & Pidgeon, 1833) | NC_023346        | Chen et al. 2012   |
| Ptychorhynchus pfisteri (Heude, 1874) | KY067440         | Zhou et al. 2016a  |
| Potomida litoralis (Cuvier, 1798) | NC_030073         | Froufe et al. 2016 |
| Solenaia oleovora (Heude, 1877) | NC_022701         | Huang et al. 2015  |
| Solenaia carinatus (Heude, 1877) | NC_023250         | Huang et al. 2013  |
| Sinobryopsis schlegeli (Martens, 1861) | HQ641406         | Unpublished        |
| Sinobryopsis cumingii (Lea, 1852) | NC_011763         | Unpublished        |
| Anodontinae |                        |                    |
| Acuticosta chinensis (Lea, 1868) | MH919390          | This study         |
| Arconia lanceolata (Lea, 1856) | KJ144818          | Wang et al. 2014   |
| Lanceolaria grayana (Lea, 1834) | NC_026686         | Unpublished        |
| Pyganodon grandis (Say, 1829) | FJ809754          | Breton et al. 2009 |
| Utterbackia peninsularis Bogon & Hoeh, 1995 | HM856636        | Breton et al. 2011 |
| Utterbackia imbecillis (Say, 1829) | HM856637         | Breton et al. 2011 |
| Lasmigona compresa (Lea, 1829) | NC_015481         | Breton et al. 2011 |
| Anodonta anatina (Linnaeus, 1758) | NC_022803         | Soroka et al. 2015 |
| Sinanodonta woodiana (Lea, 1834) | HQ283346          | Soroka et al. 2010 |
| Sinanodonta lucida (Heude, 1877) | KF667529          | Song et al. 2016   |
| Anemina arcaeformis (Heude, 1877) | KF667530          | An et al. 2016     |
| Anemina eucaphys (Heude, 1879) | NC_026792         | Xue et al. 2016    |
| Cristaria plicata (Leach, 1814) | KM233451          | Wang et al. 2016   |
| Unioninae |                        |                    |
| Lepidodesma langulata (Heude, 1874)* | NC_029491        | Zhou et al. 2016b  |
| Schistodesmus lampreyanus (Baird & Adams, 1867) | MH919388        | This study         |
| Cuneopsis pisciculus (Heude, 1874) | NC_026306         | Han et al. 2016    |
| Cuneopsis heudei (Heude, 1874) | MH919389          | This study         |
| Cuneopsis capitatus (Heude, 1874) | MH919387          | This study         |
| Nodularia douglasiae (Griffith & Pidgeon, 1833) | NC_026111      | Unpublished        |
| Unio delphinus Spengler, 1793 | KT326917          | Fonseca et al. 2017 |
| Unio pictorum (Linnaeus, 1758) | NC_015310         | Soroka et al. 2010 |
| Unio crassus Retzius, 1788 | KY290446          | Burzyński et al. 2017 |
| Unio tumidus Retzius, 1788 | KY021076          | Soroka et al. 2018 |
| Aculamprotula tortuosa (Lea, 1865) | NC_021404         | Wang et al. 2013   |
| Aculamprotula scripta (Heude, 1875) | MF991456         | Wu et al. 2017b    |
| Aculamprotula coreana (Martens, 1886) | NC_026035        | Lee et al. 2016    |
| Aculamprotula tientsinensis (Crosse & Debeaux, 1863) | NC_029210        | Wu et al. 2016     |
in IQ-TREE was used to choose the appropriate models, which additionally considers the FreeRate heterogeneity model (+R). IQ-TREE (Nguyen et al. 2015) was also used for ML tree reconstruction, and 1000 ultrafast bootstrap replicates were run to estimate branch support (Minh et al. 2013). The optimal substitution models for each partition by PartitionFinder and ModelFinder are shown in Suppl. material 1: Tables S1, S2.

Results

General features of the mitochondrial genomes

The lengths of the complete mitogenomes of *Acuticosta chinensis*, *Schistodesmus lampreyanus*, *Cuneopsis heudei*, and *Cuneopsis capitatus* were 15652bp, 15855bp, 15892bp and 15844bp, respectively. The newly sequenced four mitogenomes all contained 13 protein-coding genes, two rRNA genes, 22 tRNAs, and one female specific gene (FORF). All four F-type mitogenomes had the same pattern of gene arrangement. Among the 38 mitochondrial genes, 11 genes were encoded on the heavy chain, and the remaining 27 genes were encoded on the light chain (Figure 2).

The nucleotide composition of the *Acuticosta chinensis*, *Schistodesmus lampreyanus*, *Cuneopsis heudei* and *Cuneopsis capitatus* had obvious A+T bias (*A. chinensis*: 65.73%; *S. lampreyanus*: 64.54%; *C. heudei*: 62.45%; *C. capitatus*: 63.69%). In the base composition analysis for the four species, the A+T skews were negative, and the G+C skew were positive, indicating that the bases composition ratios of the four mitogenomes were T biased to A, and G biased to C. In invertebrate mitochondria, there are three conventional start codons: ATG, ATA and ATT, and three alternative start codons: ATC, TTG, and GTG (Wolstenholme 1992). The mitochondrial genomes of *A. chinensis* and *C. capitatus* had eleven protein coding genes which used the conventional start codons, and the remaining two used alternative start codons. *S. lampreyanus* and *C. heudei* had 12 PCG which used the common start codons, and one used the alternative start codon (Table 4).
The overlapping of neighboring genes is common in freshwater mussel mitochondria. There were three overlaps of neighboring genes in the mitochondrial genome of *Acuticosta chinensis* and *Schistodesmus lampreyanus*, and two in *Cuneopsis heudei*. The position of the largest gene overlap (8 bp) was between ND4 and ND4L. The mitochondrial genome of *Cuneopsis capitatus* only had one overlapping region between tRNA\textsubscript{Met} and ND2. There were 29 non-coding regions (NCRs) in *A. chinensis*, *C. heudei*, and *C. capitatus*, and 27 NCRs in *S. lampreyanus*. The longest NCRs of the *A. chinensis*, *S. lampreyanus*, *C. heudei*, and *C. capitatus* were 224 bp, 349 bp, 216 bp, and 323 bp, respectively; all were located between ND5 and tRNA\textsubscript{Gln} (Table 4).
**Table 4.** Structural characteristics of F-type mitochondrial genomes of *Acuticosta chinensis*, *Schistodesmus lampreyanus*, *Cuneopsis heudei*, and *Cuneopsis capitatus*. For each protein coding genes, start and stop codons and anticodons are presented in parentheses. Gene lengths are in bp.

|                     | *A. chinensis* | *S. lampreyanus* | *C. heudei* | *C. capitatus* |
|---------------------|----------------|------------------|-------------|---------------|
| Total size (bp)     | 15652          | 15855            | 15892       | 15844         |
| AT%                 | 65.73          | 64.54            | 62.45       | 63.69         |
| CG%                 | 34.27          | 35.46            | 37.55       | 36.31         |
| AT skew             | -0.18          | -0.19            | -0.21       | -0.18         |
| GC skew             | 0.28           | 0.33             | 0.33        | 0.32          |
| No. of NCR          | 29             | 27               | 29          | 29            |
| Size range of gene overlap | 1 to 8       | 1 to 8           | 1 to 8      | 1             |

| Gene                | *A. chinensis* | *S. lampreyanus* | *C. heudei* | *C. capitatus* |
|---------------------|----------------|------------------|-------------|---------------|
| **cox1**            | 1539 (TTG/TAG) | 1578 (ATA/TAG)   | 1566 (TTG/TAA) | 1542 (TTG/TAG) |
| tRNA-Asp (D)        | 63 (GTC)       | 64 (GTC)         | 64 (GTC)    | 64 (GTC)      |
| **cox3**            | 780 (ATG/TAA)  | 780 (ATG/TAA)    | 780 (ATG/TAA) | 780 (ATG/TAG)  |
| atp6                | 702 (ATG/TAA)  | 702 (ATG/TAG)    | 702 (ATG/TAG) | 702 (ATG/TAG)  |
| atp8                | 189 (ATG/TAA)  | 192 (ATG/TAA)    | 192 (ATG/TAG) | 192 (ATG/TAG)  |
| **nd1L**            | 297 (GTG/TAG)  | 279 (ATG/TAA)    | 255 (ATG/TAG) | 255 (ATG/TAG)  |
| **nd1**             | 1347 (ATT/TAA) | 1347 (ATT/TAA)   | 1347 (ATT/TAA) | 1329 (ATA/TAA) |
| **nd6**             | 489 (ATT/TAG)  | 486 (ATC/TAA)    | 507 (ATA/TAA) | 507 (ATA/TAA)  |
| tRNA-Gly (G)        | 62 (TCC)       | 63 (TCC)         | 63 (TCC)    | 63 (TCC)      |
| **nd1**             | 900 (ATA/TAG)  | 900 (ATA/TAG)    | 900 (ATA/TAG) | 900 (ATA/TAG)  |
| tRNA-Leu (L2)       | 64 (TAA)       | 64 (TAA)         | 63 (TAA)    | 64 (TAA)      |
| tRNA-Val (V)        | 64 (TAC)       | 63 (TAC)         | 63 (TAC)    | 64 (TAC)      |
| tRNA-Ile (I)        | 64 (GAT)       | 67 (GAT)         | 64 (GAT)    | 64 (GAT)      |
| tRNA-Cys (C)        | 64 (GCA)       | 62 (GCA)         | 64 (GCA)    | 61 (GCA)      |
| tRNA-Gln (Q)        | 69 (TTG)       | 70 (TTG)         | 69 (TTG)    | 69 (TTG)      |
| **nd5**             | 1728 (ATA/TAA) | 1713 (ATA/TAA)   | 1794 (ATA/TAA) | 1734 (ATG/TAA) |
| tRNA-Phe (F)        | 66 (GAA)       | 65 (GAA)         | 65 (GAA)    | 64 (GAA)      |
| **Cob**             | 1137 (ATA/TAA) | 1146 (ATT/TAA)   | 1149 (ATA/TAA) | 1020 (ATC/TAA) |
| tRNA-Pro (P)        | 64 (TGG)       | 66 (TGG)         | 64 (TGG)    | 64 (TGG)      |
| tRNA-Asn (N)        | 65 (GTT)       | 66 (GTT)         | 68 (GTT)    | 65 (GTT)      |
| tRNA-Leu (L1)       | 66 (TAG)       | 64 (TAG)         | 63 (TAG)    | 64 (TAG)      |
| **rrnL**            | 1285           | 1304             | 1302        | 1297          |
| tRNA-Tyr (Y)        | 60 (GTA)       | 61 (GTA)         | 63 (GTA)    | 63 (GTA)      |
| tRNA-Thr (T)        | 61 (TGT)       | 66 (TGT)         | 64 (TGT)    | 63 (TGT)      |
| tRNA-Lys (K)        | 68 (TTT)       | 70 (TTT)         | 70 (TTT)    | 70 (TTT)      |
| **rrnS**            | 853            | 857              | 859         | 853           |
| tRNA-Arg (R)        | 66 (TCG)       | 67 (TCG)         | 65 (TCG)    | 65 (TCG)      |
| tRNA-Trp (W)        | 65 (TCA)       | 64 (TCA)         | 63 (TCA)    | 62 (TCA)      |
| tRNA-Met (M)        | 65 (CAT)       | 65 (CAT)         | 65 (CAT)    | 65 (CAT)      |
| **nd2**             | 966 (ATG/TAA)  | 966 (ATG/TAA)    | 966 (ATG/TAA) | 966 (ATG/TAA) |
| tRNA-Glu (E)        | 63 (TTC)       | 72 (TTC)         | 68 (TTC)    | 68 (TTC)      |
| tRNA-Ser (S2)       | 68 (AGA)       | 73 (AGA)         | 68 (TCT)    | 68 (TCT)      |
| tRNA-Ser (S1)       | 64 (TGA)       | 64 (TGA)         | 64 (CGA)    | 64 (CGA)      |
| tRNA-Ala (A)        | 67 (TGC)       | 65 (TGC)         | 66 (TGC)    | 64 (TGC)      |
| tRNA-His (H)        | 65 (GTT)       | 69 (GTT)         | 69 (GTT)    | 67 (GTT)      |
| **nd3**             | 357 (ATG/TAG)  | 357 (ATG/TAG)    | 357 (ATG/TAA) | 357 (ATG/TAG) |
| **cox2**            | 681 (ATG/TAA)  | 681 (ATG/TAG)    | 681 (ATG/TAA) | 681 (ATG/TAG) |
All four mitochondria contained 22 tRNAs, including two serine tRNAs and two leucine tRNAs. The histidine tRNA and aspartate tRNA were located in the heavy chain, whereas the remaining 20 tRNAs were encoded by the light chain. The length of tRNAs differed slightly in each species (Table 4). The tRNA anticodons were the same in all species with the exception of two serine tRNAs. The anticodons of the two serine tRNAs of *A. chinensis* and *S. lampreyanus* were AGA and TGA, while those of *C. heudei* and *C. capitatus* were TCT and CGA (Table 4).

**Phylogenetic analyses**

ML and BI trees have completely congruent topologies and in general are well supported by high bootstrap and posterior probability values at almost all nodes (Figure 3). The mitogenomic dataset supports the monophyly of four Unionidae subfamilies (i.e., Unioninae, Anodontinae, Ambleminae, and Gonideinae) by both ML and BI methods. Phylogenetic analyses reveal the following relationships: (((Unioninae + Anodontinae) + Gonideinae) + Ambleminae) within the Unionidae.

Our phylogenetic analyses indicate that except for *Lepidodesma languilati* (Heude, 1874), the 21 Chinese species belong to the following three subfamilies: Unioninae (*Aculamprotula, Cuneopsis, Nodularia* and *Schistodesmus*), Anodontinae (*Cristaria, Arconaia, Acuticosta, Lanceolaria, Anemina* and *Sinoanodonta*), and Gonideinae (*Psychorhynchus, Solenaia, Lamprotula, Sinohyriopsis*). Our results support the placement of *Acuticosta chinensis* in the Anodontinae, but *Leidodesma languilati* is not placed as a member of any subfamily, but instead is the well-supported sister taxon to the monophyletic group formed by the Unioninae and Anodontinae.
Discussion

Phylogenetic relationships of subfamilies in the Unionidae

In this study, we provide a novel phylogenetic hypothesis for relationships between subfamilies in the Unionidae (Figure 4). Other phylogenetic analyses of the Unionidae have been based on selected gene regions. For example, Lopes-Lima et al. (2017a) proposed the phylogenetic relationship of the subfamily based on COI and 28S as follows: (Anodontinae + Unioninae) + (Rectidentinae + (Ambleminae + Gonideninae)). Bolotov et al. (2017a) proposed relationships based on three loci (COI, 16S and 28S), and adding more taxa: ((Anodontinae + Unioninae) + (Ambleminae + Gonideninae)) + (Rectidentinae + Pseudodontinae). Prior investigations into subfamily relationships in the Unionidae, based on complete mitochondrial genomes, seem to be consistent with these earlier studies, (Anodontinae + Unioninae) + (Ambleminae + Gonideninae) (Huang et al. 2013; Burzyński et al. 2017; Huang et al. 2018; Wu et al. 2016, 2017b). The current study is based on the mitochondrial genome sequences for the largest number of unionid species (41). By increasing the number of taxa and the amount of DNA sequences, we obtain a unique set of phylogenetic relationships: ((Anodontinae + Unioninae) + Gonideninae) + Ambleminae). Our phylogeny differs from other studies based on mitochondrial genome sequences in that the Ambleminae is the basal subfamily as opposed to the sister Gonideninae.

Bolotov et al. (2017a) proposed that the most recent common ancestor (MRCA) of the Anodontinae, Unioninae, Ambleminae, and Gonideninae likely originated in East Asia (Probability 65.8%). Under this scenario the MRCA of Anodontinae + Unioninae arose in East Asia during the Cretaceous period, whereas the MRCA of Ambleminae + Gonideninae was continuously distributed in East Asia and North America. The ancestor of the Ambleminae was most likely to originate in North America. The diversification of each subfamily occurred in the late Cretaceous (Bolotov et al. 2017a). The results of phylogenetic analyses in the current study have different evolutionary implications. Our results indicate that the Ambleminae is basal to the other three subfamilies, and its origin is therefore earlier than the other three subfamilies. Globally, eight subfamilies (Anodontinae, Unioninae, Pseudodontinae, Gonideinae, Ambleminae, Rectidentinae, Parreysiinae, and Modellnaiinae) are recognized in the Unionidae (Bolotov et al. 2017a; Lopes-Lima et al. 2017a; Whelan et al. 2011). The lack of mitochondrial genomes for Rectidentinae, Parreysiinae, Modellnaiinae, and Pseudodontinae, precluded their incorporation into this study. However, we believe that the fully resolved phylogenetic tree, with high branch support in the present study, serves as a framework for further studies on the Unionidae. Future phylogenetic analyses based on complete mitochondrial genome sequences of representatives of all the subfamilies in the Unionidae will ultimately produce well-supported phylogenetic hypotheses for the Unionidae.
Phylogeny and taxonomy of Chinese taxa

The classification of the Chinese unionid genera has been in a state of flux, different studies having placed the same genus in different subfamilies. For example, based on the presence or absence of the glochidial hooks and the type of marsupium, Wu et al. (1999a) divided the genus *Lamproula sensu lato* Simpson, 1900 into *Lamprotula sensu stricto* and *Aculamprotula* Wu, Liang, Wang & Ouyang, 1999. This distinction was later confirmed by molecular data (Zhou et al. 2007; Pfeiffer and Graf 2013; Wu et al. 2018b), but the classification of *Lamprotula* has also been disputed. Our results do not support the taxonomy of Huang et al. (2002), Zhou et al. (2007) and Ouyang et al. (2011; 2015) that placed *Lamprotula* sensu stricto in the Ambleminae. Our phylogenetic analyses instead confirm the results of Pfeiffer and Graf (2013), Lopes-Lima et al.
(2017a), Bolotov et al. (2017a; b) and Wu et al. (2018b) that Lamprotula is a member of the Gonideninae. The classification of the genus Sinohyriopsis has also been unstable. The shape of the glochidia of Sinohyriopsis cumingii (Lea, 1852) is semi-elliptical and unhooked, and resembles the typical morphology of glochidia in the Gonideninae (Wu et al. 2018a). But the marsupium of S. cumingii is restricted to the outer two demibranches of the gills (ectobranchous), whereas in other species in the Gonideninae (Lamprotula leaii (Griffith & Pidgeon, 1833) Solenaia carinatus (Heude, 1877) and Solenaia oleivora (Heude, 1877)) the marsupium includes all four demibranchs (tetrigenous) (Wu et al. 2018a). Therefore, based on anatomical features alone, the classification of the Sinohyriopsis in the Gonideninae has always been in doubt. Prior phylogenetic analyses based on one or two mitochondrial molecular markers (Huang et al. 2002; Zhou et al. 2007; Ouyang et al. 2011; 2015) placed Sinohyriopsis in the Ambleminae, However, our results indicate that Sinohyriopsis should be placed in the Gonideninae, confirming the conclusions of Lopes-Lima et al. (2017a) and Bolotov et al. (2017a, b). The placement of Aculamprotula has not been as controversial and our results place it in the Unioninae.

The genus Lepidodesma Simpson, 1896 is endemic to China and Lepidodesma languilati (Heude, 1874) is the type species. The juvenile of this species is thin and fragile, and the adult shell is robust. In addition, adults lack pseudocardinal teeth, but possess lateral teeth and the glochidia are triangular and have hooks. The breeding period is from February to August, and the type of marsupium is ectobranchous (Wu et al. 2018a). These characteristics are similar to species in the subfamily Unioninae and Anodontinae. Other characters, such as the size of the glochidia, which is large, and the tripartite water tubes (Wu et al. 2018a), indicate an affinity with the subfamily Anodontinae. The classification of Lepidodesma has alternated between these two subfamilies with some (Simpson 1900, Huang et al. 2002, Graf and Cummings 2007, Zhou et al. 2016) placing it in the Unioninae, and others (Haas 1969, Liu et al. 1979, Prozorova et al. 2005) in the Anodontinae. The results of our study indicate a novel result in which L. languilati is placed in neither of these subfamilies, but is sister to a clade that includes both the Unioninae and Anodontinae. The robust branch support values indicate that L. languilati is not a member of either subfamily, but is instead a member of another, as yet unrecognized clade or perhaps is the remnant of a once larger more diverse group. Owing to the lack of available mitochondrial genomes for representatives of the Rectidentinae, Parreysiinae, and Pseudodontinae, our study did not include these subfamilies, and we recognize that their inclusion could produce a different set of relationships.

Due to the emphasis on the morphological characteristics of the shell, malacologists have consistently supported including both Arconaia and Lanceolaria in the Unioninae (Haas 1969; Liu 1979; Graf and Cummings 2007). The shells of Arconaia and Lanceolaria are thick and have distinct hinge teeth, and the morphology of the glochidia (triangular; hooked) and type of marsupium (ectobranchous) are similar to species of the subfamily Unioninae and Anodontinae (Wu et al. 2018a). The phylogenetic relationships inferred by different molecular markers, seem to confirm the
Analysis of mitochondrial genomes resolves the phylogenetic position of these genera in the Unioninae (Huang et al. 2002; Zhou et al. 2007; Ouyang et al. 2015). However, the above-mentioned phylogenetic analyses included a limited number of taxa, and several key nodes in the phylogeny had low branch support. The results of the current study support the placement of *Arconaia* and *Lanceolaria* in the Anodontinae, confirming the results of Lopes-Lima et al. (2017a) and Wu et al. (2018b).

The genus *Acuticosta* was erected by Simpson and *Acuticosta chinensis* (Lea, 1868) was designated as the type species. Based on the marsupium, anatomy, larvae type and umbo sculpture, Simpson (1900) placed this genus in the Hyriinae. Subsequently, Chinese malacologists (Liu et al. 1979) re-classified the genus as a member of the Unioninae based on the presence or absence of hinge teeth. Prozorova et al. (2005) in a review of the bivalves in the Yangtze River drainage, placed the genus in Acuticostinae, although Graf and Cummings (2007) still maintained *Acuticosta* in the Unioninae. Molecular genetic analyses of a variety of markers by Huang et al. (2002), Zhou et al. (2007), and Ouyang et al. (2011; 2015) all indicated that *A. chinensis* was a member of the Unioninae. However, the limited taxon sampling and low branch support values in molecular phylogenetic analyses have allowed questions concerning the true affinities of *Acuticosta* to persist (Pfeiffer and Graf 2013; Huang et al. 2013; Lopes-Lima et al. 2017). Recently, Wu et al. (2018b) indicated that *A. chinensis* is a member of the Anodontinae based on mitochondrial DNA sequences of two genes. The current analysis of mitochondrial genomes provides further support for the placement of *Acuticosta* in the Anodontinae and indicates affinity of *Acuticosta* to the genera *Arconaia* and *Lanceolaria*.

Endangered status and conservation implications

China is a vast territory with a huge number of lakes and rivers. As a result, it is one of the most species-rich regions in the world (Zieritiz et al. 2017; Cai et al. 2018). However, in recent decades, freshwater mussels in China have declined drastically, and species diversity has been seriously threatened. At present, 40 species of Chinese unionids are included in the 2018 IUCN Red List, although 32 of these are categorized as data deficient or least concern. In addition, nearly half of the species included had not been evaluated. At present, advancing urbanization in the Yangtze River Basin, increasingly threatens the habitat of freshwater mussels, and conservation and management efforts targeting freshwater taxa are urgently needed.

Understanding of the phylogenetic diversity of freshwater mussels has important significance for determining the priority conservation strategies of species (Lopes-Lima et al. 2017b, 2018). This study provides support for the classification of a number of Chinese species, and lays the foundation for the future development of a more comprehensive phylogenetic based classification for freshwater unionids in China. Accurate taxonomic placement of rare and understudied species is central to many aspects of conservation as important biological characteristics (e.g., habitat preferences, reproductive traits) can be inferred from closely related taxa. Future research on Chinese...
unionids should focus on species delimitation and classification. In addition, more research is needed on understanding the basic ecology of Chinese mussels including species distributions, habitat preferences, and host fish identification.

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**Supplementary material 1**

**Supplementary Tables S1, S2**

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Data type: molecular data

Explanation note: Table S1: Partitioning strategies from PartitionFinder for mt genome dataset; Table S2: Partitioning strategies from ModelFinder for mt genome dataset.

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