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Research Article

Unraveling the Phylogenetic Relationships and Taxonomic Status of a Puzzling Freshwater Mussel Genus *Inversidens* (Bivalvia, Unionidae) through Multilocus Phylogeny and Mitochondrial Phylogenomics

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Accurate phylogenetic reconstructions are crucial for comprehending the evolutionary histories, reproductive traits, and ecological habits of organisms. The subfamily Gonideinae of freshwater mussels is currently thought to include eight tribes. However, due to inadequate taxon sampling and molecular data, the assignment of the freshwater mussel genus *Inversidens* at the tribe level has been unstable. Additionally, the lack of phylogenetic data has hindered efforts to understand the basic biology and implementation of conservation efforts of *Inversidens rentianensis*, an endemic species to China. Here, we first present the complete mitochondrial genome of *Inversidens rentianensis* and offer a detailed description of its anatomical morphology. Based on DNA sequence data from five genes (COI, ND1, 16S rRNA, 18S rRNA, and 28S rRNA) and complete mitochondrial genomes, we investigated the phylogenetic position of *Inversidens* using various analytical methods. Both the concatenated five-gene and mitogenome datasets strongly supported that *Inversidens* classified to the tribe Gonideini in Gonideinae and formed a basal clade within the tribe Gonideini. Molecular dating analysis suggested that *Inversidens* originated during the mid-Cretaceous era (102.73 Mya, 95% highest posterior density (HPD) = 72.22-137.03 Mya) and underwent diversification in the Late Paleogene era (37.92 Mya, 95% HPD = 20.39-60.59 Mya). Moreover, based on the Quantitative Assessment of Species for Conservation (QASCP), *Inversidens rentianensis* is ranked as second priority, providing valuable insights for its management and conservation efforts. Taken together, this study provides a comprehensive understanding of the systematic position and evolutionary history of *Inversidens* within the currently accepted subfamily Gonideinae classification framework. These findings establish a solid foundation for future investigations on the ecology, reproductive behavior patterns, and conservation biology of this taxonomic group.

1. Introduction

Freshwater mussels of the family Unionidae occur in freshwater habitats worldwide except Antarctica and South America, with the most diverse regions being East Asia and North America [1–3]. These bivalves are ecologically significant benthic organisms that play pivotal roles in ecosystem functioning and services [4–7]. Additionally, they also possess substantial economic value; for instance, soft body serves as a natural food source for poultry and livestock, while shell holds high ornamental and collection value [8]. Regrettably, anthropogenic activities and environmental changes have posed severe threats to the habitats of freshwater mussels, resulting in a significant decline in their biodiversity over recent decades [9–11]. In many countries, especially in North America and Europe, the number of conservation actions for these mussels has increased substantially over the latest years [12, 13]. China is a biodiversity hotspot for freshwater mussels, but limited understanding of their biology and lack of precise systematic classification hinder the implementation of conservation measures for certain taxa [14].

The freshwater mussel genus *Inversidens* Haas, 1911 was initially described as a subgenus of *Nodularia* Conrad, 1853, containing two species endemic to Japan, i.e., *Unio brandtii* Kobelt, 1879 and *Nodularia parcedentata* Haas, 1911 [15]. Later, Thiele [16] elevated it to the generic level and designated *U. brandtii* as the type species, classifying it under the subfamily Unioninae in Unionidae. Haas [17] also placed *Inversidens* in Unioninae based on shell morphology and described six species within the genus. The subsequent efforts of malacologists focused on refining the classification on the genus level and assessing the species validity [18–21].

Currently, there are three recognized species within this genus, i.e., *Inversidens brandtii* (Kobelt, 1879), *Inversidens pantoensis* (Neumayr, 1899), and *Inversidens rentianensis* Wu & Wu, 2021 [22, 23]. *I. brandtii* is restricted to Japan [21], while *I. pantoensis* and *I. rentianensis* are endemic to China [24, 25].

The classification of the upper genus for *Inversidens* based on shell morphology has been subject to uncertainty [26, 27]. With the recent advancement of molecular systematics, researchers have attempted to employ molecular fragments (COI, 16S rRNA, and 28S rRNA) from type species *Inversidens brandtii* for resolving phylogenetic positions [28–31]. At present, the taxonomic status of this genus under the subfamily Gonideinae in Unionidae has been recognized [22, 23]. However, the classification position at the tribe level has not been solved (Figure 1). Based on three-gene data, Lopes-Lima et al. [30] resolved the diversity, biogeography, and evolutionary relationships of the Far East Asian Unionidae and assigned *Inversidens brandtii* to the tribe Gonideini based on a phylogenetic context of five tribes within Gonideinae. Later, Dai et al. [32] expanded the taxonomic sampling and found that the results do not support the classification of *Inversidens* to Gonideini and considered Gonideini to be polyphyletic. Recently, a phylogenetic framework for eight tribes of Gonideinae proposed by Zieritz et al. [33] by incorporating new taxa and molecular markers (five genes) has been accepted. Therefore, the systematic status of the genus *Inversidens* in the context of the updated phylogeny of Gonideinae necessitates reassessment.

Additionally, the species *Inversidens rentianensis* Wu & Wu, 2021, which was discovered and described by our research team [25], only had limited molecular data (i.e., mitochondrial COI) and shell morphological description. This species has a limited distribution range and specific ecological requirements, exhibiting a preference for inhabiting smaller rivers and streams. The vulnerability of this distinctive habitat to both anthropogenic and natural influences makes the species vulnerable to threats [34, 35]. The progress of modern biodiversity and conservation research heavily relies on the phylogenetic information [36]. Yet the lack of phylogenetic data for many imperiled species hinders our understanding of their basic biology and the implementation of conservation efforts [37–39]. Thus, phylogenetic information on *I. rentianensis* must be gathered to ensure scientific assessment of the endangered status and development of conservation and management strategies.

To determine the phylogenetic position of *Inversidens*, we constructed a combined gene dataset (including COI-mtDNA, ND1-mtDNA, mt 16S-rRNA, 18S-rRNA, and 28S-rRNA) based on Zieritz et al.'s [33] comprehensive sampling of eight tribes within the Gonideinae and utilized various software programs with partitioning strategies to construct phylogenies. Moreover, mitochondrial genomes are known for providing significantly more informative characters for phylogenetics and have proven highly effective in resolving both shallow and deep relationships in freshwater mussels [40–43]. In order to further validate the systematic position of *Inversidens* obtained from the five-gene dataset, we also conducted phylogenetic analyses using mitochondrial genomes. Therefore, a new mitogenome from *Inversidens rentianensis* was acquired.

Herein, the purpose of this study is to (1) describe the anatomical characteristics of *Inversidens rentianensis* and decipher its female complete mitochondrial genome, (2) elucidate the phylogenetic position of *Inversidens* based on both multilocus dataset and mitogenomic dataset, and (3) infer the evolutionary history of the genus *Inversidens* by time-calibrated phylogeny of Unionoidea.

2. Materials and Methods

2.1. Sample Collection and Anatomical Feature Observation. From 2021 to 2022, we conducted multiple field expeditions to the type locality of Mianshui River in Ganzhou City, Jiangxi Province, China, for collecting *Inversidens rentianensis*. However, it was not until October 2022 that we fortuitously obtained two live specimens (Figure 2). Both voucher specimens were deposited at the Museum of Zoology, Shanxi Normal University (SXNU), China (SXNU22102201-SXNU22102202). Anatomical features of the soft body were observed by visual examination and stereoscopic microscopy.

2.2. DNA Extraction, PCR Sequencing, and Mitogenome Assembly. Based on five genes, i.e., the mitochondrial cytochrome c oxidase subunit I (COI), NADH dehydrogenase subunit 1 (ND1), and 16S rRNA, as well as the nuclear 18S rRNA and 28S rRNA gene fragments, we compiled three multilocus datasets: a mtDNA gene dataset (COI+ND1+16S), a nuDNA gene dataset (18S+28S), and a concatenated five-gene dataset (COI+ND1+16S+18S+28S). We then employed various phylogenetic methods to generate a set of phylogenetic hypotheses.

Genomic DNA from samples was extracted from dissected foot tissue using the TIANamp Marine Animals DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Five gene regions (COI, ND1, 16S rRNA, 18S rRNA, and 28S rRNA) were amplified and sequenced using the same primers from Zieritz et al. [33]. PCR conditions were performed according to the TaKaRa Ex manufacturer's protocol, including an initial denaturation step at 98°C for 10 s, followed by 35 cycles of amplification consisting of a denaturation step at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for seven minutes. Amplified PCR products were purified and sequenced by Sangon Biotech (Shanghai).

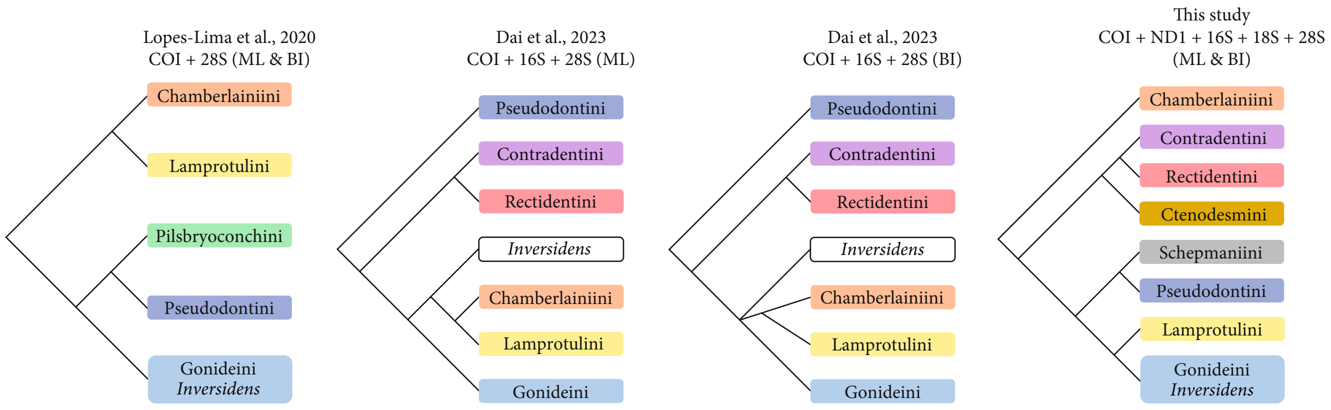


FIGURE 1: Summary of recent multilocus phylogenetic hypotheses at tribe level of Gonideinae and the position of *Inversidens*.

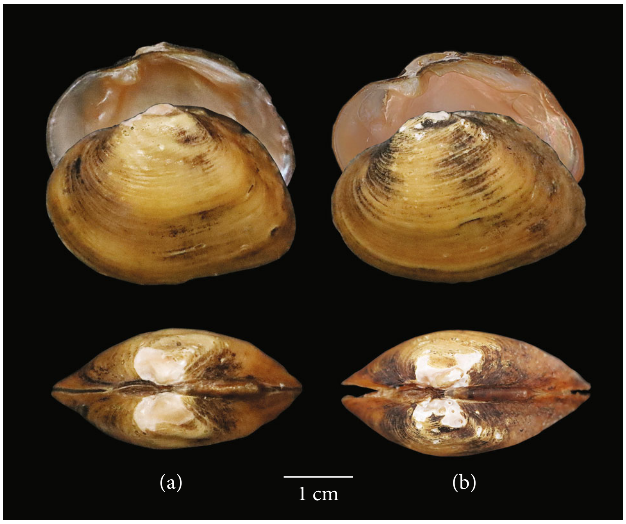


FIGURE 2: Shell images of *Inversidens rentianensis*.

Based on the dataset provided by Zieritz et al. [33], we expanded the taxa, resulting in a five-gene dataset comprising 35 Gonideinae taxa, with *Margaritifera dahurica* and *Margaritifera margaritifera* included as outgroup. The sequences of certain gene fragments from *Postolata guangxiensis*, *Ptychorhynchus pfisteri*, *Koreosolenaia sitgyensis*, and *Parvasolenaia rivularis* are newly obtained in this study (Table 1).

To further confirm the results of the multilocus datasets and clarify the phylogenetic relationship between *Inversidens* and other close relative, we sequenced the mitochondrial genome of *Inversidens rentianensis*. Qualified samples were sent to Novogene Co., Ltd. (China) for library construction and sequencing. The complete mitogenome was concatenated by Geneious (ver. 11.0, see https://www.geneious.com) [44]. Protein-coding genes were confirmed by the NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) and BLAST search (http://blast.ncbi.nlm. http://nih.gov/) analysis. Subsequently, the mitogenome genomic sequence was submitted to GenBank under accession number OR823224.

The mitogenome sequence was annotated using MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py) [45]. Sequence length, base composition, and AT content were calculated using the built-in EditSeq program in DNAstar. Strand asymmetry was determined by calculating the GC skew = $(G\% - C\%)/(G\% + C\%)$ and AT skew = $(A\% - T\%)/(A\% + T\%)$. The mitochondrial genome was visualized using GenomeVx (GenomeVx (ucd.ie)) [46] with manual modifications.

We combined the new genome with 92 Unionida mitogenomes from GenBank to construct the complete mitogenome dataset (Table 2). The nucleotide sequences of 12 protein-coding genes (12 PCGs, excluding ATP8 due to high sequence variation) and two rRNA genes were used for subsequent phylogenetic analyses.

2.3. Phylogenetic Analyses. The molecular phylogenetic reconstruction is consistent with the methods used in our previous studies [47]. In the datasets constructed above, protein-coding genes (PCGs) were aligned by built-in MUSCLE [48] with default settings implemented in PhyloSuite [49]. rRNA genes were aligned using MAFFT v7.2 [50] with the L-INS-i algorithm. Ambiguous alignment areas were trimmed by Gblocks (ver. 0.91b; see http://molevol.cmima.csic.es/castresana/Gblocks.html) [51], the parameter ribosomal gene block with a minimum length was set to 2 base pairs (bp), and allowed gap position was selected with half; the minimum length of protein-coding gene block was set to 3 bp, and allowed gap position was also selected with half. For the five-gene dataset, the COI, ND1, 16S, 18S, and 28S sequences were aligned and trimmed to lengths of 657 bp, 882 bp, 469 bp, 642 bp, and 743 bp, respectively. The sequences of various multigene datasets were concatenated using PhyloSuite v1.2.3. The mitogenomic data consisted of concatenated twelve protein-coding genes (12 PCGs) and two ribosomal RNA genes with a total length of 12,751 bp.

All built datasets were performed with partition schemes based on genes and codons. PartitionFinder (ver. 2.1.1; see http://www.robertlanfear.com/partitionfinder/) [52] was used to select Bayesian inference (BI) and BEAST analysis models for partitioning schemes. ModelFinder (ver. 1.4.2; see http://www.iqtree.org/ModelFinder/) [53] was used to select the maximum likelihood (ML) analysis models in IQ-TREE. The selection for best-fit models was based on

Table 1: List of sequences used in multilocus phylogenetic analyses.

Taxa	COI	ND1	16S rRNA	18S rRNA	28S rRNA
Unionidae Rafinesque, 1820					
Gonideinae Ortmann, 1916					
Gonideini Ortmann, 1916					
Gonidea angulata (Lea, 1838)	DQ272371	MZ695078	KF011258	MZ684047	AF400691
Koreosolenaia sitgyensis Lee et al., 2020	GQ451872	**OR842385**	GQ451859	**OR826145**	MT020817
Microcondylaea bonellii (Férussac, 1827)	KX822652	MK994772	KP218021	MZ684056	KX822609
Parvasolenaia rivularis (Heude, 1877)	KX966393	KJ434587	KX966393	**OR826147**	MG595632
Postolata guangxiensis Dai et al., 2023	OP009379	OP009366	OP020466	**OR826148**	OP020470
Ptychorhynchus pfisteri (Heude, 1874)	KY067440	MG933788	KY067440	**OR826146**	MG595562
Sinosolenaia carinata (Heude, 1877)	KX822669	MG933804	MK683025	MK683000	KX822626
Inversidens brandtii (Kobelt, 1879)	MT020598	n/a	LC455726	n/a	LC519069
Inversidens brandtii (Kobelt, 1879)	MT020597	n/a	LC455725	n/a	LC519068
Inversidens rentianensis Wu & Wu, 2021	**OR826137**	**OR842383**	**OR826140**	**OR826143**	**OR826149**
Inversidens rentianensis Wu & Wu, 2021	**OR826138**	**OR842384**	**OR826141**	**OR826144**	**OR826150**
Pseudodontini Frierson, 1927					
Bineurus loeiensis Konopleva et al., 2021	KX865879	MZ695073	KX865650	MZ684042	KX865750
Bineurus anodontinum (Rochebrune, 1882)	MW603662	MZ695074	MZ684076	MZ684043	MZ684018
Indopseudodon bogani (Bolotov et al., 2017)	MF352218	MZ695085	MF352292	MZ684064	MF352350
Indopseudodon kayinensis (Bolotov et al., 2020)	MZ678754	MZ695086	MZ684081	MZ684065	MZ684033
Pilsbryoconcha exilis (Lea, 1838)	KX051291	MZ695084	KX865646	MZ684061	KX822613
Pseudodon mekongi (Bolotov et al., 2020)	KX865861	MZ695083	KX865632	MZ684057	KX865733
Pseudodon vondembuschianus (Lea, 1840)	KP795029	MK994774	KP795052	MZ684058	MZ684028
Thaiconcha callifera (Martens, 1860)	KX865862	MZ695090	KX865633	MZ684070	KX865734
Lamprotulini Modell, 1942					
Lamprotula caveata (Heude, 1877)	KX822646	KX060991	NC_030336	MK682986	KX822603
Lamprotula leaii (Gray, 1833)	NC_023346	JQ691662	NC_023346	MF072524	MG595524
Potomida littoralis (Cuvier, 1798)	JN243905	KF011251	NC_030073	KU763289	JN243883
Chamberlainiini Bogan et al., 2017					
Chamberlainia somsakpanhai Kongim et al., 2023	KX822635	MK994770	MK994770	MZ684044	KX822592
Rectidentini Modell, 1942					
Ensidens sagittarius (Lea, 1856)	KX865950	MG025721	KX865696	MZ684046	KX865821
Hyriopsis bialata Simpson, 1900	KX051274	MW242816	MT993644	MZ684048	MT993697
Hyriopsis desowitzi Brandt, 1974	KX822644	MG025706	KU318351	MZ684049	KX822601
Rectidens sumatrensis (Dunker, 1852)	KX051314	MW242818	MW242818	MZ684066	KX822620
Contradentini Modell, 1942					
Lens contradens (Lea, 1838)	MG581991	MW242812	MT993693	MZ684053	MT993745
Lens eximius (Lea, 1856)	KX865941	MZ695075	KX865689	MZ684054	KX865812
Physunio superbus (Lea, 1843)	MG582020	MW242814	MT993689	MZ684059	MT993741
Trapezoideus foliaceus (Gould, 1843)	MH345985	MZ695091	MH346025	MZ684071	MH346005
Yaukthwa inlenensis Konopleva et al., 2019	KX865927	MZ695094	KX865681	MZ684073	KX865798
Yaukthwa paiensis Konopleva et al., 2019	MH345972	MZ695093	MH346012	MZ684074	MH345992
Yaukthwa elongatula Bolotov et al., 2019	MK372408	MZ695092	MK372456	MZ684072	MK372486
Ctenodesmini Pfeiffer et al., 2021					
Khairuloconcha lunbawangorum Zieritz et al., 2021	MN900790	MZ695080	MZ684078	MZ684050	MN902294
Khairuloconcha sahanae Zieritz et al., 2021	MZ678752	MZ695081	MZ684079	MZ684051	MZ684024
Schepmaniini Lopes-Lima et al., 2021					
Schepmania sp. 5973 Zieritz et al., 2021	MZ678755	MZ695087	MZ684082	MZ684067	MZ684035
Schepmania sp. 5974 Zieritz et al., 2021	MZ678756	MZ695088	MZ684083	MZ684068	MZ684036

Table 1: Continued.

Taxa	COI	ND1	16S rRNA	18S rRNA	28S rRNA
Margaritiferidae Henderson, 1929					
Margaritifera dahurica (Middendorff, 1850)	KJ161516	KF514426	KJ943526	AY579091	KT343747
Margaritifera margaritifera (Linnaeus, 1758)	KX550089	EF446105	KX550091	AY579103	KX550093

Bold represents the sequence from this study.

the corrected Akaike information criterion (AICc). Substitution models assigned to each partition by PartitionFinder and ModelFinder are listed in Supplementary Table S1–S4.

ML analysis was performed in IQ-TREE [54] based on generated models in ModelFinder, using 1000 ultrafast bootstraps. BI analysis was used in MrBayes v2.01 [55] with generated models in PartitionFinder. Four independent Markov chain Monte Carlo (MCMC) were run simultaneously for ten million generations, and sampling was conducted every 1000 generations, with a burn-in of 25%. We terminated the process when the average standard deviation of splitting frequency falls below 0.01. The visualization and editing of phylogenetic trees were performed by using iTOL online software (http://itol.embl.de/itol.cgi) [56].

2.4. Divergence Time Estimation. The time-calibrated phylogenetic tree was generated using BEAST v1.8.4 [57] based on the concatenated five-gene dataset. We employed the uncorrelated lognormal clock model with a priori model of birth-death speciation process for tree construction. Three reliable fossil calibrations were selected according to our previous study [58]: (1) the minimum stem age of Margaritiferidae was set to 230 Mya (exponential prior, lambda = 30), (2) the minimum age of *Margaritifera dahurica* and *Margaritifera margaritifera* for the most recent common ancestor (MRCA) was set to 34 Mya (exponential prior, lambda = 9.3), and (3) the minimum age of the most recent common ancestor (MRCA) of *Lamprotula leaii* and *Lamprotula caveata* was also set to 34 Mya (exponential prior, lambda = 9.3).

Four independent MCMC ran with 300 million generations each with sampling every 50,000 generations. Convergence and effective sample size (ESS) of parameters were checked using Tracer 1.7.1 [59]. LogCombiner v1.8.4 [57] was used to combine trees from these four runs with discarding the first 25% generations. A maximum clade credibility tree was summarized in TreeAnnotator v1.8.4 [57], with a burn-in of 25%.

3. Results

3.1. Soft-Body Anatomical Features. The fresh soft-body morphology of *Inversidens rentianensis* is depicted in Figure 3. The color of gills is milky white, and the inner gill is larger than the outer one (Figure 3(a)). The foot is butter yellow and darker than the gills (Figure 3(a)). The papillae of incurrent aperture are weakly developed, arranged in two rows, and almost stick together (Figure 3(b)); no papillae are observed in the excurrent aperture (Figure 3(c)). The pigmentation on both the incurrent and excurrent aperture is remarkable. The anal opening, located on the dorsal margin of the posterior adductor muscle, is connected to the excurrent aperture (Figure 3(d)). Labial palps are milky white and subtriangular (Figure 3(e)).

3.2. Mitochondrial Genome Structure. The mitogenome of *Inversidens rentianensis* is 15,987 bp in length (Figure 4). A +T bias has been observed in many unionid mitogenomes [60], and *I. rentianensis* genome is no exception: AT content of 60.91% (38.48% A, 22.42% T, 11.35% G, and 27.75% C). The AT skew and GC skew are 0.26% and -0.42%, respectively, indicating a bias towards A over T and C over G.

The genome contains all 37 genes commonly found in animal mtDNAs [61]: 13 protein-coding genes (PCGs), 22 tRNAs (including two *trnL* and two *trnS*), and 2 rRNAs (Figure 4). The gene arrangement pattern is consistent with species in the subfamily Gonideinae, except for *Chamberlainia somsakpanhai* [62, 63]. The gene distribution pattern in unionid mussels follows the typical arrangement, with 11 genes (*trnH*, *cox2*, *cox1*, *cox3*, *atp6*, *trnD*, *atp8*, *nad4L*, *nad4*, *nad5*, and *nad3*) located on the heavy strand and the remaining 26 genes encoded on the light strand (Figure 4). Additionally, a noncoding region of 1468 bp is observed which accounts for approximately 9.2% of the entire mitogenome.

The 13 PCGs have a total length of 10,995 bp, which accounts for 68.77% of the entire mitochondrial genome. The base composition is A, 21.44%; T, 38.73%; G, 24.09%; and C, 15.74%, with an AT content of 60.17%. The mitochondrial genome has four start codons: ATA, ATT, ATG, and TTG. Among 13 PCGs, *cox1* starts with TTG while the other genes use standard codons ATN (ATA, ATG, and ATT). The mitogenome contains 22 tRNA genes, including two *trnS* and two *trnL*. Most of tRNA genes are located on the light strand, except for the *trnH* and *trnD* (Figure 4). The length of tRNA genes varies from 52 bp (*trnA*) to 68 bp (*trnS1* and *trnN*). Both *rrnS* and *rrnL* are encoded on the light strand and are separated by *trnK*, *trnT*, and *trnY* (Figure 4), as in all the unionoid mt genomes studied so far [60, 63].

3.3. Multilocus Phylogenetic Analyses. Based on the mtDNA gene dataset, the phylogenetic trees obtained from Bayesian inference (BI) and maximum likelihood (ML) analyses show that all eight tribes in the subfamily Gonideinae are monophyletic groups, although most are weakly supported (BS < 70%, PP < 0.9). The sisterly group between *Inversidens brandtii* and *Inversidens rentianensis* is closely related to the tribe Lamprotulini, but with low support values (BS = 60% and PP = 0.62, Figure 5(a)). The BI and ML trees based on the nuDNA dataset do not form monophyly for all eight

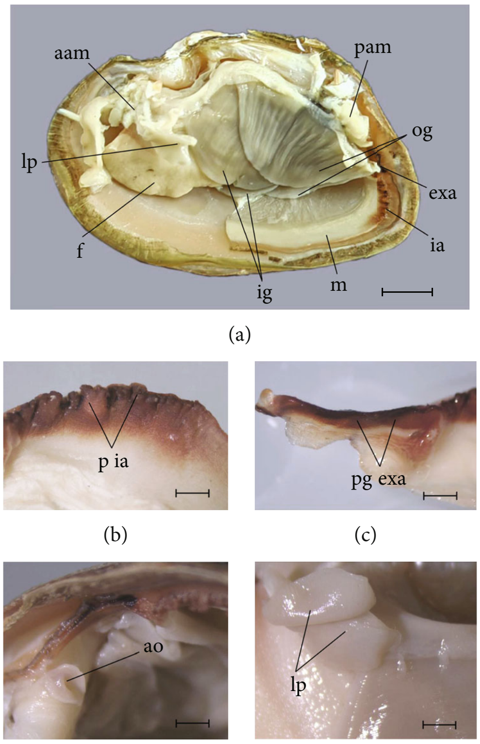
Table 2: List of sequences used in mitochondrial phylogenomic analyses.

Family	Subfamily	Tribe	Taxa	Accession number	
Unionidae	Unioninae	Nodulariini	*Cuneopsis demangei*	NC_066664	MZ571513
			Cuneopsis heudei	NC_042471	MH919389
			Cuneopsis celtiformis	MZ571520	
			Cuneopsis rufescens	NC_066663	MZ571512
			Schistodesmus sp.	NC_023806	KJ018924
			Schistodesmus spinosus	NC_066662	MZ571511
			Schistodesmus lampreyanus	NC_042470	MH919388
			Tchangsinaia piscicula	NC_026306	KP273584
			Nodularia breviconcha	MT955592	
			Nodularia douglasiae	NC_026111	KM657954
			Pseudocuneopsis capitata	NC_042469	MH919387
			Pseudocuneopsis sichuanensis	NC_066661	MZ571510
		Unionini	*Unio delphinus*	NC_033854	KT326917
			Unio pictorum	NC_015310	HM014130
			Unio crassus	NC_033976	KY290446
			Unio tumidus	KY021076	
			Unio elongatulus	MN594537	
			Unio mancus	MN594538	
		Aculamprotulini	*Aculamprotula polysticta*	MK728823	
			Aculamprotula scripta	NC_045529	KF991456
			Aculamprotula coreana	NC_026035	JX050180
			Aculamprotula tientsinensis	NC_029210	
			Aculamprotula tortuosa	NC_021404	KC109779
		Anodontini	*Alasmidonta varicosa*	NC_038155	MG938673
			Lasmigona complanata	OM736811	
			Platynaias compressa	NC_015481	HM856638
			Prolasmidonta heterodon	NC_037431	MG905826
			Utterbackia imbecillis	NC_015479	HM856637
			Utterbackia peninsularis	HM856636	
			Pyganodon grandis	NC_013661	FJ809754
			Anodonta anatina	NC_022803	KF030964
			Anodonta exulcerata	MN594533	
			Anodonta nuttalliana	MN594534	
			Anodonta cygnea	NC_036488	MG385135
			Pseudanodonta complanata	MN594535	
		Cristariini	*Anemina arcaeformis*	NC_026674	KF667530
			Anemina euscaphys	NC_026792	KP187851
			Sinanodonta tumens	LC592406	
			Sinanodonta lucida	NC_026673	KF667529
			Sinanodonta woodiana	HQ283346	
			Beringiana fukuharai	LC592410	
			Cristaria plicata	KM233451	
		Lanceolariini	*Acuticosta chinensis*	NC_042472	MH919390
			Lanceolaria gladiola	KY067441	
			Lanceolaria grayii	NC_026686	KJ495725
			Lanceolaria lanceolata	NC_023955	KJ144818
		Lepidodesmini	*Lepidodesma aligera*	OP859025	
			Lepidodesma languilati	OQ910483	

Table 2: Continued.

Family	Subfamily	Tribe	Taxa	Accession number	
	Parreysiinae	Lamellidentini	*Lamellidens marginalis*	NC_062877	MT230549
	Gonideinae	Gonideini	*Parvasolenaia rivularis*	NC_039839	KX966393
			Ptychorhynchus pfisteri	KY067440	
			Sinosolenaia carinata	NC_023250	KC848654
			Sinosolenaia oleivora	NC_022701	KF296320
			Sinosolenaia iridinea	MT477834	
			Microcondylaea bonellii	NC_044111	MK994772
			Inversidens rentianensis *	OR823224	
		Lamprotulini	*Lamprotula caveata*	NC_030336	KX060991
			Lamprotula leaii	NC_023346	JQ691662
			Lamprotula cornuumlunae	MK728822	
			Potomida littoralis	NC_030073	KT247374
			Pronodularia japanensis	AB055625	
		Pseudodontini	*Pseudodon vondembuschianus*	NC_044112	MK994774
			Pilsbryoconcha exilis	NC_044124	MK994776
		Contradentini	*Lens contradens*	MW242812	
			Physunio superbus	MW242814	
		Rectidentini	*Hyriopsis bialata*	MW242816	
			Rectidens sumatrensis	MW242818	
		Chamberlainiini	*Sinohyriopsis cumingii*	NC_011763	FJ529186
			Sinohyriopsis schlegelii	NC_015110	HQ641406
			Chamberlainia somsakpanhai	NC_044110	MK994770
	Ambleminae	Lampsilini	*Lampsilis powellii*	NC_037720	MF326971
			Lampsilis siliquoidea	NC_037721	MF326973
			Venustaconcha ellipsiformis	FJ809753	
			Lampsilis cardium	BK010478	
			Lampsilis cariosa	OM736866	
			Lampsilis ornata	NC_005335	AY365193
			Leaunio lienosus	BK010479	
			Potamilus alatus	KU559011	
			Potamilus streckersoni	ON855351	
			Potamilus leptodon	NC_028522	KT723012
			Toxolasma parvum	NC_015483	HM856639
		Popenaiadini	*Popenaias popeii*	NC_050058	MT648776
		Amblemini	*Amblema plicata*	NC_050056	MT648774
		Pleurobemini	*Elliptio complanata*	BK010477	
			Pleurobema oviforme	NC_050057	MT648775
		Quadrulini	*Quadrula quadrula*	NC_013658	FJ809750
			Uniomerus tetralasmus	BK010480	
Margaritiferidae	Margaritiferinae		*Margaritifera dahurica*	NC_023942	KF514426
			Margaritifera margaritifera	KY996745	
Hyriidae	Hyriinae		*Echyridella menziesii*	NC_034845	KU873121
Iridinidae	Iridininae		*Mutela dubia*	NC_034844	KU873120
Mycetopodidae	Anodontitinae		*Anodontites trapesialis*	KU873119	
Trigoniidae			*Neotrigonia margaritacea*	NC_034843	KU873118

*The sequence from this study.



(a)

(b) (c)

(d) (e)

Figure 3: Anatomical features of *Inversidens rentianensis* with left valve remove. (a) Overall anatomical diagram of *Inversidens rentianensis*; (b–e) close-up of apertures, anal opening, and labial palps. Abbreviations: aam: anterior adductor muscle; pam: posterior adductor muscle; exa: excurrent aperture; ia: incurrent aperture; f: foot; ig: inner gill; og: outer gill; ao: anal opening; lp: labial palps; m: mantle; p ia: papillae in incurrent aperture; pg exa: pigmentation of excurrent aperture. Scale of (a) is 5 mm, and scale of (b–e) is 1 mm.

tribes, while taxa of *Inversidens* independently form distinct clades that do not belong to any tribe (Figures 5(b) and 5(c)).

The ML and BI trees based on the concatenated five-gene dataset yield congruent topologies (Figure 6). The eight recognized tribes of the subfamily Gonideinae form monophyletic groups, with the following phylogenetic relationships: ((((Gonideini+Lamprotulini)+(Pseudodontini+Schepmaniini))+((Contradentini+Rectidentini)+Ctenodesmini))+Chamberlainiini). However, these clades are not well resolved in both ML and BI trees, as indicated by low supports at certain nodes (Figure 6; BS < 70%, PP < 0.9).

Both BI and ML phylogenetic analyses consistently support a sister group relationship between *Inversidens brandtii* and *Inversidens rentianensis*, with strong support values (BS = 100%, PP = 1.0; Figure 6). Furthermore, these two species are placed in a basal clade within the tribe Gonideini, as strongly supported by maximum likelihood bootstrap (BS) values of 97% and Bayesian posterior probabilities (PP) of 1.0 (Figure 6).

3.4. Fossil Calibrations and Molecular Dating. The tree topology obtained using the AICc partitioning scheme in BEAST does not align with our BI and ML phylogeny hypothesis at the tribe level; however, it consistently exhibits a phylogenetic relationship among taxa of Gonideini (Figures 6 and 7).

The genus *Inversidens* originates in the mid-Cretaceous (102.73 Mya, 95% HPD = 72.22-137.03 Mya). The divergence time between *I. rentianensis* and *I. brandtii* is estimated to be 37.92 Mya (95% HPD = 20.39-60.59 Mya), which occurs during the Late Paleogene (Figure 7).

3.5. Mitochondrial Phylogenomic Analyses. ML and BI trees based on the mitogenome dataset yield nearly identical topologies and are statistically well supported by 100% maximum likelihood bootstrap (BS) support values and Bayesian posterior probabilities (PP) in most nodes. Some discrepancies are observed only in specific branches, i.e., *Anodonta nuttalliana*, *Anodonta anatina*, and *Pseudanodonta complanata* (Figure S1-S2).

The phylogenetic results support the recognized four monophyletic groups of Unionidae, i.e., (Ambleminae+(Gonideinae+(Unioninae+Parreysiinae))) (Figure 8). Focusing on the subfamily Gonideinae, it is divided into six robust monophyletic clades, corresponding to six tribes with the following relationships: (Chamberlainiini+((Rectidentini+Contradentini)+(Lamprotulini+(Gonideini+Pseudodontini)))) by high support values (BS ≥ 99%, PP = 1.0; Figure 8). In both trees, *Inversidens rentianensis* is positioned as a basal clade within the tribe Gonideini with strong support values (BS = 100% and PP = 1.0; Figure 8).

4. Discussion

4.1. Systematic Position of Inversidens from Multilocus and Mitogenomic Data. The classification of Gonideinae has undergone consistent revisions in recent years by the incorporation of additional taxa [33, 64–67]. Lopes-Lima et al. [30] classified *Inversidens* in Gonideini under the phylogenetic framework of the five tribes of Gonideinae based on the (COI+28S) dataset. However, Dai et al.'s [32] phylogenetic trees inferred from the three-gene (COI+16S+28S) dataset did not support this classification and suggested that "*Inversidens* was not within any tribe of Gonideinae." Recently, Zieritz et al. [33] established and recognized a taxonomic system for eight tribes within Gonideinae based on five-gene phylogeny (COI+ND1+16S+18S+28S). Under this updated phylogenetic framework, we attempt to utilize multiple molecular datasets to address the phylogenetic location of *Inversidens*. The nuDNA trees were unable to effectively resolve the tribal relationship (Figures 5(b) and 5(c)), suggesting that nuclear genes 18S and 28S lack reliability in analyzing relationships at the tribe and lower levels within unionids. The phylogenetic relationships of all eight tribes were consistent between the mtDNA tree and the combined five-gene tree (Figures 5(a) and 6); however, the mtDNA tree exhibited limited confidence in accurately locating *Inversidens*. Therefore, we strongly support the genus *Inversidens* as a member of the tribe Gonideini and positioned in the basal clade based on the five-gene phylogeny (Figure 6, BS/PP = 97%/1.0). Additionally, our mitochondrial genome

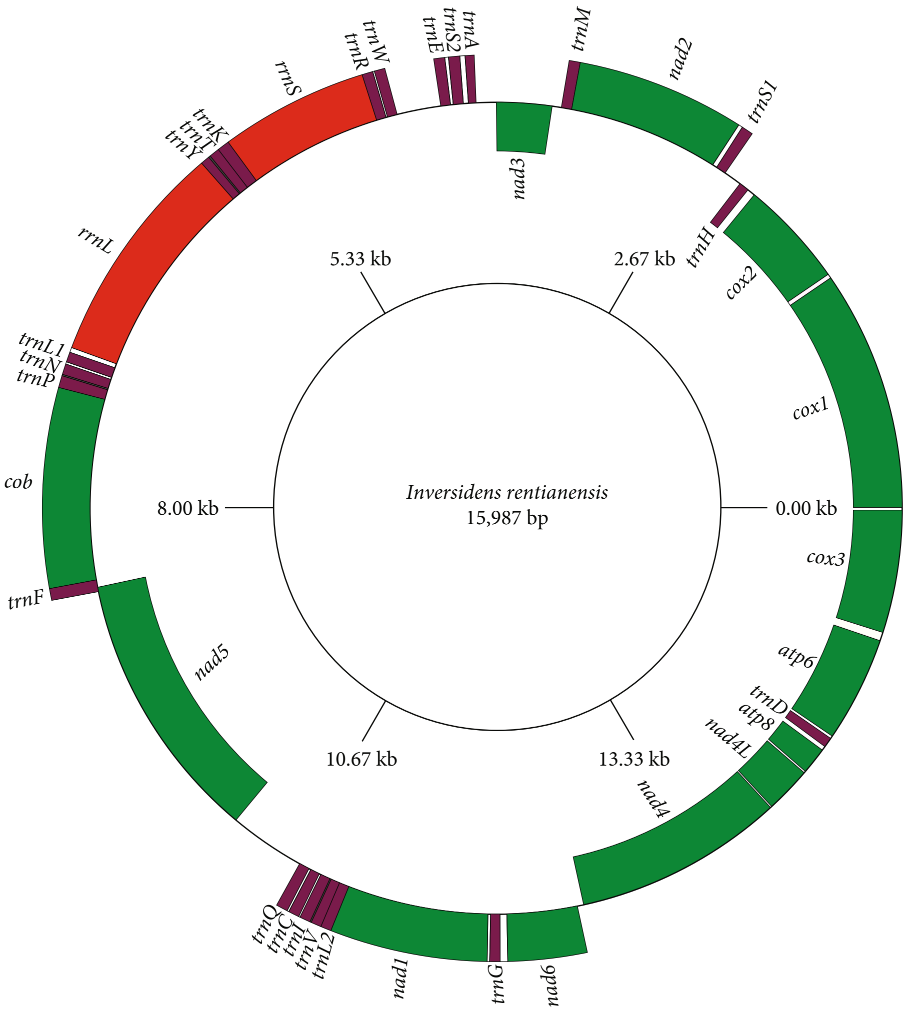
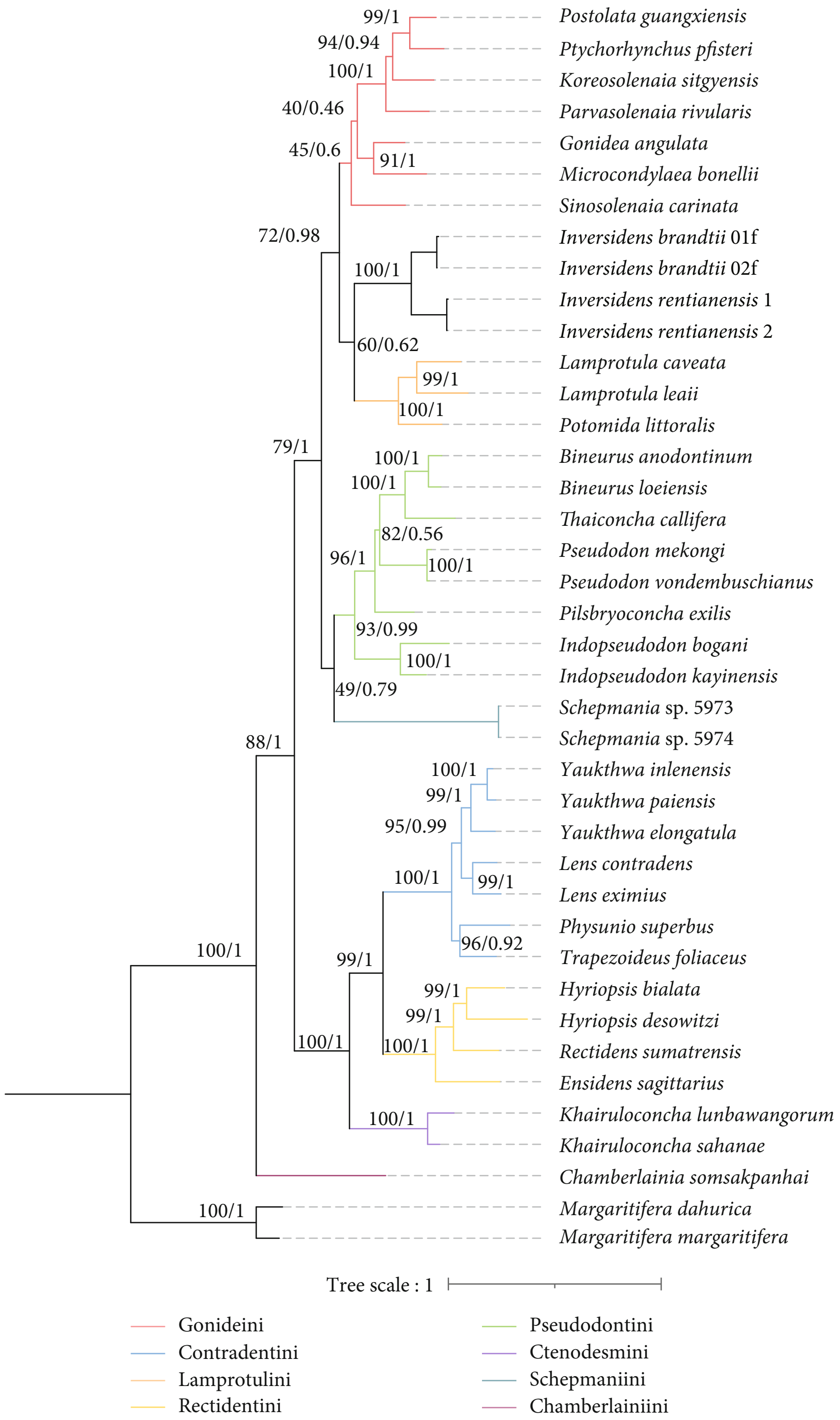


Figure 4: Gene maps of the newly generated mitochondrial genome of *Inversidens rentianensis*. Genes positioned inside the circle are encoded on the heavy strand, and genes outside the circle are encoded on the light strand. Color codes: small and large ribosomal RNAs (red), transfer RNAs (purple), and PCGs (green).

systematics further corroborate its taxonomic status (Figure 8, BS/PP = 100%/1.0).

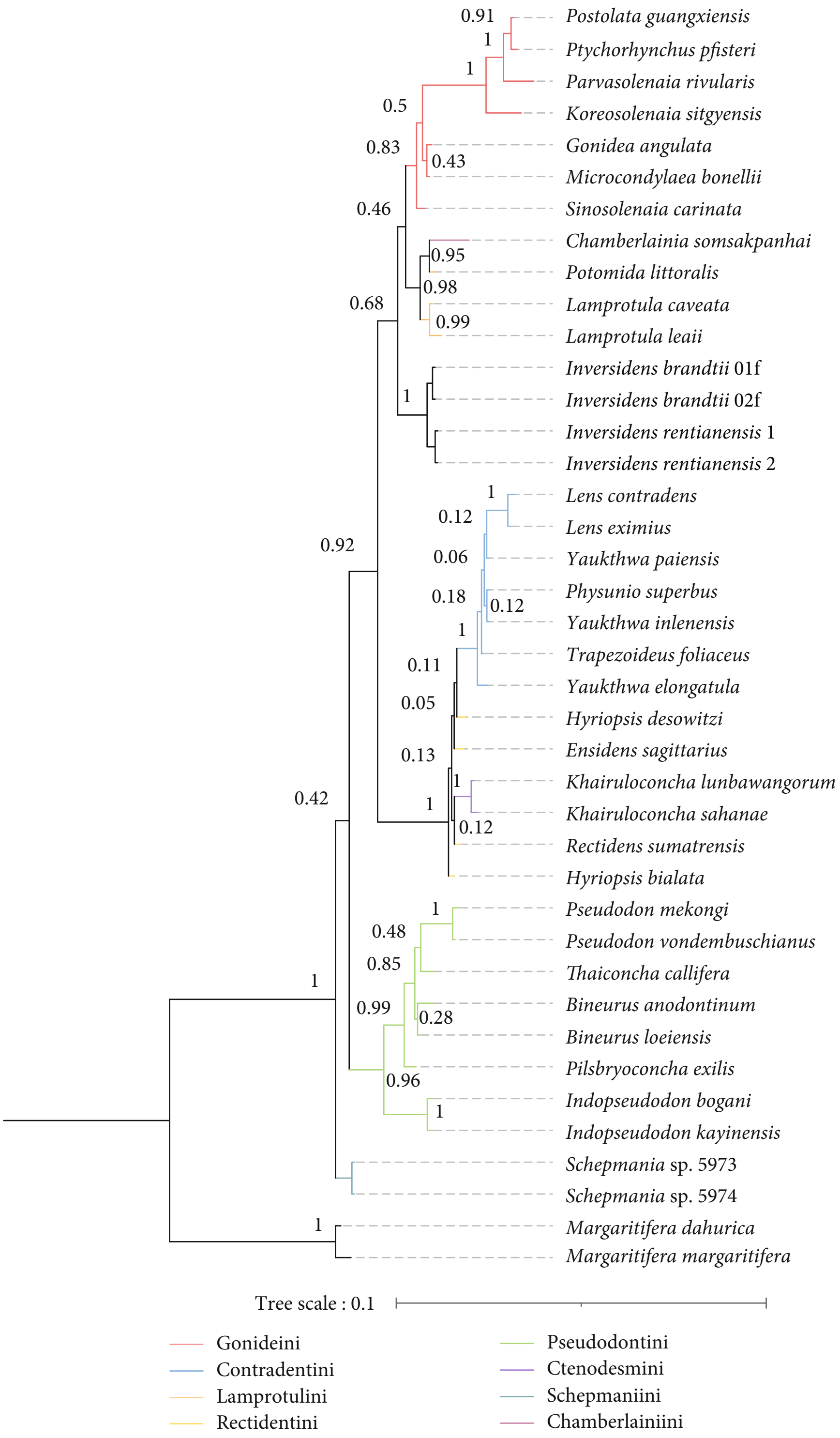
Accurate systematics is essential for comprehending the evolutionary lineage of organisms. The previous divergence time estimation for the tribe Gonideini was 63.7 Mya, with *Ptychorhynchus pfisteri* as the basal clade [41]. In this study, the basal clade group of Gonideini was determined to be *Inversidens*. By reconstructing the species differentiation time, we revised the divergence time for Gonideini to be 102.73 Mya (95% HPD = 72.22-137.03 Mya) (Figure 7). Despite we speculate that *Inversidens rentianensis* and *Inversidens brandtii*, which are endemic to China and Japan, respectively, originated in the Late Paleogene, their historical biogeography remains enigmatic due to a lack of comprehensive understanding on species diversity and distribution for this group. The recent discovery of new species in Southwest China [32, 68, 69] underscores the imperative for a more comprehensive examination of the overall diversity and distribution of freshwater mussels in other inadequately surveyed regions across China.

The accuracy of the phylogenetic framework is influenced by insufficient information loci and inadequate taxon selection [70, 71]. The phylogenetic reconstruction of eight tribes in Gonideinae that was conducted by Zieritz et al. [33] only included three taxa from the species-rich tribes Gonideini and Lamprotulini. The present study incorporated additional sampling taxa from Gonideini and Lamprotulini, which predictably revealed discrepancies in the phylogenetic relationships at the tribe level. The mitochondrial genomics in this study covered six out of the eight tribes within the subfamily Gonideinae, excluding Schepmaniini and Ctenodesmini due to unavailability of mitochondrial genome data. We found strong support for the monophyly of six tribes within Gonideinae, which is consistent with recent phylogenomic analyses utilizing anchored hybrid enrichment data [66] and mitogenomic data [32, 41, 63]. In this study, a novel phylogenetic hypothesis for the eight tribes of Gonideinae was proposed based on the five-locus dataset; however, further verification is required due to the low nodal support. To reconstruct and validate



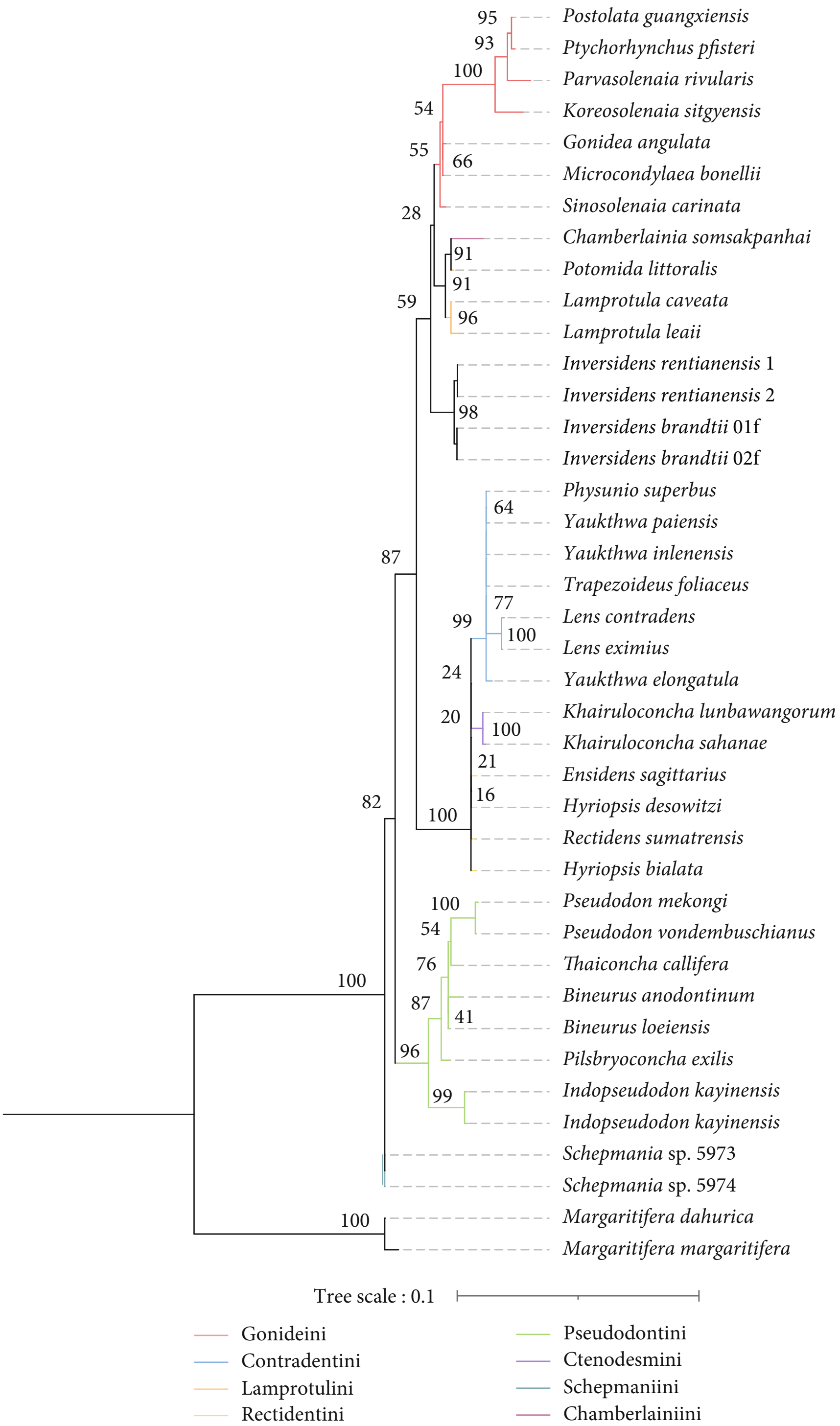
(a) ML/BI-mtDNA

FIGURE 5: Continued.



(b) BI-nuDNA

Figure 5: Continued.



(c) ML-nuDNA

Figure 5: Phylogenetic trees inferred from Bayesian inference (BI) and maximum likelihood (ML) analyses. (a) Phylogram of BI and ML tree based on the mtDNA gene dataset (COI+ND1+16S rRNA). Numbers at nodes are support values for ML (bootstrap support (BS))/BI (posterior probability (PP)). (b) Phylogram of BI tree based on the nuDNA gene dataset (18S rRNA+28S rRNA). Numbers close to the nodes indicate for PP values. (c) Phylogram of ML tree based on nuDNA gene dataset (18S rRNA+28S rRNA). Numbers close to the nodes indicate BS values. Both *Inversidens* species are shown in red font. Colored clades represent eight tribes of the subfamily Gonideinae.

deep evolutionary relationships within Gonideinae, we suggest including genome-scale data such as mitochondrial genomes from Schepmaniini and Ctenodesmini taxa.

4.2. Threats and Conservation Implications. The genus *Inversidens* currently recognizes three species, with both *I. pantoensis* and *I. brandtii* classified as data deficient on the

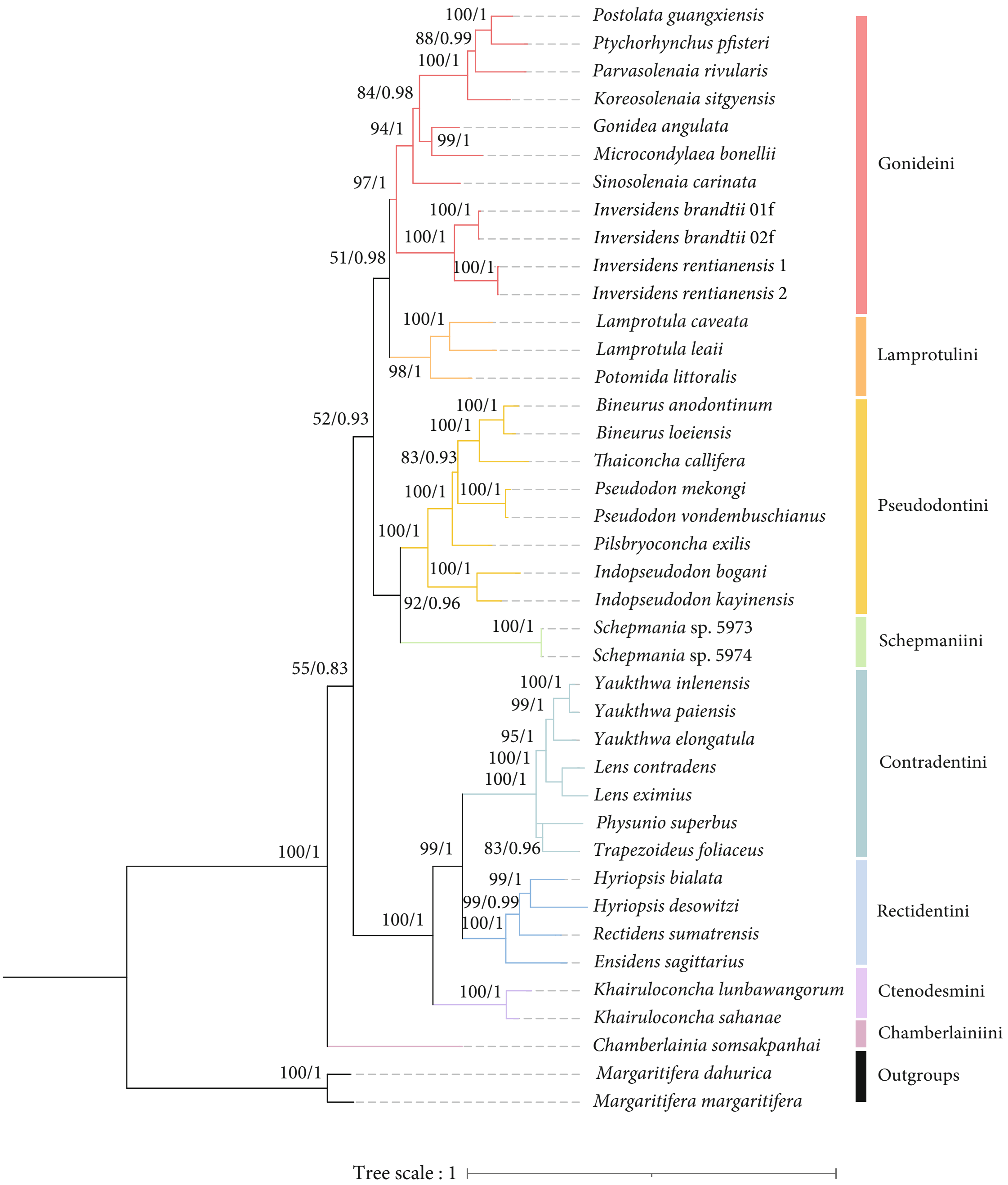


FIGURE 6: Maximum likelihood (ML) and Bayesian inference (BI) trees of the Gonideinae subfamily based on the five-gene dataset (COI +ND1+16S rRNA+18S rRNA+28S rRNA). BI tree generated by Bayesian analysis and ML tree generated by IQ-TREE analysis produce completely congruent topology. Numbers at nodes are statistical support values for ML (bootstrap support (BS))/BI (posterior probability (PP)). Color-coded clades are eight tribes within Gonideinae.

IUCN Red List, while the third species, *I. rentianensis*, remains unevaluated due to its recent discovery and description [25, 72, 73]. *I. brandtii*, an endemic species in Japan, was assessed as vulnerable based on extensive research conducted by Kondo [21] on its morphology, ecology, and reproductive biology. On the contrary, the understanding of the endemic species *I. pantoensis* and *I. rentianensis* in China is obviously insufficient. The knowledge about *I. pantoensis* is currently limited to its morphological shell description, which poses significant challenges in determining its validity due to the considerable shell morphological plasticity [74–76]. It is regrettable that the taxonomic status of this particular species has not been determined yet due to difficulties in collecting specimens for molecular examination. The species *I. rentianensis* is restricted to a narrow geographic range and primarily inhabits creeks and small streams characterized by substrata ranging from gravelly to sandy muddy compositions. Despite multiple visits to the

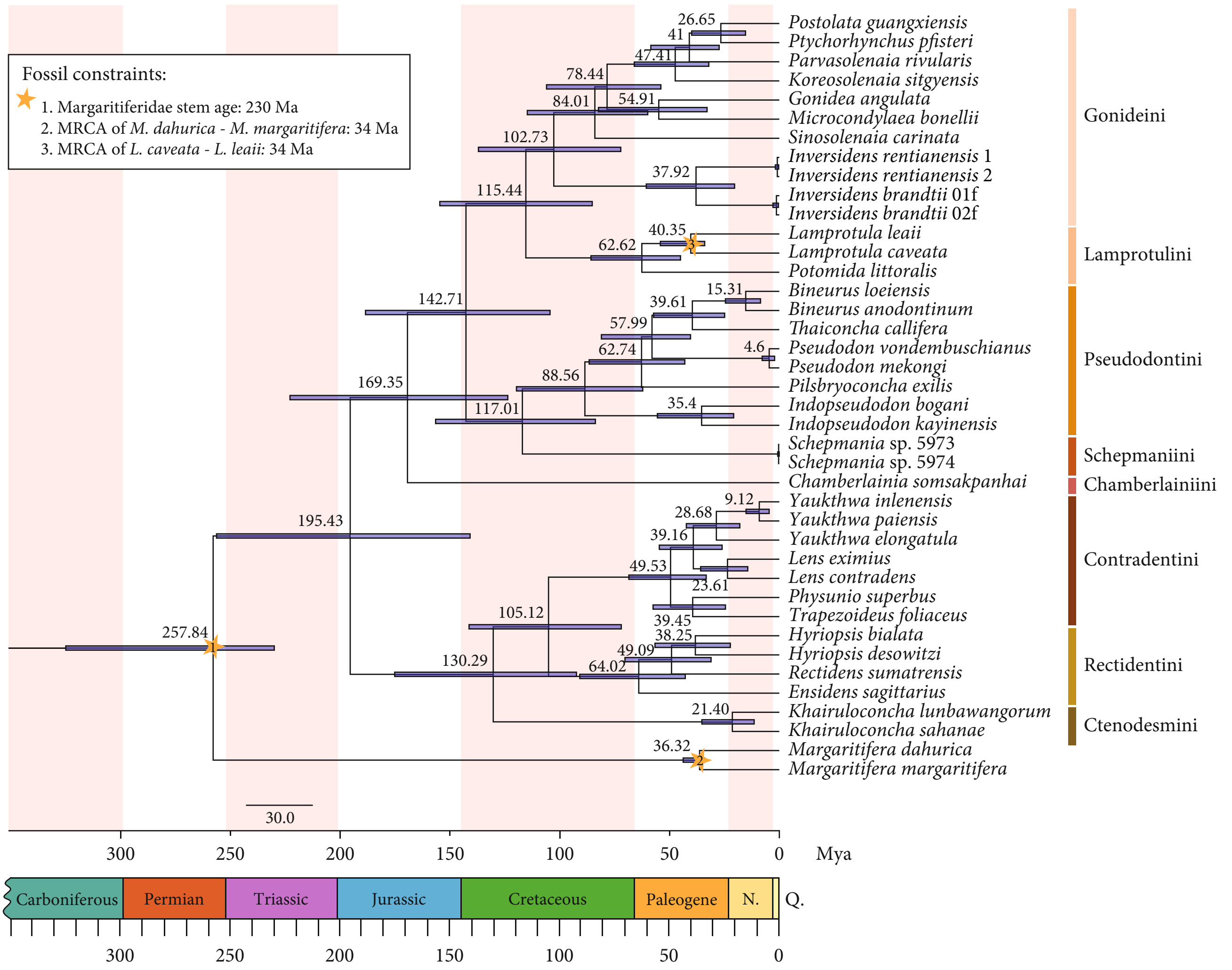
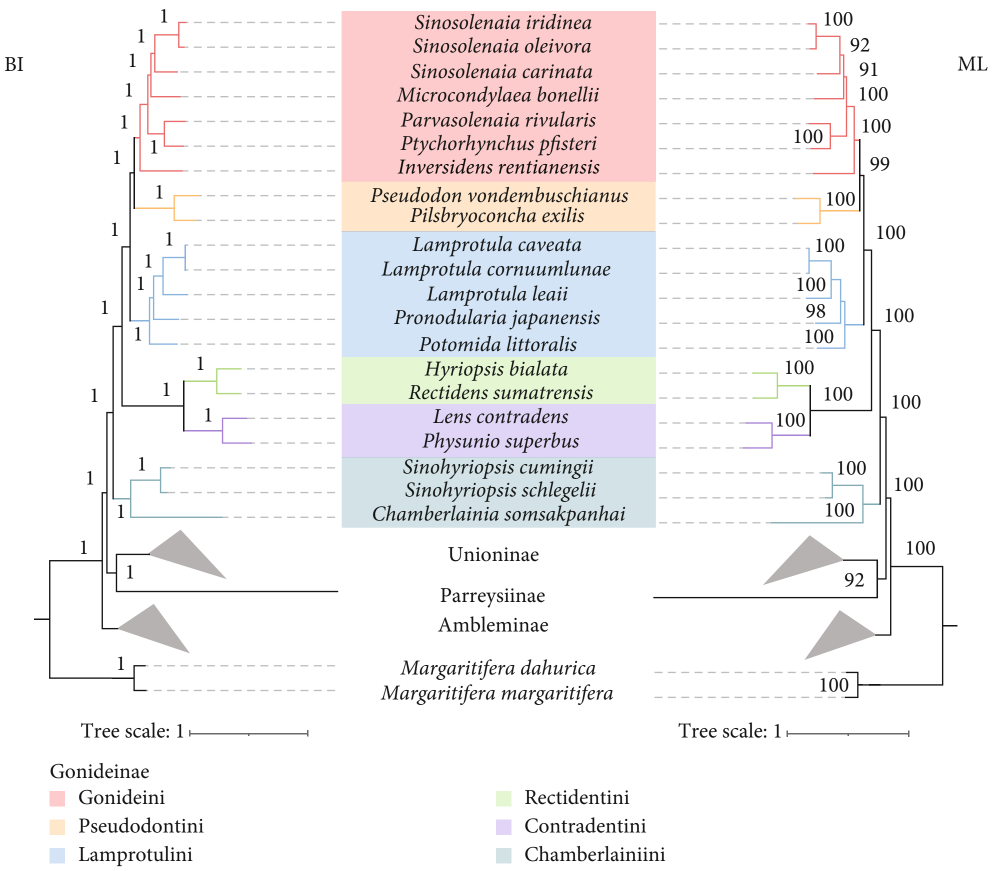


Figure 7: Fossil-calibrated phylogeny based on the five-gene dataset. Node bars represent the mean ages of 95% highest posterior density (HPD). Black numbers near nodes are mean ages (Mya). Fossil calibrations are marked by star signs. Mya: million years ago; Q: Quaternary; N: Neogene.

type locality following the publication of this new species, only two live specimens were successfully collected. Through careful examination of these invaluable specimens, our understanding of the anatomy and molecular systematics of *I. rentianensis* has been significantly enriched. Since no brooding individuals were collected in this study, we could not determine the exact brooding period and examine the glochidia of this species. The accuracy of phylogenies is crucial for comprehending the evolutionary histories, reproductive traits, and ecological habits of organisms. The breeding period of the closely related species *I. brandtii* is from May to September [18]. The specimens of *I. rentianensis* were collected in October, and the gill exhibited characteristics indicative of postegg release. Therefore, we speculated that the breeding period of *I. rentianensis* likely occurs during August and September. Recently, Liu et al. [14] established a new conservation priority method for freshwater mussels, i.e., Quantitative Assessment of Species for Conservation (QASCP). According to this conservation assessment method, our preliminary evaluation ranks *I. rentianensis* as the second priority.

The impacts of urbanization and human activities have left river habitats containing endemic freshwater mussel species vulnerable to threats and require urgent attention and protection [38, 77]. Fortunately, from 2020, Chinese government has implemented comprehensive watershed protection measures (e.g., a decade-long fishing prohibition in crucial sections of the Yangtze River) and legislation (e.g., Yangtze River Protection Law) to effectively restore aquatic ecosystems, yielding remarkable outcomes [78, 79]. The recent discovery of new species, including *Inversidens rentianensis*, highlights the crucial role that small rivers and tributaries play as sanctuaries for freshwater mussels. However, the lack of comprehensive biodiversity surveys in these tributary basins hampers our understanding of aquatic biodiversity and the factors posing threats to it. Furthermore, the conservation efforts for unionid fauna are significantly hindered by a lack of knowledge regarding their distribution, population



(a) (b)

Figure 8: Phylogenetic trees based on mitogenome dataset inferred from Bayesian inference (BI) and maximum likelihood (ML) analyses. (a) BI tree. (b) ML tree. Numbers close to the nodes for (a) indicate posterior probabilities (PP), and those for (b) indicate bootstrap support (BS). Colored backgrounds and colored clades represent six tribes of the subfamily Gonideinae. Red font indicates the species sequence from this study. Here, outgroups are not shown, and both Unioninae and Ambleminae taxa are collapsed in the phylogenetic tree, but complete trees can be found in supplementary Figure 1 and 2.

dynamics, and accurate taxonomic information pertaining to freshwater mussels. Therefore, we advocate researchers to pay more attention to the species diversity and population distribution of freshwater mussels in small tributaries. Simultaneously, we urge the government to formulate scientific protection measures aimed at safeguarding other endemic or endangered mussel species inhabiting small rivers and streams, in addition to those residing in larger rivers.

5. Conclusion

This study provides anatomical features and mitogenome data for *Inversidens rentianensis*. The mitochondrial phylogenomics and five-gene phylogeny clearly show that *Inversidens rentianensis* and *Inversidens brandtii* have a well-supported sister group relationship and form a basal clade in the tribe Gonideini, which should be classified to Gonideini in the subfamily Gonideinae. Molecular dating analysis suggests that *Inversidens* originated in the mid-Cretaceous era (102.73 Mya, 95% HPD = 72.22-137.03 Mya) and diversified during the Late Paleogene era (37.92 Mya, 95% HPD = 20.39-60.59 Mya). Additionally, preliminary assessment ranks Chinese endemic species *Inversidens rentianensis* as second priority based on the QASCP, which aids in managing and conserving this rare group. Taken together, this study shed light on the systematic position of *Inversidens*, while enriching information on the biology of *Inversidens rentianensis*, an endemic species to China. These findings pave the way for further comprehensive studies on the ecology, reproductive behavior patterns, and conservation biology of this fascinating group.

Data Availability

Sequences obtained in this study were uploaded to GenBank, and readers can find GenBank accession numbers in Tables 1 and 2.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: phylogenetic trees based on mitogenome dataset inferred from maximum likelihood (ML) analyses. Supplementary Figure 2: phylogenetic trees based on mitogenome dataset inferred from Bayesian inference (BI) analyses. Supplementary Table S1: partitioning strategies from ModerFinder and PartitionFinder for mtDNA dataset. Supplementary Table S2: partitioning strategies from ModerFinder and PartitionFinder for nuDNA dataset. Supplementary Table S3: partitioning strategies from ModerFinder and PartitionFinder for concatenated five-gene dataset. Supplementary Table S4: partitioning strategies from ModerFinder and PartitionFinder for mitogenome dataset. *(Supplementary Materials)*

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