

PHYLOGENETIC ANALYSIS OF SOME CHINESE FRESHWATER UNIONIDAE BASED ON MITOCHONDRIAL COI SEQUENCES

JIEXIU OUYANG, XIAOPING WU, SHAN OUYANG, SHAOBO LI & DAXIAN ZHAO

College of Life Sciences and Food Engineering, Nanchang University, Jiangxi, Nanchang 330031, China

Abstract In the present study, the mitochondrial COI sequences were sequenced for 43 unionid individuals, belonging to 13 species from 7 genera. Sequences were analyzed using Sequencer 4.05 software. Genetic distances were calculated using the Kimura 2-parameter model. The average intraspecific distance found was 0.02623, much lower than the average interspecific distance, which was 0.18472. The interspecific distance between *Anodonta woodiana elliptica* and *Anodonta woodiana pacifica* was only a little higher than the intraspecific distance. This suggests that *Anodonta woodiana elliptica* and *Anodonta woodiana pacifica* are not separable as either subspecies or species. A molecular phylogeny of the Chinese freshwater unionidae was constructed based on mitochondrial COI sequences. The phylogenetic trees were constructed by the minimum evolution (ME) method, in which the 13 species were divided into three groups. The first group comprised *Solenaia oleivora*, *Solenaia carinatus*, *Solenaia rivularis* and *Lamprotula caveata*. The second group included *Anodonta arcaeformis*, *Anodonta woodiana elliptica* and *Anodonta woodiana pacifica*. While *Lanceolaria gladiola*, *Lanceolaria grayana*, *Unio douglasiae*, *Lamprotula tortuosa*, *Acuticosta chinensis* and *Cuneopsis heudei* made up the third group. Phylogenetic analysis of the genus *Solenaia* shows that the Chinese endemic species *Solenaia* comprise not three species as previously supposed, but four species, one of which has not yet been formally brought forward and described. COI sequences can provide useful information for phylogenetic studies in the Unionidae, and has important implications for these animals' conservation, especially for those considered to be endangered.

Key words Unionidae, freshwater, mussels, *Solenaia*, mitochondrial DNA, phylogeny

INTRODUCTION

The Unionidae is the most abundant and widely distributed family within the freshwater bivalve Mollusca. It has at least 142 genera and more than 600 species (Graf & Cummings, 2007; Bogan, 2008). The Chinese Unionidae were divided into more than 100 species by Heude (1875), 22 genera and about 41 species by Liu (1979), and 16 genera and about 57 species by Hu (2005). Unionids have declined in both diversity and abundance of species. The primary causes of this are overexploitation, habitat loss, competition with introduced species and pollution (Anthony & Downing, 2001; Spielman *et al.*, 2004). Many species are in need of conservation. Researchers have stressed the importance of shell characters or the use of both anatomical and reproductive features to classify freshwater mussels (Balla & Walker, 1991; Zieritz *et al.*, 2010). These different schemes have sometimes led to differing and inconsistent classifications of the unionids (Hoeh *et al.*, 2001), as many species display extreme morphological plasticity. Failure to resolve inconsistencies in freshwater unionid classifications could affect the conservation of these animals.

It is essential to develop an appropriate protocol for obtaining a consistent classification, or hypothesis for phylogenetic relationships. When we understand genetic variation in the group it will be easier to develop strategies to maintain this variation and conserve threatened taxa. Advances in molecular technology have provided new techniques to classify and analyse phylogenetic relationships in these animals. The 5' end of the mitochondrial cytochrome oxidase subunit I (COI) gene was proposed as a 'barcode' for all animal species. The use of DNA barcoding (Hebert *et al.*, 2003), whereby the sequence from a standardized region of the COI gene is used to identify species, provides a way to analyze phylogeny consistently. Given a validated data set of sequences obtained from morphologically identified specimens, an unknown individual may be identified rapidly and cost-effectively. The COI gene has been tested as a tool for species identification, biodiversity analysis and discovery for many animals (Smith *et al.*, 2006; Roe *et al.*, 2001; Campbell *et al.*, 2008; Chong *et al.*, 2008; Jia *et al.*, 2009; Graf & Cummings, 2010; Mock *et al.*, 2010).

In this study, we sought to investigate phylogenetic relationships in a selection of Chinese unionids and to test a classification based on partial sequences of COI genes. These data would also

Table 1 Species used for phylogenetic analysis in the present study.

Species	Specimen number	Localities of samples
<i>Acuticosta chinensis</i>	2	Poyang Lake in Jiangxi Province
<i>Anodonta arcaiformis</i>	2	Poyang Lake in Jiangxi Province
<i>Anodonta woodiana elliptica</i>	4	Poyang Lake in Jiangxi Province
<i>Anodonta woodiana pacifica</i>	2	Poyang Lake in Jiangxi Province
<i>Cuneopsis heudei</i>	1	Poyang Lake in Jiangxi Province
<i>Lamprotula caveata</i>	4	Poyang Lake in Jiangxi Province
<i>Lamprotula tortuosa</i>	1	Poyang Lake in Jiangxi Province
<i>Lanceolaria gladiola</i>	2	Poyang Lake in Jiangxi Province
<i>Lanceolaria grayana</i>	2	Poyang Lake in Jiangxi Province
<i>Solenia carinatus</i>	3	Poyang Lake in Jiangxi Province
<i>Solenia oleivora</i>	6	Hong Lake in Hubei
	5	Poyang Lake in Jiangxi Province
<i>Solenia rivularis</i>	6	Poyang Lake in Jiangxi Province
<i>Unio douglasiae</i>	3	Poyang Lake in Jiangxi Province

provide a useful evolutionary framework for future interspecific and intraspecific studies of Chinese freshwater unionids.

MATERIALS AND METHODS

Collection and DNA extraction Specimens were collected at different locations from Poyang Lake in Jiangxi Province and from Hong Lake in Hubei Province. Since freshwater unionids exhibit aggregated distributions in suitable habitats, study sites in a lake were not selected at random. Our research objectives do not include determinations of abundance or demographic trends, so did not employ traditional quantitative sampling techniques (Strayer & Smith, 2003). Depending on water depth, collecting required wading, snorkeling or dredging on the lake bed. Collected unionids were returned to the laboratory rapidly and stored at -80°C until processed. Table 1 lists the species, specimen numbers and locality data.

Shells were opened by inserting reverse pliers between the valves, and mantle tissue was removed for DNA extraction. Whole genomic DNA was extracted from mantle tissue in fresh or frozen specimens using standard proteinase K/SDS digest (Roe & Lydeard, 1998) extraction methods followed by phenol/chloroform isolation and ethanol precipitation.

PCR amplification and sequencing Mitochondrial DNA sequences were obtained from an amplified

segment of the *COI* gene using the polymerase chain reaction (PCR). The primers were *COI*-22me (5'-GGTCAACAAATCATAAAGATATTGG-3') and *COI*-700dy (5'-TCAGGGTGACCAAAAAA TCA-3') (Elderkin *et al.*, 2007). Thermal cycling consisted of the following steps: an initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 1 min, 42°C annealing for 1 min, 72°C extension for 2 min, and a final extension at 72°C for 7 min. PCR products were isolated on 0.8% agarose gels and delivered to Shanghai Sangon Biological Engineering & Technology Service Co. Ltd. (Shanghai, China) for sequencing. This was performed using the amplification primers with an ABI-PRISM3730 automatic DNA sequencer.

Data analysis The sequences were edited and analyzed using a Sequencer 4.05 (Gene Codes Corporation), and were aligned using Clustal X1.83 software (Thompson *et al.*, 1997). Phylogenetic trees were constructed by the minimum evolution (ME) method with MEGA4 software (Tamura *et al.*, 2007).

RESULTS AND ANALYSIS

Sequence analysis of the *COI* The size of the *COI* PCR amplification product ranged from 644 bp to 685 bp. Sequence alignment of the *COI* gene portions yielded 580 bp homological fragments for 43 individuals. Two hundred and thirty sites were polymorphic. The total number of mutations

Table 2 Average AT and GC contents of COI PCR product in the present study, 13 species among 580bp homologous fragments,

Species	AT (%)	GC (%)
<i>Acuticosta chinensis</i>	60.7	39.3
<i>Anodonta arcaiformis</i>	58.9	41.1
<i>Anodonta woodiana elliptica</i>	60.4	39.4
<i>Anodonta woodiana pacifica</i>	59.9	40.1
<i>Cuneopsis heudei</i>	59.5	39.5
<i>Lamprotula caveata</i>	55.8	44.2
<i>Lamprotula tortuosa</i>	58.3	41.7
<i>Lanceolaria gladiola</i>	59.5	40.5
<i>Lanceolaria grayana</i>	57.9	42.1
<i>Solenaiia carinatus</i>	55.2	44.8
<i>Solenaiia oleivora</i>	57.0	43.0
<i>Solenaiia rivularis</i>	54.9	45.1
<i>Unio douglasiae</i>	59.7	40.3
Average	58.3	41.7

was 354, accounting for 61.03%. Transitions pairs (si) were 46, while transversions pairs (sv) were 37, and R (si/sv) was 1.3. Table 2 shows AT and GC contents of COI PCR amplification products in 13 species, with AT contents ranging from 54.9% to 60.7%, while GC contents ranged from 39.3% to 45.1%. The average contents of AT (58.3%) are significantly higher than those of GC (41.7%).

Genetic distance analysis of the Unionidae Based on the Kimura 2-Parameter model, intraspecific and interspecific distance were calculated. The largest intraspecific genetic distance (Table 3) was 0.07599 among *Unio douglasiae* individuals. The largest interspecific genetic distance was 0.24697 between *Anodonta woodiana pacifica* and *Solenaiia carinatus*. The average intraspecific distance was 0.02623, much lower than the average interspecific distance, which was 0.18472. The results suggest that COI is a useful genetic marker for identification and phylogenetic analysis of mussel species. The interspecific distance (0.05542) between *Anodonta woodiana elliptica* and *Anodonta woodiana pacifica* was found to be very low, only a little higher than the intraspecific distance (0.03737) of *Anodonta woodiana pacifica*. This suggests that *Anodonta woodiana elliptica* and *Anodonta woodiana pacifica* are not distinct and should be regarded as a single species.

Phylogenetic analysis of the Unionidae Using COI sequences as a molecular marker, a phylogenetic tree of the Chinese freshwater unionids was constructed using ME methods (Fig. 1). Tree topologies showed that the surveyed Unionidae comprised three groups. The first group was composed of *Solenaiia oleivora*, *Solenaiia carinatus*, *Solenaiia rivularis* and *Lamprotula caveata*. The second group included *Anodonta arcaiformis*, *Anodonta woodiana elliptica* and *Anodonta woodiana pacifica*. While *Lanceolaria gladiola*, *Lanceolaria grayana*, *Unio douglasiae*, *Lamprotula tortuosa*, *Acuticosta chinensis* and *Cuneopsis heudei* comprised the third group. The first group belongs to subfamily Ambleminae, the second group to subfamily Anodontinae, while the third group belongs to subfamily Unioninae. Thus the results were to some extent in accordance with a more traditional morphological taxonomy (Liu, 1979; Huang *et al.*, 2002; Wei, 2004; Zhou *et al.*, 2007). However, there were some differences. The phylogenetic analysis suggested that Anodontinae was the sister to Unioninae, and furthermore, both were sister to the Ambleminae.

Phylogenetic analysis of the genus Solenaiia *Solenaiia* is an endemic Asian genus, mainly distributed in China, India and Myanmar. In China, some species are cultivated and raised commercially. The Chinese genus *Solenaiia* has 4 recorded species: *Solenaiia oleivora* (syn. *Mycetopus oleivora*, *M. armatus*, *M. recognitus*, *M. similis*, *M. succineus*, *M. viridis*, *M. coeruleus*), *Solenaiia carinatus* (syn. *Mycetopus carinatus*), *Solenaiia triangularis* (syn. *Mycetopus triangularis*) and *Solenaiia rivularis* (syn. *Mycetopus rivularis*) (Heude, 1875; Liu, 1979). Three of these are still widespread, *Solenaiia carinatus*, *Solenaiia oleivora* and *Solenaiia triangularis* (Heude, 1875; Liu, 1979; Wu, 1991; Hu, 2005). *Solenaiia rivularis* is a much rarer species and has received comparatively little attention, with few references in the literature. The other three species are federally listed as endangered by the IUCN (International Union for Conservation of Nature). There is clearly an urgent need for effective conservation planning. In our research, 5 specimens of *Solenaiia oleivora*, 3 specimens of *Solenaiia carinatus* and 6 specimens of *Solenaiia rivularis* were collected from Poyang Lake in Jiangxi Province, while 6 specimens of *Solenaiia oleivora* were collected from Hong Lake in Hubei Province. We were unable to collect

Table 3 Interspecific genetic distance (below the diagonal) and intraspecific genetic distance (in the diagonal) of 13 species based on Kimura 2-Parameter model.

Species	<i>Acuticosta chinensis</i>	<i>Anodonta arcaeformis</i>	<i>A. woodiana elliptica</i>	<i>A. woodiana pacifica</i>	<i>Cuneopsis heudei</i>	<i>Lamprotula caveata</i>	<i>L. tortuosa</i>	<i>Lanceolaria gladiola</i>	<i>Solenia carinatus</i>	<i>S. oleivora</i>	<i>Lanceolaria grayana</i>	<i>Solenia Rivularis</i>	<i>Unio douglasiae</i>
<i>Acuticosta chinensis</i>	0.00693												
<i>Anodonta arcaeformis</i>	0.18158	0.00346											
<i>A. woodiana elliptica</i>	0.16661	0.18227	0.00202										
<i>A. woodiana pacifica</i>	0.16302	0.19543	0.05542	0.03737									
<i>Cuneopsis heudei</i>	0.14420	0.20376	0.18911	0.20281									
<i>Lamprotula caveata</i>	0.18061	0.19514	0.18759	0.19202	0.17429	0.01133							
<i>L. tortuosa</i>	0.15174	0.19127	0.17975	0.17621	0.15852	0.17931							
<i>Lanceolaria gladiola</i>	0.14126	0.18122	0.17120	0.16981	0.16336	0.17660	0.12680	0.02468					
<i>Solenia carinatus</i>	0.15403	0.21023	0.19147	0.19063	0.17959	0.22242	0.19509	0.12476	0.04587				
<i>S. oleivora</i>	0.17404	0.21048	0.22720	0.24697	0.17630	0.15137	0.19955	0.18671	0.21473	0.06979			
<i>Lanceolaria grayana</i>	0.19514	0.21693	0.21677	0.22778	0.19647	0.16414	0.22040	0.21572	0.22508	0.15158	0.00115		
<i>Solenia rivularis</i>	0.20724	0.22091	0.21600	0.20959	0.17546	0.16247	0.19816	0.19110	0.21501	0.13948	0.15303	0.00994	
<i>Unio douglasiae</i>	0.16595	0.22856	0.19993	0.20353	0.13692	0.17689	0.16594	0.16071	0.18844	0.19984	0.2256	0.20066	0.07599

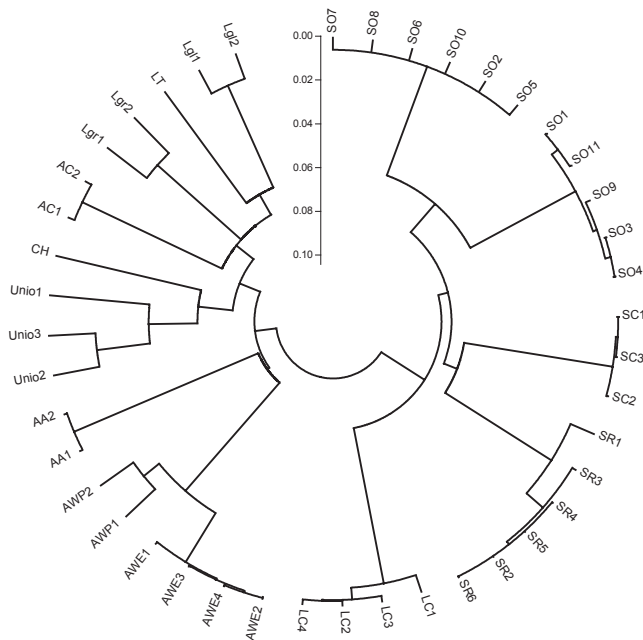


Figure 1 The phylogenetic tree using the ME methods based on the mt COI gene sequences of Chinese freshwater Unionidae: *Acuticosta chinensis* (AC); *Anodonta arcaiformis* (AA); *Anodonta woodiana elliptica* (AWE); *Anodonta woodiana pacifica* (AWP); *Cuneopsis heudei* (CH); *Lamprotula caveata* (LC); *Lamprotula tortuosa* (LT); *Lanceolaria gladiola* (Lgl); *Lanceolaria grayana* (Lgr); *Solenia carinatus* (SC); *Solenia oleivora* (SO); *Solenia rivularis* (SR); *Unio douglasiae* (Unio).

Solenia triangularis, which is mainly found in Anhui Province (Hu, 2005).

From measurements of genetic distance in these mussels, we found the average intraspecific distance to be 0.02696, much lower than the average interspecific distance of 0.18974, in the three species. However, the intraspecific distance of *Solenia oleivora* was 0.06979, much higher than the rest. This is explained by sampling from two different lakes and suggests that the populations in these lakes differ genetically i.e. two species are present. The phylogenetic tree shows the same result. From these results it is clear that an additional, undescribed, Chinese *Solenia* exists.

DISCUSSION

Phylogeny of the Unionidae This study is the first to evaluate the phylogeny of the Chinese freshwater Unionidae based on mitochondrial COI sequences. Few other studies have used this technique for Chinese freshwater unionids.

Our molecular tree supported the relationship and further agreed with Zhou (2007) and Huang (2002) that *Lamprotula caveata* was a close ally of the *Solenia* species, and should be assigned to subfamily Ambleminae. *Lamprotula tortuosa* was shown to be very close to *Aculamprotula* (Wu, 1998), and assigned to the subfamily Unioninae. In the present study, Anodontinae was the sister to Unioninae, and furthermore, both of them were sister to Ambleminae, which was supported by the studies of Huang (2002). However, Zhou (2007) has claimed that the Unioninae is the sister to the Ambleminae, with both of them sister to the Anodontinae. The goal of our work was to put the phylogeny of the Chinese freshwater unionids on a molecular foundation.

Examination of the Chinese genus Solenia *Solenia* represents a neglected genus of unionids and little is known about their taxonomic and conservation status; perhaps due to the fact that they have a relatively restricted distribution and abundance. The research reports on *Solenia* have been very few (Deein *et al.*, 2003). Only two species, *Solenia oleivora* and *Solenia triangularis*, can be seen on the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=165459>). In the present study, molecular data indicated that '*Solenia oliveivora*' collected from two lakes comprised two distinct species rather than one, making a total of five not four, species now known from China. The unknown fifth species has not yet been formally brought forward. Our work is the first report dealing with the detailed phylogeny of Chinese *Solenia*. It should contribute towards knowledge of this group and assist their conservation.

REFERENCES

- ANTHONY JL & DOWNING JA 2001 Exploitation trajectory of a declining fauna: a century of freshwater mussel fisheries in North America. *Canadian Journal of Fisheries and Aquatic Sciences* **58**: 2071–2090.
- BALLA SA, WALKER KF 1991 Shape variation in the Australian freshwater mussel *Alathyria jacksoni* Iredale (Bivalvia, Hyriidae). *Hydrobiologia* **220**: 89–98.
- BOGAN AE 2008 Global diversity of freshwater mussels (Mollusca, Bivalvia) in freshwater. *Hydrobiologia* **595**: 139–147.

- CAMPBELL DC, JOHNSON PD, WILLIAMS JD, RINDSBERG AK, SERB JM, SMALL KK & LYDEARD C 2008 Identification of 'extinct' freshwater mussel species using DNA barcoding. *Molecular Ecology Resources* **8**: 711–724.
- CHONG JP, BRIM-BOX JC, HOWARD JK, WOLF D, MYERS TL & MOCK KE 2008 Three deeply divided lineages of the freshwater mussel genus *Anodonta* in western North America. *Conservation Genetics* **9**: 1303–1309.
- DEEIN G, UNAKORNSAWAT Y, RATTANADAENG P, SUTCHARIT C, KONG IM BO & PANHA S 2003 A new species of *Soleinaia* from Thailand (Bivalvia: Unionidae: Ambleminae). *The Natural History Journal of Chulalongkorn University* **3**: 53–58.
- ELDERKIN CL, CHRISTIAN AD, METCALFE-SMITH JL & BER DJ 2008 Population genetics and phylogeography of freshwater mussels in North America, *Elliptio dilatata* and *Actinonaias ligamentina* (Bivalvia: Unionidae). *Molecular Ecology* **17**: 2149–2163.
- GRAF DL & CUMMINGS KS 2007 Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoidea). *Journal of Molluscan Studies* **73**: 291–314.
- GRAF DL, CUMMINGS KS 2010 Comments on the value of COI for family-level freshwater mussel systematics: A reply to Hoeh, Bogan, Heard & Chapman. *Malacologia* **52**: 191–197.
- HEBERT PDN, CYWINSKA A, BALL SL, WAARD JR DE 2003 Biological identifications through DNA barcodes. *Proceedings of the Royal Society B* **270**: 313–321.
- HEUDE RP 1875 *Conchyliologie fluviatile province de Nanking et de la Chine centrale*. F Savy, Paris.
- HOEH WR, BOGAN AE & HEARD WH 2001 A phylogenetic perspective on the evolution of morphological and reproductive characteristics in the Unionoidea. *Ecological studies* **145**: 257–280.
- HU ZQ 2005 Geographical distribution of endemic species of Chinese freshwater bivalves. *Chinese Journal of Zoology* **40**: 80–83. (In Chinese).
- HUANG YY, LIU HZ, WU XP & OUYANG S 2002 Testing the relationships of Chinese freshwater Unionidae (Bivalvia) based on analysis of partial mitochondrial 16S rRNA sequences. *Journal of Molluscan Studies* **68**: 359–363.
- JIA MJ, LI JL, NIU DH & BAI ZY 2009 Sequence variation of COI gene in ten populations of *Cristaria plicata* from the Middle and Lower Yangtze River. *Chinese Journal of Zoology* **44**: 1–8. (In Chinese).
- LIU YY 1979 *Freshwater mollusk economic fauna of China*. Science Press, Beijing. 134 pp. (In Chinese).
- LIU YY & WU XP 1991 The further description of *Soleinaia carinatus* (Lamellibranchia: Unionidae). *Acta Zootaxonomica Sinica* **16**: 122–123. (In Chinese).
- MOCK KE, BRIM-BOX JC, CHONG JP, HOWARD JK, NEZ AD, WOLF D & GARDNER RS 2010 Genetic structuring in the freshwater mussel *Anodonta* corresponds with major hydrologic basins in the western United States. *Molecular Ecology* **19**: 569–591.
- ROE KJ, HARTFIELD PD & LYDEARD C 2001 Phylogeographic analysis of the threatened and endangered superconglutinate-producing mussels of the genus *Lampsilis* (Bivalvia: Unionidae). *Molecular Ecology* **10**: 2225–2234.
- ROE KJ & LYDEARD C 1998 Molecular systematics of the freshwater mussel genus *Potamilus* (Bivalvia: Unionidae). *Malacologia* **39**: 195–205.
- SMITH MA, WOODLEY NE, JANZEN DH, HALLWACHS W & HEBERT PD 2006 DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proceedings of National Academy of Sciences USA* **103**: 3657–3662.
- SPIELMAN D, BROOK BW & FRANKHAM R 2004 Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 15261–15264.
- STRAYER DL & SMITH DR 2003 *A Guide to Sampling Freshwater Mussel Populations*. Monograph 8, American Fisheries Society, Bethesda, Maryland.
- THOMPSON JD, GIBSON TJ, PLEWNIAK F, JEANMOUGIN F & HIGGINS DG 1997 The CLUSTAL_X windows interface. Flexible strategies for multiple-sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.
- TAMURA K, DUDLEY J, NEI M & KUMAR S 2007 MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.
- WEI KJ 2004 *Studies on genetic diversity and phylogeny of Chinese Unionidae (Mollusca: Bivalvia)*. Huazhong Agricultural University, China. 130pp. (Doctoral Dissertation, in Chinese).
- WU XP 1998 *Studies on freshwater shellfish in middle and lower reaches Yangtze River*. Institute of Hydrobiology, Chinese Academy of Sciences. 45pp. (Doctoral Dissertation, in Chinese).
- WU XP 1991 The further description of *Soleinaia carinatus* (Lamellibranchia: Unionidae). *Acta Zoologica Sinica* **16**: 122–123. (In Chinese).
- ZHOU CH, OUYANG S, WU XP & LI M 2007 Phylogeny of the genus *Lamprotula* (Unionidae) in China based on mitochondrial DNA sequences of 16S rRNA and ND1 genes. *Acta Zoologica Sinica* **53**: 1024–1030. (In Chinese).
- ZIERITZ A, HOFFMAN JI, AMOS W & ALDRIDGE DC 2010 Phenotypic plasticity and genetic isolation-by-distance in the freshwater mussel *Unio pictorum* (Mollusca: Unionoidea). *Evolutionary Ecology* **24**: 923–938.