GENETIC DIVERSITY OF FIVE FRESHWATER MUSSELS IN GENUS *ANODONTA* (MOLLUSCA: BIVALVIA) REVEALED BY RAPD ANALYSIS

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Abstract: Unionidae are an important group of benthic freshwater species. Due to the convergence phenomenon within freshwater mussels, there is still much controversy in the classification of Chinese Unionidae. In China, most studies on freshwater mussels emphasized resource investigation, biology and morphology, while little has been done in genetics, and particularly not in population genetic structure as well as genetic diversity. In order to further understand the status of genetic diversity of different species, random amplified polymorphic DNA (RAPD) markers were used to detect genetic diversity of populations in five species of the genus *Anodonta*: *Anodonta arcaeformis*, *A. arcaeformis flavotincta*, *A. fluminea*, *A. woodiana woodiana* and *A. w. pacifica*. DNA extraction method was based on phenol-chloroform and extracted genomic DNA from the adductor muscle and mantle tissues. Sixteen random primers were used for RAPD amplification and the polymorphism of amplified loci were analyzed. The results demonstrated that the percentage of amplified polymorphic loci for various populations ranged from 34.5% to 62.8%, the mean Shannon's genetic diversity indices ranged from 0.2021 to 0.3552, and the mean intra-population Nei's genetic distance ranged from 0.1386 to 0.1713. In all populations of the five species, the genetic diversity for *A. arcaeformis* was the largest, and that of *A. fluminea* was the lowest. The inter-population genetic distance between *A. w. woodiana* and *A. w. pacifica* was 0.3186, so they can be considered as two sister species at the genetic angle.

Key words: Genus *Anodonta*; Chinese unionidae; Genetic diversity; RAPD analysis
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Unionidae (Bivalvia) are distributed in freshwaters, and represent a significant taxon of benthic community[1]. In China, freshwater mussels are abundant resources[2]. Since 1949, substantial investigations on the unionid fauna had been undertaken in China[3—8]. With reference to overseas research[9, 10], a preliminary reorganization on the Unionidae was performed according to some classification characteristics such as shell shape, larvae characteristics, and breeding habit[11]. Due to the serious convergence of freshwater mussels, apparent variation of shape during their ontogenesis and variation of shell shape with habitats, some difficulties were encountered in the species identification (in particular the genus *Anodonta*) and some correlation studies. With the pollution level aggravating in inland waters such as lakes and rivers, as well as the habitat degradation, mussel populations are being artificially intervened and destroyed in recent years. The germplasm of the mussel resources were also partly destroyed. However, few reports are related to the study of the genetic structure of the populations and the genetic diversity of freshwater mussels.

RAPD (Random amplified polymorphic DNA) analysis is a technique based on the polymerase chain reaction (PCR) amplification of discrete regions of the genome with short oligonucleotide primers of arbitrary sequence[12, 13]. The method is simple and quick to perform, and most importantly, no prior knowledge of the genetic make-up of the organism in question is required[14].

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This technique has been used extensively to detect genetic diversity in aquatic animals such as fishes[15] and marine bivalves[16—18]. RAPD analysis also has been used to evaluate genetic diversity in freshwater bivalves, but mainly used with harmful invasive species zebra mussels[19] in North America and Europe, and only one analysis on the genetic diversity of *Hyriopsis cumingii*, a freshwater bivalve, was reported in China[20].

The objectives of this study were to (a) determine population genetic diversity levels of five local freshwater mussels, *Anodonta arcaeformis*, *A. arcaeformis flavotincta*, *A. fluminea*, *A. woodiana woodiana* and *A. w. pacifica*, which belongs to genus *Anodonta* in China, and (b) analyze their species validity. The knowledge obtained can be applied to the conservation of germpasm resources and taxonomic classification of these freshwater mussels in China.

1 Materials and methods

1.1 Sampling Specimens from populations of five species of the genus *Anodonta* were collected live from the Suya Lake in Henan Province and Yezhi Lake in Hubei Province respectively. Three to ten individuals were randomly sampled from each population (Tab. 1). Adductor muscle and mantle tissues were removed from each mussel and immediately placed in liquid nitrogen, and stored at −80 ℃ until further utilized.

Tab. 1 Sampling locations and sample sizes of *Anodonta* species used in this study

Species	Sampling location	Sample size (N)
A. a. flavotincta	Suya Lake, Henan	10
A. arcaeformis	Yezhi Lake, Hubei	10
A. fluminea	Suya Lake, Henan	3
A. w. woodiana	Suya Lake, Henan	9
A. w. pacifica	Yezhi Lake, Hubei	10

1.2 DNA extraction Approximately 150 mg of adductor muscle or mantle tissue of each individual was dissected, followed by genomic DNA extraction using the SDS-phenol-chloroform method[21]. Concentrations of extracted DNA were spectrophotometrically determined by using BioPhotometer 6131 (eppendorf, Germany).

1.3 RAPD analysis and agarose gel electrophoresis Eighty decanucleotide primers in groups of OPI、OPP、OPG、OPM purchased from the Biostar Company (Shanghai, China) were screened. Sixteen selected primers were used for determination of genetic diversity (Tab. 2). RAPD-PCR was carried out in a 25μl reaction volume containing 10mM of Tris-HCl (pH 8.3), 50mM of KCl, 2mM of $MgCl_2$, 0.001% of gelatin, 200μM of each dNTPs (dATP, dCTP, dGTP, and dTTP), 0.2μM of each primer, 30ng of genomic DNA, and 1.5U of *Taq* DNA polymerase (Opromega company, Shanghai). A negative no-DNA control was run with all of the gradients except DNA for each reaction. Reactions were carried out in a PTC-200 DNA Engine (MJ Research, Inc.), the optimized amplification profile was an initial denaturation for 4 min at 94 ℃, followed by 45 cycles composed of denaturation at 94 ℃ for 1min, annealing at 37 ℃ for 1min, and extension at 72 ℃ for 2min. The final extension was performed at 72 ℃ for 7minutes. The amplified products were electrophoretically analyzed through a 1.5% agarose gel and visualized by using GeneGenius Bio Imaging System (SynGene, England).

Tab. 2 Codes and sequences of the Operon random primers used in this study

Primer codes	Sequence (5′ to 3′)	Primer codes	Sequence (5′ to 3′)
OPI04	CCGCCTAGTC	OPP10	TCCCGCCTAC
OPI10	ACAACGCGAG	OPP16	CCAAGCTGCC
OPI11	ACATGCCGTG	OPG03	GAGCCCTCCA
OPI14	TGACGGCGGT	OPG15	ACTGGGACTC
OPI19	AATGCGGGAG	OPG17	ACGACCGACA
OPP02	TCGGCACGCA	OPM02	ACAACGCCTC
OPP06	GTGGGCTGAC	OPM04	GGCGGTTGTC
OPP08	ACATCGCCCA	OPM12	GGGACGTTGG

1.4 Data analysis Each RAPD fragment was treated as an independent character. Sizes of each RAPD band were estimated by comparison with 200bp DNA ladder and recorded in a binary matrix to represent the absence (0) or presence (1) of a particular band.

The POPGENE computer package[22] was used to calculate Shannon's index of phenotypic diversity for RAPD diploid data according to $H_0 = -\sum \pi_i \log_2 \pi_i$, where π_i represents the frequency of the presence or absence of the amplified fragment, and percentage of polymorphic loci was calculated by formula $\%P = (B/P) \times 100$, where B and P represent the number of amplified

bands and polymorphic loci, respectively.

The similarity index between individuals was calculated as $S_{xy}=2N_{xy}/(N_x+N_y)$, where N_x and N_y represent the number of RAPD bands in individuals x and y, respectively, and N_{xy} represents the number of shared bands between individuals[23]. Between-sample similarity was calculated as the average of all possible comparisons of individuals across samples i and j using the same equation. Genetic distances between pairs of samples (D_{ij}) were converted from the index of similarity between samples using the equation $D_{ij}=1-S_{ij}$[24]. The average genetic distances were obtained with RAPD istance 2.00 package. Phylogenetic relationships between investigated samples of Chinese mussels in the genus *Anodonta* were constructed by a neighborjoining (NJ) method and an unweighted pair-group method with arithmetic mean (UPGMA) using NEIGHBOR implemented in PHYLIP Version 3.57[25].

2 Results

2.1 RAPD patterns and percent of polymorphic loci

A total of 925 RAPD fragments ranging from 300 bp to 2500 bp in length were consistently generated. Parts of RAPD patterns for *A. arcaeformis*, *A. a. flavotincta* and *A. fluminea* were showed in figures 1 to 2, and parts of RAPD patterns for *A. w. woodiana* and *A. w. pacifica* in figures 3 to 4.

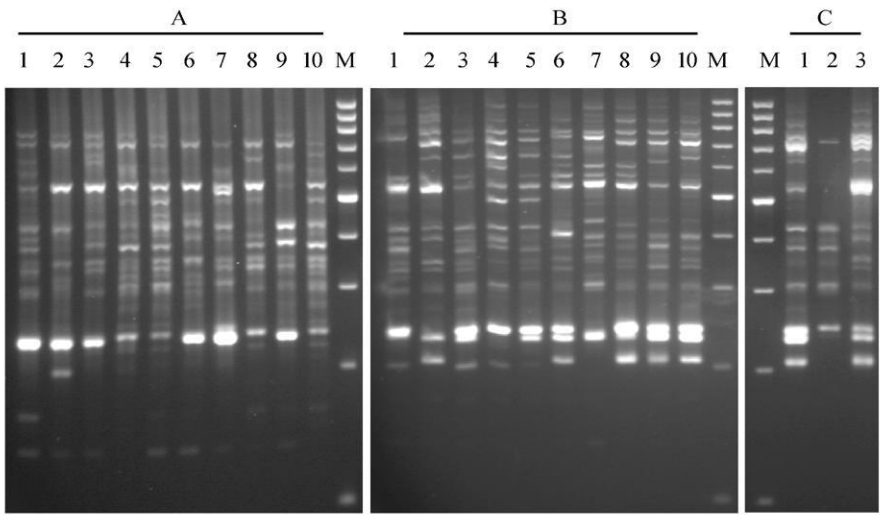

Fig. 1 RAPD patterns for *A. arcaeformis*, *A. a. flavotincta* and *A. fluminea* using the primer OPI04

A: *A. arcaeformis*; **B**: *A. a. flavotincta*; **C**: *A. fluminea*; **M**: 200bp DNA Ladder (200~2000bp); 1~10: No. of individuals

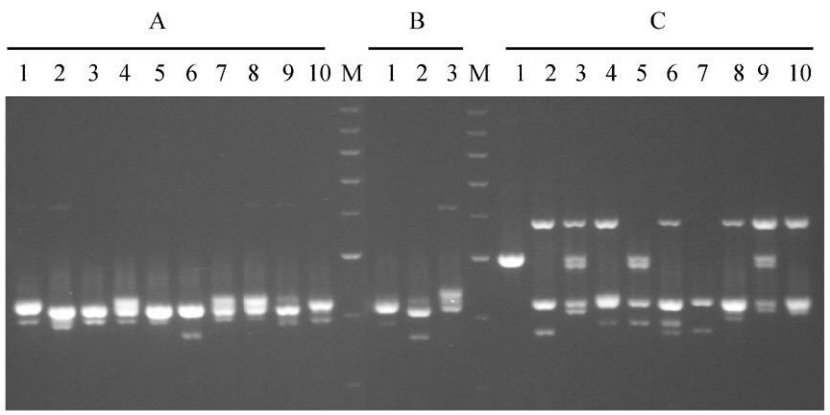

Fig. 2 RAPD patterns for *A. a. flavotincta*, *A. fluminea* and *A. arcaeformi* using the primer OPI11

A: *A. a. flavotincta*; **B**: *A. fluminea*; **C**: *A. arcaeformis*; **M**: 200bp DNA Ladder (200~2000bp); 1~10: No. of individuals

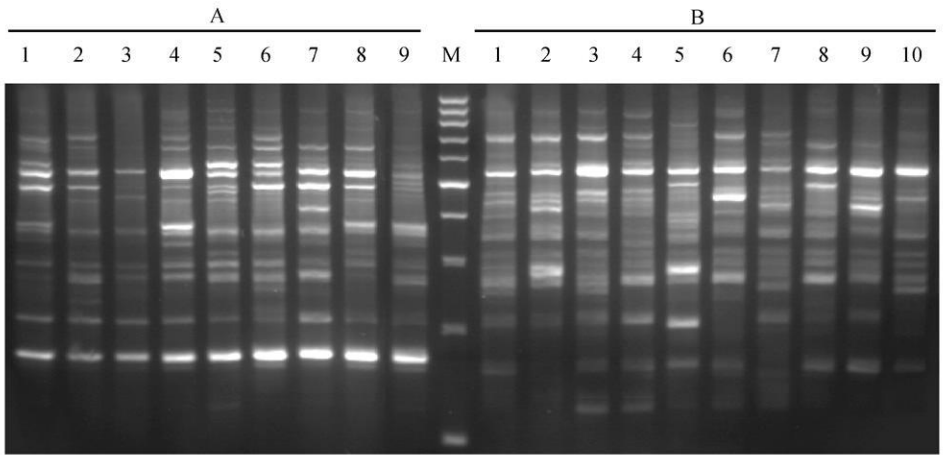

Fig. 3 RAPD patterns for *A. w. woodiana* and *A. w. pacifica* using the primer OPI04

A: *A. w. woodiana*; **B**: *A. w. pacifica*; **M**: 200bp DNA Ladder (200~2000bp); 1~10: No. of individuals

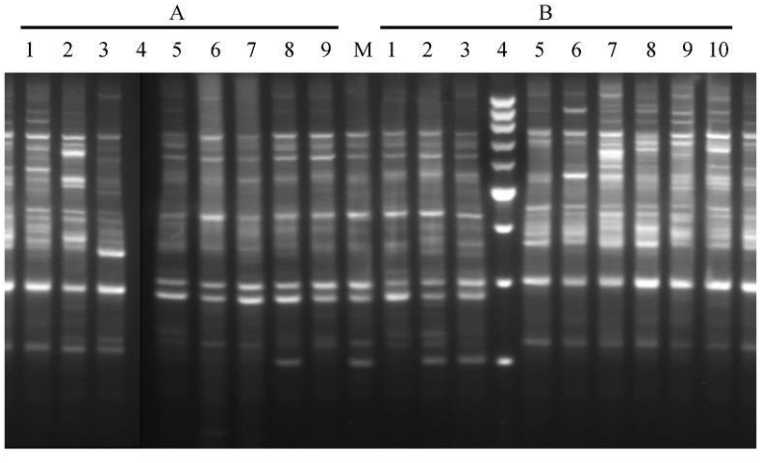

Fig. 4 RAPD patterns for *A. w. woodiana* and *A. w. pacifica* using the primer OPP16

A: *A. w. woodiana*; **B**: *A. w. pacifica*; **M**: 200bp DNA Ladder (200~2000bp); 1~10: No. of individuals

The number of RAPD-amplified bands and percentage of polymorphic loci generated with various primers were indicated in Tab. 3. In total, 16 random primers yielded a total of 196, 171, 189, 183 and 186 reproducible bands for *A. a. flavotincta*, *A. fluminea*, *A. arcaeformis*, *A. w. woodiana* and *A. w. pacifica*, respectively. On average, the percents of polymorphic loci of five species were 62.8%, 34.5%, 61.9%, 55.7% and 59.7%, respectively. The number of RAPD bands scored per primer ranged from 5 to 17, 5 to 15, 6 to 16, 7 to 17 and 7 to 19, respectively for five species.

Tab. 3 Number of RAPD-amplified bands and percentage of polymorphic loci for populations of five species in genus *Anodonta*

Primers	Species														
	A. a. flavotincta			*A. fluminea*			*A. arcaeformis*			*A. w. woodiana*			*A. w. pacifica*		
	B	*P*	%*P*	*B*	*P*	%*P*	*B*	*P*	%*P*	*B*	*P*	%*P*	*B*	*P*	%*P*
OPI04	17	14	82.4	14	6	42.9	13	8	61.5	17	7	41.2	19	15	78.9
OPI10	8	2	25.0	8	3	37.5	6	2	33.3	7	3	42.9	10	5	50.0
OPI11	5	4	80.0	5	3	60.0	6	5	83.3	13	5	38.5	13	7	53.8
OPI14	14	7	50.0	11	5	45.5	16	12	75.0	9	3	33.3	8	5	62.5
OPI19	14	9	64.3	11	6	54.5	13	7	53.8	10	7	70.0	10	6	60.0

Continued

Primers	Species														
	A. a. flavotincta			*A. fluminea*			*A. arcaeformis*			*A. w. woodiana*			*A. w. pacifica*		
	B	*P*	%*P*	*B*	*P*	%*P*	*B*	*P*	%*P*	*B*	*P*	%*P*	*B*	*P*	%*P*
OPP02	8	3	37.5	7	2	28.6	8	5	62.5	11	4	36.4	11	6	54.5
OPP06	14	10	71.4	10	3	30.0	15	11	73.3	13	13	100.0	10	5	50.0
OPP08	16	14	87.5	15	5	33.3	14	9	64.3	7	4	57.1	7	4	57.1
OPP10	13	8	61.5	12	5	41.7	12	8	66.7	8	5	62.5	10	7	70.0
OPP16	12	8	66.7	12	3	25.0	12	7	58.3	10	3	30.0	13	6	46.2
OPG03	11	5	45.5	10	2	20.0	10	6	60.0	10	8	80.0	12	8	66.7
OPG15	13	8	61.5	12	5	41.7	12	9	75.0	15	11	73.3	11	7	63.6
OPG17	15	9	60.0	13	5	38.5	12	6	50.0	13	7	53.8	14	8	57.1
OPM02	10	6	60.0	9	1	11.1	12	9	75.0	12	7	58.3	11	6	54.5
OPM04	14	10	71.4	10	1	10.0	15	7	46.7	13	8	61.5	12	9	75.0
OPM12	12	6	50.0	12	4	33.3	13	6	46.2	15	7	46.7	15	7	46.7
Total	196	123	62.8	171	59	34.5	189	117	61.9	183	102	55.7	186	111	59.7

1) *B*: Number of amplified bands; 2) *P*: Number of polymorphic loci; 3) %*P*: Percent of polymorphic loci

2.2 Shannon's index of phenotypic diversity (H_0)

Tab.4 presents the estimates of Shannon's phenotypic diversity for populations of five species for RAPD variation. The mean RAPD H_0 for *A. a. flavotincta*, *A. fluminea*, *A. arcaeformis*, *A. w. woodiana* and *A. w. pacifica* were 0.3472, 0.2021, 0.3552, 0.3223 and 0.3474, respectively, with *A. arcaeformis* having the higher H_0 (0.3552) and *A. fluminea* the lower (0.2021). RAPD H_0 also varied among primers within population.

Tab. 4 Shannon's phenotypic diversity index (H_0) of RAPDs for populations of five species in genus *Anodonta*

Primers	Species				
	A. a. flavotincta	*A. fluminea*	*A. arcaeformis*	*A. w. woodiana*	*A. w. pacifica*
OPI04	0.5188	0.2919	0.3955	0.1870	0.4603
OPI10	0.1602	0.2556	0.2078	0.2034	0.2709
OPI11	0.4342	0.3267	0.4527	0.2341	0.3504
OPI14	0.3054	0.2539	0.4602	0.1939	0.4139
OPI19	0.3061	0.2787	0.2910	0.4272	0.3432
OPP02	0.2306	0.1944	0.3484	0.2105	0.2649
OPP06	0.3510	0.1634	0.4270	0.5804	0.2858
OPP08	0.5111	0.1998	0.3797	0.3780	0.3282
OPP10	0.2669	0.2156	0.3427	0.3004	0.4317
OPP16	0.4354	0.1532	0.3440	0.1895	0.2513
OPG03	0.2570	0.1157	0.2946	0.5008	0.4198
OPG15	0.3620	0.2328	0.4596	0.4795	0.3898
OPG17	0.2999	0.2148	0.3310	0.3207	0.3230
OPM02	0.3669	0.0757	0.4262	0.3246	0.2848
OPM04	0.4238	0.0682	0.2333	0.3429	0.4733
OPM12	0.3259	0.1931	0.2890	0.2837	0.2668
Sum	5.5549	3.2332	5.6827	5.1566	5.5580
Mean	0.3472	0.2021	0.3552	0.3223	0.3474
SD	0.09914	0.07379	0.07895	0.12289	0.07495

2.3 Genetic distances and phylogenetic relationships

The average genetic distances and similarity coefficients between individuals within populations (Tab.5) and the average genetic distances between different populations of five species (Tab.6) were calculated from RAPDs. The values of average similarity coefficients and genetic distances within populations ranged from 0.8266 to 0.8614 and from 0.1386 to 0.1713, respectively. The average genetic distance within *A. fluminea* (0.1386) was lower than that within other species, and that of *A. arcaeformis* (0.1734) was the largest. The genetic distance between *A. a. flavotincta* and *A. fluminea* was the lowest (0.1774), but there were no significant differences among the average genetic distances (D) within *A. a. flavotincta* and *A. fluminea*, and the genetic distance between these two populations as revealed by ANOVA ($P>0.05$).

Phylogenetic relationships among populations of five species in the genus *Anodonta* are shown in Fig.5, and were analogous in the NJ and UPGMA trees constructed from genetic distances.

Tab.5 Average genetic distance and average similarity coefficient within populations of five species

Species	Average genetic distance ($D \pm SD$)	Average similarity coefficient ($S \pm SD$)
A. a. flavotincta	0.1515±0.01974	0.8485±0.01974
A. fluminea	0.1386±0.02452	0.8614±0.02452
A. arcaeformis	0.1734±0.02852	0.8266±0.02852
A. w. woodiana	0.1464±0.01722	0.8536±0.01722
A. w. pacifica	0.1483±0.02294	0.8517±0.02294

Tab.6 Genetic distance between different populations of five species

Species	Aaa	Af	Aa	Aww	Awp
A. a. flavotincta (Aaa)	0.0000				
A. fluminea (Af)	0.1774	0.0000			
A. arcaeformis (Aa)	0.2632	0.2758	0.0000		
A. w. woodiana (Aww)	0.5665	0.5719	0.5735	0.0000	
A. w. pacifica (Awp)	0.5778	0.5692	0.5820	0.3168	0.0000

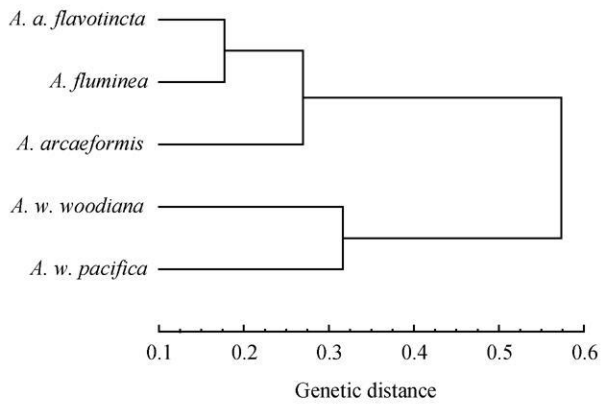

Fig.5 A UPGMA dendrogram based on genetic distance, showing the relationships among five Chinese *Anodonta* taxa

3 Discussion

The percentage of polymorphic loci and the genetic distance between individuals were usually used as indices to measure the genetic diversity of populations. The results of the RAPD analysis for five *Anodonta* species showed that the percentage of polymorphic loci of RAPD-amplified bands for different populations ranged from 34.5% to 62.8%, the average Shannon's phenotypic diversity within populations from 0.2021 to 0.3552, and the average intra-population genetic distance from 0.1386 to 0.1713. The genetic diversity of five *Anodonta* species was consistently measured by these three indices. The lower genetic diversity of *A. fluminea* might be due to its smaller specimen size. Compared with the same indices of two marine bivalves based on RAPD analysis, the average Shannon's phenotypic diversities of four populations (0.3223—0.3552) of five *Anodonta* species, except for *A. fluminea*, were higher than those of three populations of *Pinctada martensii* (0.174—0.266)[18], and the average intra-population genetic distances of these five species were between those of three *P. martensii* populations (0.312—0.358)[18] and those of four *Crassostrea talienwhanensis* populations (0.0755—0.0924)[17]. So the data of this study could be used as relative indices to measure the genetic diversity of these five *Anodonta* species, and as controls to monitor its change further.

Due to the greater morphological variation of shell in the genus *Anodonta*, there are some disputes in the aspects of species classification. Heude[9] recorded 51 *Anodonta* species in China, and Hass[26, 27] thought that there were only two species (subspecies) in China: *A. w. woodiana* and *A. arcaeformis*. Consequently, the Chinese *Anodonta* genus was classified and arranged again by Zhang et al.[6] and Liu et al.[11], with ten species (subspecies) being retained, and *A. pacifica* being regarded as a subspecies of *A. w. woodiana*, namely *A. w. pacifica*. In order to study the genetic relationships of three oysters *C. talienwhanensis*, *Alectryonella plicatula* and *Crassostrea gigas*, Liu et al.[17] calculated the genetic distances among these three species based on RAPD analysis (0.333—0.400) and compared them with the lower limit of distance between species (0.2)[28], and considered them as three sister species belonging to the genus *Crassostrea*. According to the results of this study, the genetic distance between *A. w. pacifica* and *A. w. woodiana* was apparently greater than the lower limit of distance between species, so they can be considered as two sister species of the genus *Anodonta* at the genetic angle. With regard to shapes, the adult shell of two species presents obvious differences in the length of hinge, ratio of shell length and shell height, and shape of posterodorsal margin[11]. However, since there is no apparent geographic isolation in their distributions, it seems advisable to recover the species status of *A. w. pacifica* and its specific name: *A. pacifica*.

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