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Genetic Diversity Variations of Soft-Shelled Turtle (*Pelodiscus sinensis*) Inferred from Microsatellite Approaches

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Key words
***Pelodiscus sinensis*, Microsatellite markers, Genetic diversity**

ABSTRACT

We examined the genetic diversity and genetic relationship of Yangtze River population with spotted (YS) and without spotted (YWS) population and five representative breeding populations, consisting of Huaihe (HH), Yellow River (YR), Taiwan (TW), Wubie (WB) and Japanese (JP) soft-shell turtle (*Pelodiscus sinensis*) in mainland China. The microsatellite markers were used to analyze the genetic diversity of seven populations of *Pelodiscus sinensis*. The results showed that the 10 microsatellite markers had high polymorphism, with average alleles number (N_a), expected heterozygosity (H_e), polymorphic information content (*PIC*) and Shannon information index (*I*) being equal to 14.3, 0.71, 0.68 and 1.79, respectively. The *He* of the seven populations was between 0.4768 and 0.7556, and the *PIC* was between 0.4363 and 0.6864, indicating that there were differences in genetic diversity among all populations. The level of genetic diversity was YWS> WB > TW > YS> YR > HH > JP. The value of genetic differentiation index (F_{st}) ranged from 0.00013 to 0.32091, indicating that all populations had different degrees of differentiation. The F_{st} values of these 10 microsatellite markers were all greater than 0.05, with an average value of 0.1297, which was consistent with the genetic differentiation among the populations. Molecular variation analysis (AMOVA) showed that inter-population variation accounted for 11.47%, inter-individual variation within a population accounted for 14.3% and intra-individual genetic variation accounted for 74.22% of total variation. The phylogenetic tree showed that the YS, YWS and WB populations were clustered together, TW and JP populations were clustered together, and HH and YR were clustered together, indicating that the YS, YWS and WB populations were closely related. The genetic structure analysis showed that all populations were divided into three theoretical populations. In addition, the results of genetic structure and phylogenetic analysis were consistent, indicating that YS, YWS and WB populations, HH and YR populations, and JP and TW populations had a close genetic relationship. The molecular markers are considered the effective tools for determining genetic diversity and population differentiation of *P. sinensis*.

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INTRODUCTION

The Chinese soft-shelled turtle is commonly known as soft-shelled turtle or tortoise and belongs to the Class Reptilia, order Testudine, family Trionychidae and genus *Pelodiscus*. It is an important aquaculture species in many countries (Zhang *et al*., 2016). With the improvement of people's living standards and the intake of a healthy diet, Chinese Soft-shelled turtles are widely consumed because of their delicious taste and high nutritional and medicinal

values, which promotes the rapid development of its breeding industry. Chinese soft-shelled turtles are mainly distributed in the vast areas of China except Xinjiang, Tibet and Qinghai (Yang *et al*., 2011). Due to climate differences, Soft-shelled turtles in different regions have variations in their shape and growth performance, forming different geographical populations. Anhui province is located in the Yangtze River and Huaihe River basins. Some indigenous populations of soft-shelled turtles have been cultured in the Huaihe River, Yangtze River and other water systems in the Anhui province. For instance, there are Chinese soft-shelled turtles on both sides of the Huaihe River (Liang *et al*., 2017). There is a unique group in the Yangtze River Basin in Anhui Province, commonly known as "Plum Blossom Soft-shelled Turtle" and "Yangtze River Soft-shelled Turtle". At the same time, the breeding of Chinese soft-shelled turtles of this population has been included in the major scientific and technological projects of Anhui Province. However, the research on the status of the germplasm resources of this population is beneficial to the protection, excavation, development and utilization of the germplasm resources of soft-shelled turtles, and lays the foundation for the selection and breeding of new populations of high-quality and improved varieties. Molecular markers are germplasm resource identification technology that directly reflect genetic variation at the DNA level and are not restricted by the environment and gene expression. The molecular marker is the best identification source and the advantages of abundant quantity and genetic stability (Huang *et al*., 2005).

Currently, for the genetic diversity, molecular marker techniques used are mainly random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple repeat sequence (SSR) (Kong *et al*., 2012; Ma and Pan, 2011). Among them, SSR molecular markers are widely applied in the genetic diversity of aquatic organisms due to their characteristics of simple operation, high polymorphism and good repeatability. Molecular markers are suitable for the genetic diversity research among the biological species with close relationship research (Liu and Cordes, 2004; Chistiakov *et al*., 2006; Kong *et al*., 2012; Ma and Pan, 2011; Sun *et al*., 2015).

There have been some reports on SSR molecular markers in the study of the genetic diversity of soft-shelled turtles. For instance, Liu *et al*. (2012) used microsatellite molecular markers to analyze the genetic diversity of Dongting, Yellow River, Huangsha, Japan, and Dongting-Yellow River hybrid progenies populations of Chinese soft-shelled turtle. For diversity analysis, Wang *et al*. (2020) used microsatellite markers to analyze the genetic diversity of the Huaihe population of soft-shelled turtles. There is no related report on the genetic diversity and genetic structure analysis of the Yangtze River soft-shelled turtle.

In this study, SSR molecular markers were used to analyze the population of the Yangtze River soft-shelled turtle and Japanese soft-shelled turtle, Taiwan soft-shelled turtle, Yellow River soft-shelled turtle, and black soft-shelled turtle. Among numerous varieties of molecular markers, microsatellite markers have been widely applied in the study of animal genetic diversity because of their co-dominant, widespread distribution, high polymorphism, easy to recognition etc. The comparative analysis of the genetic background of the population was carried out in order to provide a reference for the in-depth development of high-quality Yangtze River soft-shelled turtle breeding. The genetic diversity and population variation of seven geographic populations of *P. sinensis* were investigated using 10 microsatellite loci in this study.

MATERIALS AND METHODS

Experimental materials

The seven Chinese Soft-shelled turtle populations (a total of 99 samples) required for this experiment were provided by three Chinese soft-shelled turtle breeding companies in Anhui Province. Yangtze River Chinese soft-shelled turtle with spot (YS) and Yangtze River population without spot (YWS) populations were collected from the Ma'anshan Chunsheng Ecological Agriculture Co., Ltd. Similarly, the Japanese population of Chinese soft-shelled turtle (JP) was also collected from Ma'anshan Chunsheng Ecological Agriculture Co., Ltd.; population of the Huaihe Chinese soft-shelled turtle (HH), Yellow River population (YR) and Taiwan population Chinese soft-shelled turtle (TW) were collected from the Anhui Xijia Agricultural Development Co., Ltd.; Wubie population (WB) of Chinese soft-shelled turtle was collected from Anhui Heishen Ecological Agriculture Co., Ltd. Fifteen healthy soft-shelled turtles were randomly selected from each group, and the individual body weight ranged from 250-500 g. About 1 g of leg muscle tissue was taken from each group, placed in liquid nitrogen for 10 minutes, and stored at -80 °C for later use.

Microsatellite primer

The 10 pairs of microsatellite marker primers were obtained from the literature (Zhang *et al*., 2011, 2013). According to the difference in primer amplification size, the upstream 5′ ends of the primers were labeled with fluorescent dyes FAM (blue) and HEX (green), respectively. All the primers were synthesized by Shanghai Sangon Bioengineering Co., Ltd. The fluorescent labeling primers are listed in Table I.

Table I. Characteristics of the 10 microsatellite loci identified in this study.

Locus	Repeat sequence	(5'-3') Primer sequence (5'-3')	Annealing temperature (°C)
P-04	(CA)9	F: FAM-CATGGTCTAGGCAGTGCTGA R: GAGAGAACAGCCTCGCTGA	60
P-05	(TG)9	F: FAM-GCCACGTACTCGTGGTTCAT R: GGAGGCTGTTTTCACGACTG	58
P-06	(AC)8	F: FAM-GCACCAGGAAAGAGTCAAGAA R: CAGCCCGAGAACATCAGAAT	58
P-09	(AC)16	F: HEX-CAACCCAACTCTGCAGACAC R: GAATTGCATGGAAGGCAGAT	62
P-10	(GT)9	F: HEX-CAACCCAACTCTGCAGACAC R: GAATTGCATGGAAGGCAGAT	62
P-12	(CA)10	F: FAM-ACGCAGGACCAAGAGTGAGG R: TGTGCCACTCCCCGTATTGT	58
PS-04	(GT)10	F: HEX-AGTGAACCTTGCACATCCCAG R: TCCAGTGAAGGTTCCAGACA	58
PS-11	(AC)5(AG)11	F: HEX-ACCAGTCAGGAAAGTTGACAC R: GCCAGTTTACCAAGAGATGGA	62
HLJZHB076	(CA)19	F: HEX-CACCGATTTCAGCACTAGCA R: GGAGAAAGCTGGTGCCTATG	60
Pb07	(AC)14(CT)19	F: FAM-AAGAGCAACTACACGATT R: CTTTCCAGAGCCTTTTAC	55

Genomic DNA extraction of Chinese soft-shelled turtle

According to the instructions of Ezup Column Animal Tissue Genomic DNA Drawer Kit (B518251) OF Shanghai Sango Bioengineering Co., Ltd., DNA was extracted from the soft-shelled turtle's muscle tissue. The DNA quality and concentration were detected by 1% agarose gel electrophoresis and Merrill Hengtong UV spectrometer (SMA4000), and the DNA concentration of each sample was adjusted to 50 ng/μL, and stored at -20 °C.

PCR reaction conditions and product detection

PCR reaction system: 2.5 μL of 10×buffer (containing $MgCl_2$), 1 μL of dNTP (10 μmol/L), 1 μL of forward and reverse primers (10 μmol/L), 50 ng of genomic DNA, 2.5 μL of Taq enzyme, and supplemented with ddH_2O to final volume 25 μL.

PCR reaction program: pre-denaturation at 95 °C, for 3 min; denaturation at 94 °C for 30 sec; annealing at 60 °C for 30 sec; extension at 72 °C for cycle 10 times; denaturation at 94 °C for 30 sec; annealing at 55 °C for 30 s; extension at 72 °C for 35 cycles; and post-extension at 72 °C for 8 min.

Entrusted Shanghai Sangon to complete short tandem repeat (short tandem repeat, STR) genotyping. The 373XL sequencing analyzer (ABI Company, USA) was used for STR sequence analysis, and the genotype of each individual locus was judged according to the difference in the molecular weight of the amplified bands.

Data statistics and analysis

For genotype analysis, each marker in each individual by STR typing, sort alphabetically according to the order of bands from small to large. The Popgene Version 1.32 software was used to calculate the number of alleles (Na), effective number of alleles (Ne), Shannon index (I), observed heterozygosity (Ho), expected heterozygosity (He), genetic distance between populations (Da), genetic similarity (S). Polymorphism information content (PIC) was calculated by Cervus 3.0 software. Arlequin 3.1 software was used to calculate the genetic differentiation index (FST) between populations and population molecular variance analysis (AMOVA). We used MEGA X and Structure 2.3.1 software (Evanno *et al*., 2005) to construct the phylogenetic tree and the population genetic structure, respectively. We used the online website http://clumpak.tau.ac.il/ to calculate the best K value (theoretical population size) analysis and population genetic structure map construction.

RESULTS

Microsatellite locus amplification results

Table II showed the analyzed results of 10

microsatellite markers on 7 soft-shelled turtle populations. The results showed that a total of 142 alleles were detected and the number of alleles ranging from 5 to 25, among which the number of alleles at the P-05 site was the least, the number of alleles at the HLJZHB076 site was the largest, and the average allele number was 14. The observed heterozygosity Ho value ranged from 0.3232 to 0.8687, with an average value of 0.54. The effective allele number Ne value ranged from 1.61 to 11.36, with an average value of 5.37. The expected heterozygosity He value ranged from 0.3808 to 0.9120, with an average value of 0.71. The average Shannon information index I value ranged from 0.7162 to 2.7336, with an average value of 1.79. The polymorphic information content PIC values of the 10 microsatellite loci ranged from 0.3371 to 0.9054, with an average value of 0.68. Among them, the PIC values of 8 microsatellite loci in the 7 geographical populations of Chinese soft-shelled turtles were greater than 0.5, which were highly polymorphic loci. The PIC values of the two microsatellite loci (P-05 and P-06) were between 0.25 and 0.5, which were moderately polymorphic loci. The PIC results indicated that the selected loci could be used for genetic diversity analysis of soft-shelled turtle populations.

The results of the F test showed that among the 10 microsatellite loci, except P-09 locus which was negative, the inbreeding coefficients (Fis) of other loci were all positive, indicating a high degree of inbreeding. The average genetic differentiating index (Fst) of all loci is 0.1297, two loci (P-12 and PS-04) are more genetically differentiated (Fst>0.15), and the Fst values of other loci are between 0.05 and 0.15, indicating that the degree of genetic differentiation is moderate.

Population genetic diversity

Table III shows the parameters related to the genetic diversity of seven soft-shelled turtle populations. The population with the largest Na, Ne and I values was the Yangtze River soft-shelled turtle (with spots), the population with the smallest Na value was the yellow River soft-shelled turtle population, and the population with the smallest Ne and I values was the Japanese soft-shelled turtle population. The maximum value of Ho and He was for the Taiwan soft-shelled turtle population, and the minimum value was for the Japanese soft-shelled turtle population. The maximum value of PIC was the Yangtze soft-shelled turtle (without spots), and the minimum value was the Japanese soft-shelled turtle. The PIC values of Yangtze soft-shelled turtle (with spots) populations were all greater than 0.5, indicating that the genetic polymorphisms of Taiwan soft-shelled turtle, black soft-shelled turtle and Yangtze soft-shelled turtle were higher than those of Huaihe soft-shelled turtle, Yellow River soft-shelled turtle and Japanese soft-shelled turtle.

Population differentiation and genetic distance

The Fst values among the seven distinct Chinese soft-shelled turtle populations are delineated in Table IV, ranging from 0.00013 to 0.32091. In accordance with Wright's guidance, the most minimal level of genetic differentiation was observed between the HH and YR populations (Fst = 0.00013). Notably, there was a paucity of genetic differentiation among the YS and YWS, YS and TW, and YS and WB populations, with Fst values consistently below 0.05. Conversely, the population pairs demonstrating the most substantial degree of genetic

Table II. Gene flow and genetic diversity parameters among 10 microsatellite loci of *Pelodiscus sinensis.*

Site Locus	N_a	N_e	H_o	H_e	I	PIC	F_{IS}	F_{st}	N_m
P-04	11	3.19	0.3737	0.6870	1.5780	0.6583	0.3574	0.1327	1.6345
P-05	5	1.85	0.3939	0.4596	0.8447	0.4063	0.0231	0.1396	1.5411
P-06	6	1.61	0.3030	0.3808	0.7162	0.3371	0.1822	0.0644	3.6317
P-09	19	11.36	0.8687	0.9120	2.6056	0.9054	-0.0131	0.0587	4.0100
P-10	15	10.15	0.8182	0.9014	2.4530	0.8932	0.0189	0.0702	3.3108
P-12	8	2.72	0.3232	0.6328	1.2615	0.5822	0.3011	0.2278	0.8475
PS-04	12	3.44	0.4949	0.7097	1.5485	0.6653	0.0032	0.2841	0.6298
PS-11	20	5.47	0.5758	0.8172	2.1779	0.8003	0.1925	0.1259	1.7362
HLJZHB076	25	10.78	0.8021	0.9072	2.7336	0.9008	0.0135	0.1126	1.9703
Pb07	22	3.16	0.4545	0.6835	1.9669	0.6752	0.2365	0.1056	2.1164
Mean	14.30	5.37	0.54	0.71	1.79	0.68	0.1188	0.1297	1.6777

N_a, Observed number of alleles; N_e, Effective number of alleles; *I*, Shannon's Information index; H_o, Observed heterozygosity; H_e, Expected heterozygosity; *PIC*, Polymorphic information content; F_{is}, Within-population inbreeding coefficient; F_{st}, Fixation index; N_m, Gene flow.

Table III. Genetic diversity of seven populations of *Pelodiscus sinensis.*

Population	Average N_a	Average N_e	Average H_o	Average H_e	Average I	Average PIC
Huaihe strain	5.3000	2.9065	0.5100	0.5417	1.0828	0.4791
Yellow River strain	4.9000	2.8755	0.5067	0.5577	1.0784	0.4930
Japanese strain	5.1000	2.5556	0.4667	0.4768	0.9773	0.4363
Taiwan strain	6.1000	4.0253	0.6556	0.7556	1.4932	0.6717
Wubie strain	7.5000	4.5953	0.5133	0.7317	1.5945	0.6748
Yangtze River strain without spot	8.5000	5.1834	0.5667	0.7354	1.6734	0.6864
Yangtze River strain with spot	9.1000	6.0862	0.5933	0.7154	1.7000	0.6707

For abbreviations see Table II.

Table IV. Pairwise genetic differentiation Fst-statistics estimates among seven populations of *Pelodiscus sinensis.*

Strains	Huaihe strain	Yellow River strain	Japanese strain	Taiwan strain	Wubie strain	Yangtze River strain without spot	Yangtze River strain with spot
Huaihe strain	0.00000						
Yellow River strain	0.00013	0.00000					
Japanese strain	0.32091*	0.28787*	0.00000				
Taiwan strain	0.15431*	0.13007	0.11996*	0.00000			
Wubie strain	0.14100*	0.12601*	0.20143*	0.07534*	0.00000		
Yangtze River strain without spot	0.14675*	0.12405*	0.08522*	0.02896	0.03928*	0.00000	
Yangtze River strain with spot	0.13191*	0.10232*	0.14327*	0.03093*	0.03051*	0.00250	0.00000

Note: * means significant difference ($p<0.05$).

differentiation (Fst > 0.25) were the JP and HH populations, as well as the JP and YR populations. Moreover, notable genetic divergence (0.15 < Fst < 0.25) was observed between the HH and TW populations, as well as the JP and WB populations. The remaining population pairs (TW and YR, TW and JP, WB and HH, WB and YR, WB and TW, YWS and HH, YWS and YR, YWS and JP, YS and HH, YS and YR, YS and JP) exhibited intermediate levels of genetic differentiation (0.05 < Fst < 0.15).

Table V. AMOVA analysis of molecular variances of microsatellites among seven populations of *Pelodiscus sinensis.*

Source of variation	Sum of squares	Variance components	Percentage of variation (%)
Among populations	92.61	0.41584	11.47
Among individuals within populations	342.58	0.51887	14.31
Within individuals	266.00	2.69084	74.22
Total variation	701.19	3.62555	

The results of AMOVA analysis (Table V) showed that 11.47% of the total variation was inter-population variation, 14.31% was inter-individual variation within a group, and 74.22% was intra-individual genetic variation. The results showed that the genetic variation of soft-shelled turtles mainly came from among the individuals.

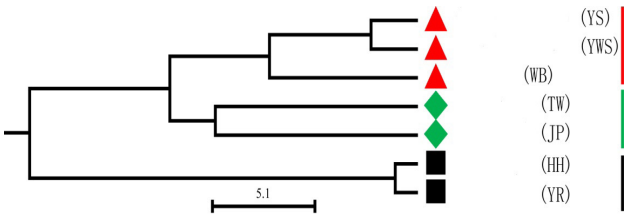

Fig. 1. A UPGMA-Phylogenetic clustering tree constructed on the microsatellite genotypes showing relationships among seven populations of *Pelodiscus sinensis*. The dendrogram is based on Nei's genetic distance derived from allele frequencies of 10 microsatellite markers. Numbers above branches indicate percentage of bootstrap values obtained from 1000 re-sampling cycles.
YS, Yangtze River population with spotted, YWS, Yangtze River population without spotted; Population, WB, Wubie, TW, Taiwan, JP, Japanese, HH, Huaihe, YR, Yellow River.

Table VI. Genetic similarity (above diagonal) and genetic distance (below diagonal) of seven populations of *Pelodiscus sinensis*.

Strains	Huaihe strain	Yellow River strain	Japanese strain	Taiwan strain	Wubie strain	Yangtze River strain without spot	Yangtze River strain with spot
Huaihe strain	****	0.9787	0.5025	0.6810	0.7095	0.6954	0.7336
Yellow River strain	0.0215	****	0.5583	0.7242	0.7347	0.7392	0.7907
Japanese strain	0.6881	0.5829	****	0.8191	0.6308	0.8795	0.7771
Taiwan strain	0.3842	0.3227	0.1995	****	0.7232	0.8567	0.8634
Wubie strain	0.3432	0.3083	0.4607	0.3241	****	0.8492	0.8797
Yangtze River strain without spot	0.3632	0.3022	0.1284	0.1546	0.1635	****	0.9557
Yangtze River strain with spot	0.3098	0.2349	0.2522	0.1468	0.1282	0.0453	****

Table VI shows that the genetic distances among the populations ranged from 0.0215 to 0.6881. The populations with the farthest genetic distance (0.6881) and the lowest genetic similarity (0.5025) were the Huaihe soft-shelled turtle and Japanese soft-shelled turtle.

The populations with the closest genetic distance (0.0215) and the highest genetic similarity (0.9787) were the Huaihe and Yellow River soft-shelled turtle populations. The results of the clustering tree in Figure 1 show that the populations of the Huaihe soft-shelled turtle and the Yellow River soft-shelled turtle are grouped into one branch, the Japanese soft-shelled turtle and the Taiwanese soft-shelled turtle are grouped into one branch, and the black soft-shelled turtle group is grouped into the same group as the Yangtze soft-shelled turtle (with spots) and the Yangtze soft-shelled turtle (without spots).

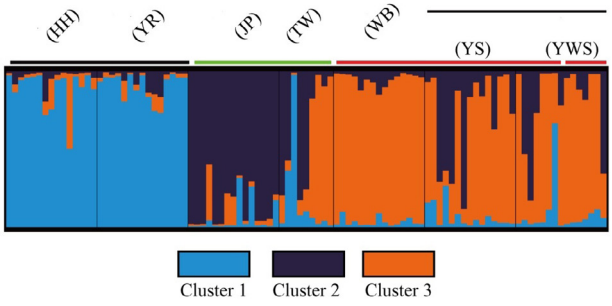

Fig. 2. Genetic structure map for the seven populations of *Pelodiscus sinensis* (K=3).
For abbreviation, see Figure 1.

Population genetic structure

This study adopted the method of Evanno *et al.* (2005), Kopelman *et al.* (2015), and set the length of Burn-in period and K value (theoretical group number) to 100000 and 1-14 respectively, each K value repeated 10 times. When the calculated K value is 3, Delta K is the largest, indicating that all individuals can be divided into 3 theoretical groups (Fig. 2). In can be seen from Figure 2 that the Huaihe and Yellow River soft-shelled turtle populations cluster 1 accounted for the majority; the Japanese soft-shelled turtle population cluster 2 accounted for the majority; The soft-shelled turtle population formed three populations with relatively independent genetic structure, and there was a small degree of mixing phenomenon. The Taiwanese soft-shelled turtle populations, Cluster 2 and Cluster 3, accounted for the majority, showing a great similarity with the Japanese soft-shelled turtle population and the Japanese soft-shelled turtle population, indicating that it had a close genetic relationship with the Japanese soft-shelled turtle population and the black soft-shelled turtle population. The genetic structure of the spotted and non-spotted soft-shelled turtles in the Yangtze River was similar, and the population Cluster 3 accounted for the majority, showing a similar genetic structure to the soft-shelled turtle.

DISCUSSION

Population genetic diversity (PGD)

The PGD not only reflects the survival, adaptability and evolution potential of species, but also serves as the main basis for evaluating the status of biological germplasm resources (Zhou *et al.*, 2015). Heterozygosity and polymorphism information content are the main indicators to measure the genetic diversity of a population. PIC can usually reflect the polymorphism and variation degree of a certain locus, and the content is directly proportional to the variation degree (Shete *et al.*, 2000). Some literature pointed out that when pic>0.5 in the population, it is highly polymorphic; when 0.25<PIC<0.5, it is moderate polymorphism; when PIC<0.25, it is low

polymorphism (Botstein *et al*., 1980).

In this study, the average PIC of the selected microsatellite loci was 0.68 except for two moderately polymorphic loci. The rest populations were highly polymorphic, and indicating that selected 10 microsatellites can be used to assess the genetic diversity and genetic structure of soft-shelled turtles. Among the Chinese soft-shelled turtle populations, the PICs of WB, TW, YWS and YS were examined greater than 0.5, indicated a high degree of polymorphism. Conversely, the HH and YR populations exhibited lower PIC values of 0.4791 and 0.4930, both falling below the 0.5 threshold, yet remaining in proximity to the boundary indicative of a substantial degree of polymorphism. This finding aligns with observations made in the study by Zhang *et al*. (2013). According to findings, the Huaihe soft-shelled turtle and Yellow River soft-shelled turtle populations may have undergone multiple generations of breeding in the farm. The JP population displayed a PIC value of 0.4363, similar to the findings reported in the study by Liu *et al*. (2012) for the JP population, indicative of a moderate level of genetic polymorphism. The results showed that compared with the Japanese soft-shelled turtle population, the 10 microsatellite loci had higher polymorphisms in other Chinese soft-shelled turtle populations, which could provide rich genetic information.

Heterozygosity is an important indicator for evaluating the genetic diversity of a population. Because the observed heterozygosity (Ho) is easily affected by the sample size, the expected heterozygosity (He) is usually used to reflect the genetic diversity of the population. The higher heterozygosity, the richer genetic diversity of the population, the stronger its adaptability to the environment (Qin *et al*., 2013). Studies have shown that when heterozygosity is between 0.5 and 0.8, the population has high genetic diversity (Takezaki and Nei, 1996). The results of this study show that, except for the Japanese soft-shelled turtle population, the expected heterozygosity of other Chinese soft-shelled turtle populations is between 0.5067 and 0.6556. It indicated that except for the Japanese soft-shelled turtle population, the other six Chinese soft-shelled turtle populations had high genetic diversity and good breeding potential. The state of population genetic variation can be judged according to the deviation index d= (Ho-He)/He. When d is close to zero, it means that the genotype distribution is close to a balanced state; when d>0, there are excess heterozygotes; when d <0, the heterozygote is missing. The d values of all soft-shelled turtle populations in this study were less than 0, indicating that there was a loss of heterozygosity in all soft-shelled turtle populations, which may be due to inbreeding in the population during the breeding process, resulting in homozygous populations, resulting in a decrease in heterozygosity. Therefore, in the breeding process of soft-shelled turtles, we should try our best to avoid mating among populations with the same genetic background and expand the breeding range. In addition, Heterozygosity decreased may also be caused by null alleles, because when studying the genetic analysis of mullet populations, blue crab populations and copper fish populations, it was found that null alleles would cause decreased heterozygosity (Shu *et al*., 2011; Wang *et al*., 2015; Hu *et al*., 2017).

Population genetic differentiation

The genetic similarity coefficient is an important indicator of the degree of genetic variation between the populations. The larger the similarity coefficient, the closer the genetic relationship between the populations and the lower the genetic variability (Plotsky *et al*., 1993).

The phylogenetic tree constructed in this study based on the results of microsatellite analysis shows that the phylogenetic relations between the Yangtze River soft-shelled turtle and the black soft-shelled turtle is the closest, followed by the Taiwan soft-shelled turtle and the Japanese soft-shelled turtle, and the farthest genetic relationship with the Huaihe soft-shelled turtle and the Yellow River soft-shelled turtle. Except for the first report of soft-shelled turtles, the genetic relationship results of other soft-shelled turtle populations are basically consistent with previous results (Zhang *et al*., 2011, 2013; Wang *et al*., 2020).

In addition, the genetic differentiation index (Fst) is often used to measure the degree of genetic differentiation among the populations. The Fst values among the seven soft-shelled turtle populations in this study ranged from 0.00013 to 0.32091, indicating that there are different degrees of differentiation among the populations.

Among them, the genetic differentiation level between Huaihe soft-shelled turtle and Yellow River soft-shelled turtle is at a low level and inconsistent with the moderate level of genetic differentiation between two populations as reported by Wang *et al*. (2020), which may be caused by differences between microsatellite loci in the study. In addition, the populations of Yangtze soft-shelled turtles, Taiwan soft-shelled turtles and black soft-shelled turtles were also at a low level of genetic differentiation, indicating that the germplasm resources of these populations were well preserved during the breeding process, resulting in less gene exchange among them. At the same time, the results of molecular analysis of variance showed that most of the genetic variation came from among the individuals.

Population genetic structure

The structure software was an ideal tool for stimulating the genetic composition of individuals without considering

the population sample size (Evanno *et al*., 2005). The software was used to analyze the genetic structure of *Pelteobagrus vachelli* (Zheng *et al*., 2020), *Micropterus salmoides* (Sun *et al*., 2019) and *Procambarus clarkia* (Li *et al*., 2020; Sun *et al*., 2015). The analysis of the genetic structure of soft-shelled turtles with this software had not been reported before.

This study found that all groups were divided into 3 ideal groups through the software analysis. The studied populations were divided into 3 theoretical groups, which is basically consistent with the clustering results, indicating that the 7 soft-shelled turtle populations come from 3 sub-groups. From the composition of the three subgroups, they are divided according to geography or river system. The similar genetic structure between Yangtze River soft-shelled turtle and black soft-shelled turtle and between Huaihe soft-shelled turtle and Yellow River soft-shelled turtle shown that there was a certain gene exchange between two populations. Hence, it may be related to the mutual exchange between parents or seedlings of two populations during the breeding process. In this study, all the soft-shelled turtle populations had better conservation of germplasm resources during the breeding process. However, when compared with other soft-shelled turtle populations, we recognized the Yangtze soft-shelled turtle population had great breeding potential, and further investigations are required. In addition, based on the analysis of microsatellite markers, a wealth of genetic diversity of soft-shelled turtles has been obtained. It also provides an important reference for the selection and breeding of Chinese soft-shelled turtles and the protection of germplasm resources.

CONCLUSION

This study on genetic diversity and population association in seven populations of *P. sinesis* and established that high allelic and genetic diversity were available in these populations. The high genetic diversity could be applied in breeding programs, decreasing the prerequisite of gathering wild turtles for intake. Accordingly, these populations will also contribute to the conservation of wild broods of *P. sinensis*.

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IRB approval

The study was approved by the Animal Experimentation Welfare Ethics Committee of Anhui Academy of Agricultural Sciences (AAAS) (No. AAAS2023-5).

Ethical statement

All the individuals in this research work we handled carefully, we neither harm nor damage to the animal. The study was conducted in accordance with the Declaration of Anhui Academy of Agricultural Sciences, Hefei, China and the protocol was approved by the Ethics Committee of AAAS (No. AAAS2023-5).

Animal welfare statement

In this research work, we all the authors declared that we had not involved any harm or threat to animal.

Conflicts of interest

The authors have declared no conflict of interest.

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