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Development of polymorphic microsatellite markers and the population genetic structure of the half-fin anchovy, *Setipinna taty*

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ABSTRACT. Microsatellite markers for the half-fin anchovy *Setipinna taty* were developed from the enriched $(CA)_{15}$ genomic library, and they were used for the population genetic studies of the half-fin anchovy from Chinese coastal areas. Samples were collected from five localities of the East China Sea and the Yellow Sea. Eleven simple sequence repeat markers were used to assess genetic differentiation in 30 individuals at each locality. As a result, 59 alleles were recorded over all loci with an average of 5.36 alleles per locus. Observed and expected heterozygosities ranged from 0.27 to 0.73 and 0.50 to 0.89, respectively. Analysis of molecular variation indicated that the variation within individuals was high (70.68%), while variations of individuals within and among populations were low (22.47 and 6.85%). The phylogenetic tree showed that these populations could be divided into two clusters: populations of the East China Sea, which came from Ninghai, Xiangshan and Zhoushan, and populations of the Yellow Sea, which were from Yantai and Weihai. It revealed that significant geographic structure existed in this species. All of the results indicated that high genetic diversity existed in the half-fin anchovy from different

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geographic populations. This conclusion was consistent with the classification based on morphological and physiological characteristics.

Key words: Population genetic structure; Genetic diversity; Microsatellite markers; *Setipinna taty*

INTRODUCTION

The half-fin anchovy, *Setipinna taty* (Clupeiformes, Engraulidae), which is a commercially important near-shore species, is widely distributed in the Indian Ocean, the western Atlantic Ocean, and all of the coastal areas of China. Generally, the half-fin anchovy attains an average length of 15 cm and weight of 25 g. It inhabits shallow seas with a depth around 4-5 m. The optimal living temperature ranges from 5° to 28°C. The half-fin anchovy feeds on plankton and crustaceans (Gu, 1990). In the past decades, the yield of *S. taty* has gradually increased, while the yield of traditional commercial fish, such as the yellow croaker, has declined (Xiong et al., 2009). The half-fin anchovy has become an important fishery protein source in China.

Considerable researches about *S. taty* have shown that the resources of half-fin anchovy have been destroyed because of overfishing in the East China Sea (Jiao et al., 2001) and the Yellow Sea (Xu et al., 2003) of China in recent years. As is well known, the half-fin anchovy plays an important role in the food chain. They eat zooplankton and are prey for larger fish (Sheng and Wei, 1992). Once the resource of half-fin anchovy is greatly destroyed, the ecological system would be affected and difficult to restore. Therefore, it is important to protect germplasm resource of half-fin anchovy and further study its genetic diversity.

To date, most of studies on *S. taty* are related to the ecological aspects of the species (Song et al., 2010, 2011), but little is known about its genetic characteristics. The study of genetic diversity can provide specific measures to protect the germplasm resource. Additionally, detailed analyses of the levels and spatial distribution of genetic diversity are vital to develop effective conservation and management for this species (Hedrick and Miller, 1992). Therefore, it is necessary to determine the level of genetic variation within and among populations of *S. taty*.

Development of molecular markers provides the repertoire for assessing genetic diversity at the DNA level in marine organisms (Reif et al., 2003). In particular, simple sequence repeat (SSR) markers are potential tools for large-scale DNA fingerprinting of *S. taty* genotypes because of their high level of polymorphism detected (Smith et al., 1997), automated analysis systems (Mitchell et al., 1997), and high accuracy and repeatability (Heckenberger et al., 2003). Nevertheless, the development and genetic diversity of SSR molecular markers within and among the populations of *S. taty* was not studied. To investigate the genetic characteristics of *S. taty*, we first developed polymorphic microsatellite markers using the fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) method (Zane et al., 2002). Then, we used the markers for the next following research of genetic diversity and population structure.

The objective of our study is to assess the genetic diversity within populations and differentiation between populations of the half-fin anchovy from the East China Sea and the Yellow Sea using microsatellite markers that we developed. This research can make possible to identify genetically distinct populations within the species. In addition, we can analyze the hereditary constitution and use the results of this study to protect the germplasm resources of *S. taty*.

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MATERIAL AND METHODS

Sample collection

Based on geographic origin and ecotype, a total of 150 samples of *S. taty* were collected from five locations that could be divided into two geographic areas: the East China Sea, which contained three populations (Zhoushan, Ninghai and Xiangshan) and the Yellow Sea, which contained the other two populations (Yantai and Weihai) (Figure 1). Five samples per population were used to develop and characterize the SSR markers. Then, 30 wild individuals were selected per population for the genetic diversity and population structure study. The tissue samples were obtained from fin clips of *S. taty* and preserved in 95% ethanol, and finally stored at -20°C.

Figure 1. Sample sites for the half-fin anchovy.

DNA extraction, microsatellite screening and SSR analysis

Total DNA was extracted from 150 individuals using the standard phenol-chloroform method, with some modification. Microsatellite loci enriched from genomic libraries were constructed following the process of FIASCO method. Briefly, genomic DNA was digested using *Mse*I restriction enzyme (NEB), and DNA fragments of approximately 200-1000 bp were purified, and ligated to adapters OligoA (5'-TACTCAGGACTCAT-3') and OligoB (5'-GACGATGAGTCCTGAG-3'), and amplified using *Mse*I-N (5'-GATGAGTCCTGAGTAAN-3'). The amplified products containing microsatellite sequences were captured by hybridizing with $(CA)_{15}$ biotin-labeled probes. The microsatellite

enrichment was separated using streptavidin magnetic beads according to Glenn and Schable's methods (Glenn and Schable, 2005), with minor modifications. Finally, the separated fragments were cloned into the pMD19-T vectors (TaKaRa, Dalian, China) using ultra-competent *Escherichia coli* DH5α (Tiangen, Beijing, China). Positive clones were screened by polymerase chain reaction (PCR) with M13-F/M13-R and sequenced on an ABI 3730 automated sequencer. From the 60 sequenced clones, 54 (90%) sequences contained microsatellites that had no less than seven dinucleotide repeats. A total of 33 pairs of primers were designed using the Primer Premier 5.0 software (Lalitha, 2000).

PCR amplifications were carried out in 15-μL volumes, which contained 10 μL sterilized water, 1.5 μL 10X PCR buffer (containing 1.5 mM Mg^{2+}), 1.2 μL 2.5 mM dNTPs, 0.6 μL forward and reverse primers, 1 μL diluted DNA template, and 0.1 μL 5 U *Taq* DNA polymerase (Tiangen). Thermal cycling conditions were implemented as follows: 94°C for 5 min, following by 30 cycles of 94°C for 40 s, annealing temperature for 30 s, and 72°C for 40 s, followed by 1 cycle of 72°C for 5 min and then holding at 4°C. PCR amplifications were performed on ABI 9700. Amplified products were denatured for 8 min at 96°C and separated on 6% denaturing polyacrylamide (19:1 acrylamide:bis-acrylamide) gels using silver staining. Denatured pBR322 DNA/*Msp*I molecular weight marker (Tiangen) was used as the size standard to the lengths of PCR products to identify alleles.

Data analysis

The number of alleles per locus (N_A), expected heterozygosity (H_E), observed heterozygosity (H_O) and polymorphism information content (PIC) were estimated using the POPGENE software package (Yeh and Yang, 1997). Gene flow (N_m) and Shannon's indices (I) were also calculated by the same software to characterize the genetic diversity and the distribution of the variation. An unbiased test of the exact test statistic was calculated using a Markov chain method (the Markov chain parameters used were 100,000 steps). All results for multiple tests were corrected using Bonferroni's correction (Rice, 1989).

A hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed using ARLEQUIN to calculate genotypic linkage disequilibrium between these loci and estimate how the variation was partitioned among populations. Based on Nei's standard genetic distance (Nei, 1972), a neighbor-joining (NJ) tree was constructed using the FigTree software. The population structure was determined using the Bayesian model-based clustering by implementing the STRUCTURE software (Evanno et al., 2005). Individuals were grouped into a predefined number of K clusters ($2 \leq K \leq 9$), with 100 independent runs for each value (Pritchard and Wen, 2004).

RESULTS

Development and characterization of microsatellite markers

The details of developing and characterizing microsatellite loci and variability measures across 30 individuals of the half-fin anchovy are summarized in Table 1. In total, 11 of 33 loci were successfully amplified and shown to be polymorphic. All 11 sequences containing microsatellite loci were deposited in GeneBank (accession Nos. JF502223-JF502233). N_A for each locus ranged from 2 to 10, with an average of 5.273. H_E and H_O ranged from 0.124 to 0.861 and

0.133 to 0.759, respectively. The remaining 22 loci were monomorphic and failed to amplify. Three loci (Seta-33, Seta-56 and Seta-H79) significantly deviated from the Hardy-Weinberg equilibrium (HWE) in the sampled population after Bonferroni's correction, possibly because of the presence of null alleles. Null alleles were detected in five loci (Seta-33, Seta-56, Seta-79, Seta-H1, and Seta-H79) and stuttering errors were found in locus Seta-56, but no evidence of allelic dropout was found in any of the loci using MICRO-CHECKER (Van Oosterhout et al., 2004). According to pairwise tests, significant linkage disequilibrium was found between Seta-79 and Seta-H23.

Table 1. Details of locus name, primer sequences, repeat sequence, allele size range, number of alleles (N_A), expected and observed heterozygosities (H_E/H_O), Hardy-Weinberg equilibrium (HWE), and GenBank accession numbers of 11 polymorphic microsatellite loci.

Locus	GenBank	Primer sequence (5'-3')	Tm (°C)	Repeat motif	Size range (bp)	N_A	HWE (P)	Null	H_O/H_E
Seta-33	JF502223	F: CTTGAGCGGCTATTGTTT R: AGGGTGCTTGAGTTTATTTC	52	$(AC)_7G(CA)_6$	199-227	10	0.000*	1	0.571/0.832
Seta-56	JF502224	F: AACAGCCTAATTTCAATC R: ATGCCATAGTCTCCTCAG	52	$(TG)_{12}CGTA(TG)_6$	160-190	7	0.000*	1	0.240/0.702
Seta-79	JF502225	F: TAACCCCAGCCCATTG R: GCTAGACCTCCTCCAGTG	52	$(GT)_8\ldots(GT)_{11}$	188-194	2	0.007	1	0.240/0.499
Seta-H1	JF502226	F: ATGACCTGACCTGTAGATTAC R: AGCCAATAAGCACTGAAGAT	60	$(TG)_{13}$	190-216	9	0.005	1	0.567/0.861
Seta-H8	JF502227	F: AGCGATAACATTCTTGACTG R: CAAACAGGACAACTGAAACT	50	$(TG)_{14}$	132-210	7	0.440	0	0.600/0.708
Seta-H23	JF502228	F: AAGATCAACAGAGGCTGAC R: CTCCGCTAATATCCACCAT	50	$(GT)_{11}$	143-149	3	0.169	0	0.321/0.335
Seta-H36	JF502229	F: GTCATCTGCCTGGGTGAT R: CAAGAACTGTGCCCTATGC	58	$(AC)_8$	118-122	2	0.467	0	0.444/0.384
Seta-H38	JF502230	F: TAACCACAGGAGCAGTAC R: CACTCACCAACAGTCACA	58	$(GT)_6\ldots(TG)_9$	120-122	2	0.737	0	0.133/0.124
Seta-H39	JF502231	F: CTTAGATGGCTGGTGGATAT R: AATTGTGTCAGACTTGTCAG	55	$(CA)_9$	233-261	6	0.047	0	0.759/0.807
Seta-H79	JF502232	F: CAGGTGTCACCACATTGT R: CAGGAGTAGAGTAGAGGAGTA	58	$(TG)_{11}\ldots(TG)_6$	156-182	6	0.000*	1	0.241/0.680
Seta-H94	JF502233	F: TCCATCGGCTGTGTAGTC R: TGAGACCCTCTTTCTGACA	55	$(CA)_{25}$	176-184	4	0.018	0	0.400/0.593

*Significant deviation from Hardy-Weinberg equilibrium.

Genetic diversity and population structure

The 11 polymorphic microsatellite loci developed in *S. taty* were used in studies of the genetic variation and population structure. The genetic information for loci is summarized in Table 2. A total of 59 alleles were detected, and the mean numbers of alleles per locus was 5.36. All of the 11 loci detected possessed a high level of polymorphism, with the effective number of alleles per locus (N_E) ranging from 1.99 at Seta-H38 to 8.51 at Seta-H39.

Genetic diversity parameters for each population, based on allelic frequencies, are presented in Table 3. N_E varied from 3.182 to 4.429, with an average of 3.763. H_O ranged from 0.384 to 0.620, with an average of 0.484. H_E was slightly higher than H_O, ranging from 0.600 to 0.730, with a mean value of 0.664. The average *I* was 1.295 at the population level. We did not observe significant departures from HWE at most loci and in most of the populations sampled nor did we find consistent departure patterns across the loci and populations (Table 4). The significant deviations from HWE in the case of 3 loci (Seta-33, Seta-H1 and Seta-H79) in the Yantai population might be because of the presence of null alleles.

Table 2. Relative measurements on expected heterozygosity (H_E), observed heterozygosity (H_O), observed number of alleles (N_A), effective number of alleles (N_E), gene flow (N_m), and polymorphism information content (PIC) among populations of *Setipinna taty.*

Locus	H_O	H_E	F_{ST}	N_A	N_E	N_m	PIC
Seta-H36	0.38	0.64	0.23	3	2.74	0.83	0.5602
Seta-H38	0.27	0.50	0.27	2	1.99	0.66	0.3736
Seta-H39	0.73	0.89	0.05	10	8.51	5.23	0.8690
Seta-H1	0.62	0.88	0.04	10	8.39	5.98	0.8711
Seta-H23	0.38	0.59	0.21	3	2.40	0.96	0.5141
Seta-H94	0.40	0.67	0.06	4	3.04	3.75	0.6085
Seta-33	0.47	0.85	0.04	7	6.40	5.82	0.8238
Seta-79	0.27	0.50	0.01	2	2.00	29.93	0.3744
Seta-H8	0.70	0.77	0.02	5	4.38	17.56	0.7338
Seta-56	0.59	0.80	0.06	6	5.04	4.29	0.7727
Seta-H79	0.52	0.75	0.03	7	3.96	7.56	0.7107
Mean	0.49	0.71	0.08	5.36	4.44	2.81	0.6556

Table 3. Details on effective number of alleles (N_E) per population, observed heterozygosity (H_O), expected heterozygosity (H_E), and Shannon's information index (I) of genetic variation within populations of *Setipinna taty* based on eleven SSR loci.

Population	N_E	H_O	H_E	I
Zhoushan	3.182	0.446	0.628	1.186
Ninghai	3.473	0.384	0.600	1.214
Yantai	4.429	0.620	0.730	1.434
Weihai	4.013	0.552	0.712	1.384
Xiangshan	3.718	0.417	0.648	1.256

Table 4. Inbreeding coefficients (f) and expected heterozygosities (H_E) across the eleven SSR loci in five populations.

Population	Parameter	Seta-H36	Seta-H38	Seta-H39	Seta-H1	Seta-H23	Seta-H94	Seta-33	Seta-79	Seta-H8	Seta-56	Seta-H79
Zhoushan	f	-0.1254	-0.2250	0.0016	0.5330	0.2641	0.6908	0.3727	0.3993	-0.1846	0.2814	0.6433
	H_E	0.2488	0.4082	0.7598	0.8412	0.4853	0.6220	0.7675	0.4994	0.7503	0.6958	0.7009
Ninghai	f	0.8014	0.8383	0.5193	0.1344	0.3412	0.3898	0.6468	0.4570	-0.0225	0.2095	0.1283
	H_E	0.3472	0.2061	0.8609	0.8472	0.1626	0.5673	0.7928	0.4911	0.7606	0.7497	0.6883
Yantai	f	0.0947	0.2457	-0.0119	-0.0902	0.0840	0.2195	0.1994	0.6571	0.0491	0.2562	0.0418
	H_E	0.6628	0.4861	0.8894	0.8561	0.6550	0.7321	0.8476	0.4861	0.7361	0.8067	0.7306
Weihai	f	0.4426	0.0834	-0.0811	0.3792	-0.0415	0.2246	0.4225	0.3993	0.0850	0.1441	0.2771
	H_E	0.6578	0.4728	0.8633	0.8055	0.5761	0.6687	0.8359	0.4994	0.7650	0.7789	0.7839
Xiangshan	f	-0.1309	0.7115	0.2463	0.3891	0.5220	0.3803	0.4897	0.3304	0.4866	0.1959	0.3493
	H_E	0.5240	0.2311	0.8354	0.8769	0.4184	0.5565	0.7984	0.4978	0.7936	0.7551	0.7093

For the five populations, the average PIC per locus ranged from 0.3736 to 0.8711 (Table 2), of which, four loci indicated middle polymorphism ($0.25 < PIC < 0.5$), while the other seven loci were all high polymorphism ($PIC > 0.5$) (Shete et al., 2000). The genetic variations between populations were estimated using the Wright's theory of hierarchical *F*-statistics (F_{ST}) (Wright, 1965). Values of F_{ST} ranged from 0.01 at locus Seta-79 to 0.27 at locus Seta-H38, with an average value of 0.08 (Table 2). This suggested that this species exhibited most of its genetic variation within its population.

Significant genetic heterogeneity among the five populations was indicated by AMOVA (Table 5). The fixation index (F_{ST}) was 0.068. AMOVA suggested that genetic variation mainly occurred within individuals (70.68%), while that among individuals within populations and

among populations was 22.47 and 6.85%, respectively. The genetic distances among the five populations had overt differences, and they ranged from 0.0468 between Yantai and Weihai to 0.3972 between Yantai and Zhoushan (Table 6). On the basis of the inter-population genetic distance, an NJ tree was constructed (Figure 2). The samples from Zhoushan, Ninghai, and Xiangshan locations were segmented into one cluster, while the other two populations were divided into the other cluster. In conclusion, among different populations, the samples not only could be distinguished from each other but also could be easily separated between the two sea areas, the East China Sea and the Yellow Sea.

Table 5. Analysis of molecular variance among and within the five populations.

Source of variation	Variation components	Percentage of variation	F statistic	P
Among populations	0.1124	6.85	0.0685	P < 0.001
Within populations	0.3688	22.47	0.2412	P < 0.001
Within individuals	1.1600	70.68	0.2932	P < 0.001

Table 6. Genetic identity and genetic distance in five populations of *Setipinna taty.*

	Zhoushan	Ninghai	Yantai	Weihai	Xiangshan
Zhoushan		0.6722	0.8172	0.7748	0.7032
Ninghai	0.2018		0.8625	0.8206	0.8011
Yantai	0.3972	0.2218		0.9543	0.8626
Weihai	0.3522	0.1977	0.0468		0.9022
Xiangshan	0.2552	0.1479	0.1484	0.1029	

Below diagonal is the genetic distance and above diagonal is the genetic identity.

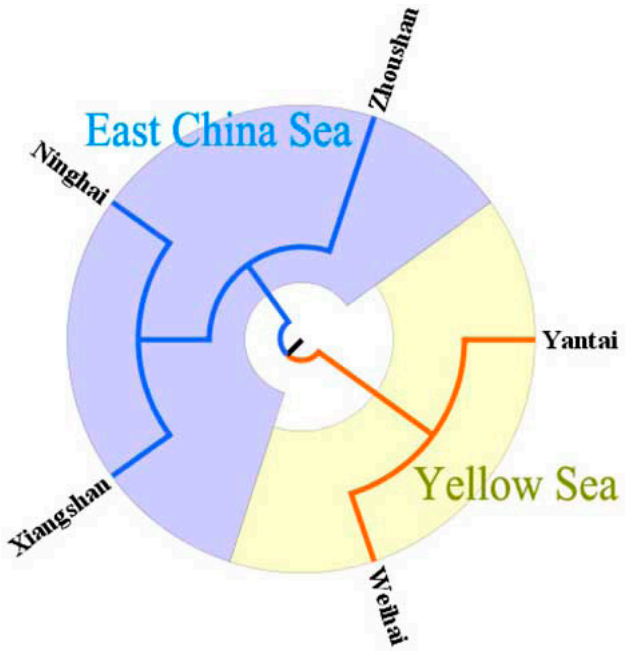

Figure 2. Neighbor-joining tree analysis based on Nei's genetic distance among five populations of *Setipinna taty*.

The results of the structure analysis as K = 2 are shown in Figure 3. We used the K values from two to seven to perform a calculation for the cluster analysis, and we found that when the K value was 2, the five populations were divided into two clusters. This result was consistent with the geographical distribution of the 5 populations: the half-fin anchovy from Zhoushan, Ninghai and Xiangshan belonged to the East China Sea, and those from Yantai and Weihai pertained to the Yellow Sea. Using the method developed by Evanno et al. (2005), we also identified two clusters as the most probable solution.

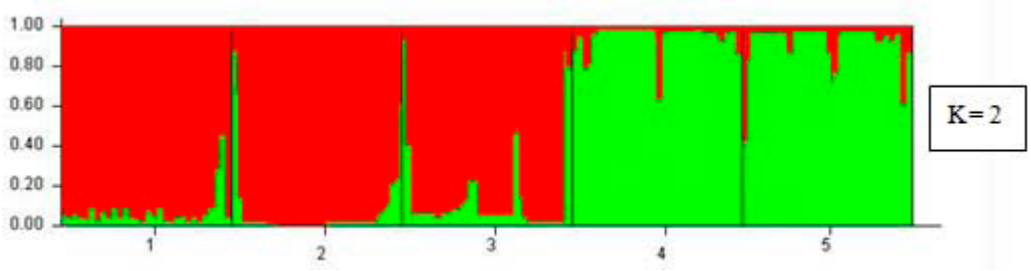

Figure 3. Substructure of five *Setipinna taty* populations from the East China Sea and the Yellow Sea. 1, 2, 3, 4, 5 stand, respectively, for Ninghai, Xiangshan, Zhoushan, Yantai, and Weihai.

DISCUSSION

In this report, we detected that the value of H_E (0.71) at the population level could indicate the genetic diversity; this result suggest that the degree of DNA polymorphism and the genetic diversity in this species were very high. In addition, our study showed that genetic analysis based on allelic and genotypic frequencies according to the heterozygosity excess test was much better than the heterozygosity excess test only. Additionally, the average value of the PIC (0.656) also indicated the high genetic diversity in *S. taty*.

Generally deviation from HWE was due to the heterozygosity excess, which was followed in our study at most of the 11 loci in the five populations. Selection, population mixing, and nonrandom mating could be employed to account for the deviation from HWE. The high average N_E in the population suggested that the genetic variation condition was good, while a population with less genetic differentiation should receive more attention and enhanced protection. The populations of Weihai and Yantai had higher average N_E values than the other three populations. We should pay more attention to the populations of Zhoushan, Ninghai and Xiangshan because the low N_E indicated that they had low evolution ability.

Based on the Nei's genetic distance, the NJ tree analysis indicated that the samples from Zhoushan, Ninghai, and Xiangshan locations were obviously separated from the other two populations (Yantai and Weihai). This showed that the populations of Zhoushan, Ninghai, and Xiangshan, which came from the East China Sea, had their own unique evolutionary pathways that were unlike the other two populations from the Yellow Sea. This result indicated that high genetic diversity existed in two different geographic populations of *S. taty* species.

When the K value was 2, the five populations were divided into two clusters: the three populations of Zhoushan, Ninghai and Xiangshan clustered and were separated from the other two populations, indicating that the East China Sea populations made up a gene pool that was

different from that of the two populations from the Yellow Sea. The graph indicated that the geographic isolation affected the evolution of the population directly, so they had their own gene pool and became two subspecies.

The genetic data obtained from *S. taty* based on microsatellite markers indirectly reflected the adaptive genetic diversity. Low levels of genetic diversity at loci might reflect the losses of genetic diversity that could influence the fitness. Preserving variation might help to inhibit the dissolution of locally well-adapted phenotypes (Godt et al., 1996; Ueno et al., 2005; Jones and Gibson, 2011). It is important to note that a species should be maintained within its ecological community, and more genetic diversity research related to adaptive traits and the knowledge about ecosystem functions and species interactions would be helpful to understand how the species could adapt to changing conditions.

In summary, we first developed microsatellite markers for *S. taty* and used them in population genetic studies of this species. The isolation of the SSR markers provided the material for the analysis of genetic diversity and population structure. All of the indexes in our research indicated that high genetic diversity existed in different geographic populations of *S. taty.* five populations from two geographical regions had high genetic variations, which could be used to the further protection of the resources of this species.

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