



Microsatellite Analysis of the Influence of *Sebastes schlegelii* Restocking on the Genetic Structure of Stocks in Rongcheng Bay

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Abstract

The black rockfish *Sebastes schlegelii*, a valuable commercial fish, is one of the most important fish species for marine fishery stock enhancement in China. In this study, genetic diversity of the hatchery-released stock and two restored-natural stocks (SSNAC1 and SSNAC2) of *S. schlegelii* from Rongcheng Bay, Yellow Sea, China was analyzed using 15 microsatellite DNA loci, and the genetic differentiation between natural and hatchery-released stocks was also investigated. The mean effective number of alleles (A_e) in the hatchery-released stock was 3.6, the observed heterozygosity (H_o) was 0.506, and the expected heterozygosity (H_e) was 0.606, while the A_e , H_o and H_e values in the SSNAC1 and SSNAC2 stocks were 2.9 and 3.9, 0.381 and 0.577, and 0.569 and 0.692, respectively. The F_{ST} values between the hatchery-released and natural stock and the natural stocks before and after restocking were 0.055 and 0.013, respectively. The results showed that there was low differentiation between the natural and hatchery-released stocks even though the hatchery stock displayed slightly higher genetic diversity and heterozygosity than the natural stock. Furthermore, there was no differentiation on genetic diversity indices between the *S. schlegelii* natural stock before release and the mixed stocks after release in the short term. However, larger differentiation was found between the natural stock and restored-natural stock SSNAC2. Consequently, more detailed surveys are required to elucidate the long-term genetic effects of the stock enhancement. The present study would be helpful for the future stock enhancement strategy of black rockfish.

Keywords Black rockfish · SSR marker · Population structure · Stock enhancement · China

Introduction

Demand for seafood will continue to rise until 2050 as a result of population growth. Nevertheless, global fish stocks have steadily declined since the 1980s through factors such as over-fishing, habitat degradation, and global warming (Botsford et al. 1997; Nellemann 2009). Nowadays, the stocks of many fish species have become depleted. Data show that 35.4% of fish stocks were estimated to be over-fished (FAO 2022). Fisheries will not easily improve even if we reduce fishing and instate better management. The release of hatchery-reared juveniles into the wild has been used as a tool to recover marine fish stocks along with the augmentation of an existing fishery to enable larger catches to be taken (Blankenship and Leber 1995; Welcomme and Bartley 1998; Leber 2004; Bell et al. 2005, 2008). These hatchery-reared juveniles were released into the wild for three distinct purposes: restocking, stock enhancement, and sea ranching (Bell et al. 2008). Then, more and more species and mass of marine organisms have been released into

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the wild in recent years. More than 190 billion aquatic animals were released nationwide between 2016 and 2020 in China (Lin et al. 2023). However, the pros and cons of this approach have been widely debated (Blaxter 2000; Hedgecock and Coykendall 2007; Lorenzen et al. 2012; Camp et al. 2017).

There are growing concerns about the genetic disturbance to natural populations with stock enhancement activities (Kitada 2018). Most of the individuals used for stock enhancement came from the cultured stock. As the broodstock individuals used to produce hatchery-released juveniles are usually from limited numbers, genetic diversity often decreases, resulting in observed genetic differences. The farmed stock often differs genetically from its natural counterpart, showing reduced genetic diversity (Champagnon et al. 2012; Lorenzen et al. 2012). Application of hatchery individuals to reverse the decline of marine fisheries is likely to reduce the genetic diversity of the mixed stock after release, and may even erode the long-term genetic diversity of the wild stock through mating with wild fish (Hedgecock and Coykendall 2007). Therefore, stock enhancement activities can reduce the overall amount of genetic variation in the stocked species, leading to a loss of adaptive potential (Christie et al. 2012). Negative genetic impacts of stock enhancement have been documented in some fish species (Laikre et al. 2010; Hagen et al. 2021). The long-term extensive hatchery release program in red sea bream (*Pagrus major*) has affected the genetic diversity of wild populations, even if the genetic effects were low and appeared to remain within Kagoshima Bay (Hamasaki et al. 2010). Romo et al. (2006) found that there were significant pairwise differences between the wild captive broodstock and the tentative recaptured groups in spotted halibut (*Verasper variegatus*) based on the F_{ST} estimator. Although the genetic risks associated with these releases are well known (Bell et al. 2005; Lorenzen et al. 2012), they have rarely been monitored (Laikre et al. 2010). Thus, the consequences of genetic interactions between hatchery-reared and wild individuals remain poorly understood and are of great concern in conservation genetics. For each released fish species, the genetic variability of the released stock and the genetic divergence of the restored stock after release should be monitored to elucidate the genetic effects of such activities on wild stocks and ensure a responsible stock enhancement program (Romo et al. 2006; Ward 2006; Lorenzen et al. 2012).

The black rockfish (*Sebastes schlegelii*) is an important commercial fish inhabiting the coasts of Asia and is considered a promising target fish species for stock enhancement. In Japan (Yoshida et al. 2005) and Korea (An et al. 2009), stock enhancement programs for this species started in the 1980s and mid-1990s, respectively. In China,

hatchery-reared juveniles have been released into coastal waters every year since 1995 to recover the wild resources (Wang et al. 2020). According to incomplete statistics, a total of 3.86 million seeds were released in the coastal region of Shandong Province, China in 2007 and 2008, 3.85 million seeds in 2010, and approximately 1 million juveniles in 2012 (Lü et al. 2014). And from 2015 to 2019, over 30 million *S. schlegelii* were released annually in Shandong (Liu et al. 2022). Although there are a few papers reported on the population genetic diversity and differentiation of this species (Yoshida et al. 2005; An et al. 2009; Zhang et al. 2016), the genetic variability of released rockfish and recipient stocks has only been examined by Wang et al. (2020) and Liu et al. (2022). In 2009, more than 100 thousand hatchery-reared juvenile black rockfish were released into the sea of Rongcheng Bay, Shandong Province, China, as part of the National Special Research Fund for Non-Profit Marine Sector project. The present study examines the genetic variability of the hatchery-released stock in Rongcheng Bay as well as the divergence between the natural and the mixed restored-natural stocks. The aim of the study is to assess the potential genetic impact of hatchery-reared fish on the wild population. This work will provide basic data to ensure a healthy and sustainable restoration of *S. schlegelii* in Rongcheng Bay. The genetic data of local stocks will establish a baseline that could provide valuable suggestions for future fish stock enhancement strategy.

Materials and Methods

Sample Collection and DNA Extraction

Mature female spawning fish were collected from the natural coastal habitat of Yantai City, Shandong, China, adjacent to Rongcheng Bay, and then were transferred to an aquaculture farm (Taihua Marine Technology Co., Ltd, Yantai, Shandong, China). After the female fish spawned in May 2009, the hatchery-reared individuals were reared until release. The hatchery-reared *S. schlegelii* individuals were released into Rongcheng Bay in Oct. 2009. Before stock enhancement began, 60 individual specimens were collected from the hatchery stock for release (SSR). These individuals were stored at -20°C after anesthesia with tricaine methanesulfonate (MS222). Approximately 1 and 1.5 years after the release, 2 restored-natural stock samples (SSNAC1 and SSNAC2) were collected using motorized bottom trawls and cage nets from same location in Sept. and Nov. 2010 ($n=60$) and Feb. and May 2011 ($n=60$), respectively. The sampling locations ($122^{\circ}39'11''$ - $122^{\circ}46'16''\text{E}$, $37^{\circ}21'32''$ - $37^{\circ}13'41''\text{N}$) are shown in Fig. 1. The 52 natural individuals, captured in Rongcheng Bay in May and Aug.

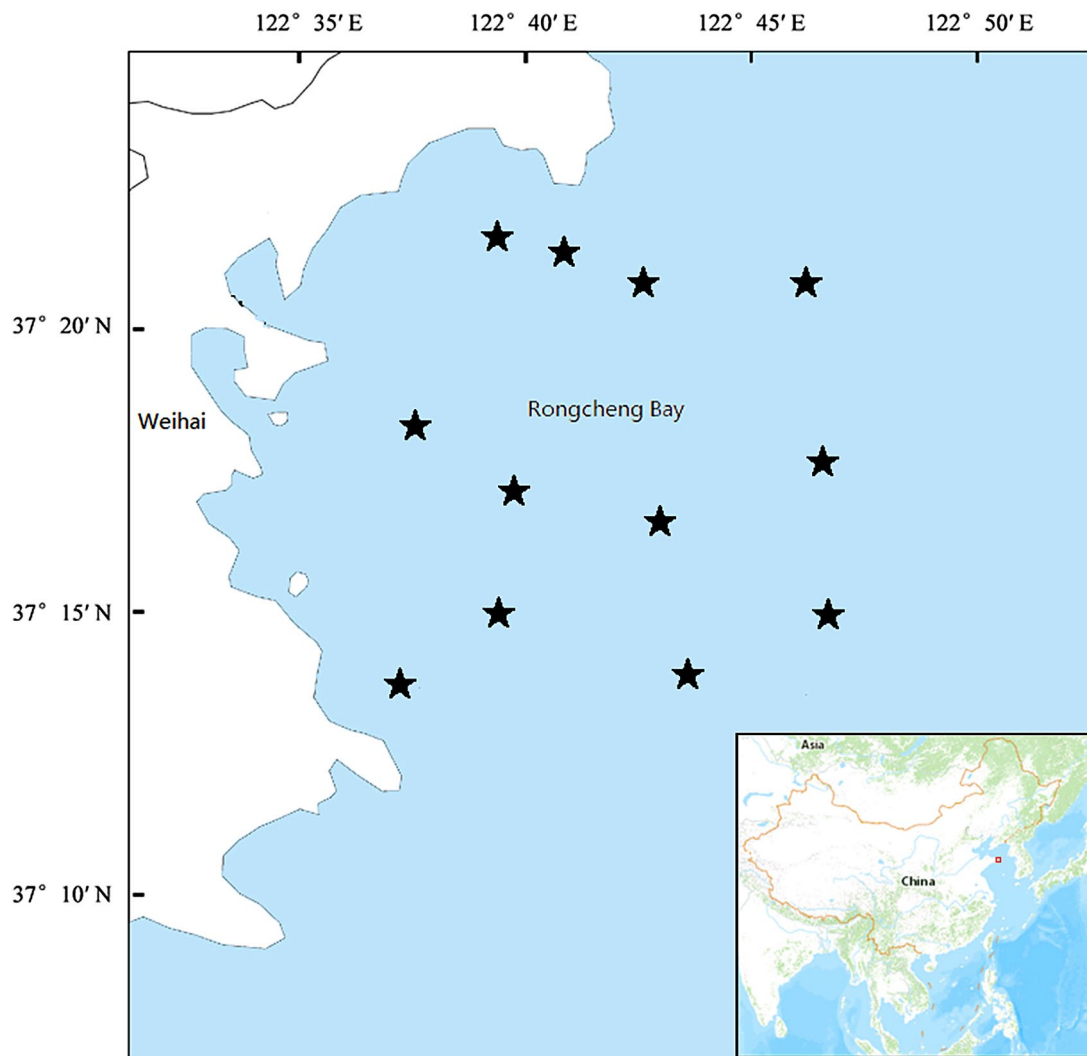


Fig. 1 Map of Rongcheng Bay showing sampling sites. The red box indicates the study location on the inset map; the asterisks indicate the sampling sites on the main map

2009, as reported in our previous study (Wang et al. 2012), refer to the natural population (SSNB).

Muscle tissue of the dorsal region of the fish was excised from each specimen. Total genomic DNA was extracted from the tissue samples using rapid salt-extraction of genomic DNA according to Aljanabi and Martinez (1997). The extracted DNA was checked using 1% agarose gel electrophoresis and then stored at -20°C for PCR amplification. The study was approved by the Ethics Committee of the Institute of Oceanology, Chinese Academy of Sciences.

Microsatellite Analysis

A total of 15 previously published microsatellite loci of *S. schlegelii*: KSs2A, KSs3, KSs6, KSs7, KSs11B, KSs12B, KSs17, KSs18A, KSs20, KSs26 (An et al. 2009) and Ssc12, Ssc23, Ssc51, Ssc69, Ssc71 (Yoshida et al. 2005) were

selected for analysis. The primers were synthesized by Sangon Biological Engineering Technology & Services CO. Ltd (Shanghai, China).

PCR were carried out in 25 μL volume comprising 1x PCR buffer [10 mmol/L Tris-HCl (pH 9.0) and 50 mmol/L KCl], 100 ng template DNA, 1.5 mmol/L MgCl_2 , 100 $\mu\text{mol/L}$ dNTPs, 10 pmol/L of each forward and reverse primers, and 1 U of Taq DNA polymerase (Promega, USA). The thermal cycling conditions for each locus were as follows: 7 min at 94°C , followed by 35 cycles of 94°C for 30 s, annealing for 45 s at the specific annealing temperature of the primers and extension at 72°C for 45 s, with a final extension temperature of 72°C for 10 min. Details of the primer sequences and specific annealing temperatures are available in An et al. (2009) and Yoshida et al. (2005). The PCR products were added to an equal volume of loading buffer. Three microliters of each PCR product mixture were

electrophoresed on a 12% nondenaturing polyacrylamide gel for 3 h at 300 V with a DYCZ-24B vertical electrophoresis tank and a DYY-6B constant voltage electrophoresis instrument (Liuyi Instrument Factory, Beijing, China). The DNA fragments were visualized using the modified silver staining method (Xu et al. 2002). Alleles were designated according to the PCR product size relative to a molecular size marker (M1041 50–500 bp, Dongsheng Biotech, China) in combination with Quantity One software (Bio-Rad). Minimum 2%, blindly scored, and sample repeats were performed within and across plates to reduce genotyping errors. Reruns were conducted for approximately 30% of all individuals to ensure reproducibility of allele scoring, and these replicates yielded identical results for each trial.

Microsatellite DNA data from fifty-two *S. schlegelii* individuals as SSNB were taken from our previous work (Wang et al. 2012).

Data Analysis

Measurements of genetic diversity within stock: standard genetic diversity parameters, including number of alleles (A), effective alleles (A_e), observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated using GenAlEx 6.5 (Peakall and Smouse 2012). Allelic richness (A_r) was calculated by using the rarefaction method based on a minimum sample size of 52 individuals with FSTAT v.2.9.3.2 (Goudet 2001). Deviations from linkage disequilibrium and Hardy-Weinberg equilibrium (HWE) for each microsatellite locus were tested by using the GENEPOP v.4.0 package (Saitoh and Rousset 2008), and significance was adjusted by applying the sequential Bonferroni correction. The polymorphic information content (PIC) was calculated according to Botstein et al. (1980) using the program Cervus v.3.0.6 (Kalinowski et al. 2007). ADZE v.1.0 (Szpiech et al. 2008) was used to determine the number of private alleles with standardized sample sizes. Micro-Checker software (Van Oosterhout et al. 2004) was used to search for null alleles at loci.

Measurements of genetic differentiation among stocks: estimation of Nei's unbiased genetic distance (Nei 1978), genetic identity, and the Principal component analysis (PCA) of *S. schlegelii* samples were performed by using GenAlEx 6.5. Population structure was analyzed using the model-based Bayesian clustering method in STRUCTURE v.2.3.4 (Pritchard et al. 2000; Falush et al. 2003), which assigned individuals to K populations. STRUCTURE was run for $K=1-4$ with a burn-in length of 100,000 and a run of 500,000 steps. All runs were repeated in triplicate for each K and the results were consistent across runs. The most probable K value was inferred by calculating ΔK (Evanno et al. 2005). To assess the extent of differentiation within

and among stocks, pairwise F_{ST} was determined as an estimator of inbreeding using FSTAT v.2.9.3.2 (Goudet 2001) with the analysis of variance method of Weir and Cockerham (1984). The Markov chain method (10,000 dememorization steps, 100 batches, 5000 iterations) was utilized to obtain unbiased estimates of the exact P-value. SMOGD v. 1.2.5 (Crawford 2010) was used to calculate the G_{ST} (recommended by Jost 2008) in order to validate the F_{ST} results (bootstrap replicates = 1000).

Estimations of effective population size of *S. schlegelii* stocks were performed in NeEstimator v. 1.3 (Do et al. 2014) by using the linkage disequilibrium and heterozygote excess methods. Bottleneck 1.2 software (Cornuet and Luikart 1996) was used to detect the presence of bottlenecks using the infinite allele model (IAM), stepwise-mutation model (SMM), and two-phased model of mutation (TPM) (Luikart and Cornuet 1998). The significant heterozygosity excess was tested using the Wilcoxon signed-rank test. Estimation of full- and half-sib family pairs in 3 *S. schlegelii* stocks was performed in kingroup v.2 (Konovalov et al. 2004). Here, 2 restored-natural stocks from Rongcheng Bay (SSNAC1 and SSNAC2) were combined into one stock (SSNAC) to be compared with the SSR and SSNB stocks.

Results

Population Genetic Variation

The measures of genetic diversity calculated from the observed allele distribution for each stock are presented in Table 1. In the studied stocks, the average number of alleles per stock per locus varied from 4.7 to 6.0. The average effective allelic numbers (A_e) and allelic richness values (A_r) ranged from 2.9 to 3.9 and 4.7 to 5.9, respectively. The overall observed heterozygosity (H_o) was lower than expected (H_e), indicating heterozygote deficiency. Null alleles were detected at loci Ssc12, Ssc51, Ssc69, KSs11, KSs12, KSs17, and KSs18. The values of the expected heterozygosity ranged from 0.569 to 0.692. And the lowest average expected heterozygosity was in the SSNAC1 stock (0.569), while the highest was in the SSNAC2 stock (0.692). The mean observed heterozygosity values showed similar results. The lowest value was in the SSNAC1 stock (0.381), whereas the highest was in the SSNAC2 stock (0.577). Most microsatellite markers in this study were unconfirmed to HWE. In which, the heterozygote of Ssc71 was excess and the heterozygotes of the others were deficient. Overall, the hatchery SSR stock presented a slightly higher genetic diversity than the natural SSNB stock. And the mixed stock SSNAC1 exhibited almost similar genetic diversity to the SSNB stock before release.

Table 1 Genetic variation at 15 microsatellite loci in the four *Sebastes schlegelii* stocks

Stock	Locus	A	Ae	Ar	Ho	He	Fis	HWE	PIC	Stock	Locus	A	Ae	Ar	Ho	He	Fis	HWE	PIC
SSNB ¹	Ssc12	6	3.6	6.0	0.385	0.731	0.477	**	0.689	SSNAC1	Ssc12	7	4.1	7.0	0.367	0.762	0.521	**	0.724
	Ssc23	6	3.6	6.0	0.615	0.731	0.160	**	0.680		Ssc23	5	2.4	5.0	0.417	0.591	0.297	**	0.553
	Ssc51	7	5.2	7.0	0.481	0.816	0.413	**	0.781		Ssc51	7	5.2	7.0	0.417	0.815	0.491	**	0.781
	Ssc69	5	4.4	5.0	0.327	0.782	0.584	**	0.737		Ssc69	5	3.9	5.0	0.400	0.752	0.470	**	0.702
	Ssc71	4	2.2	4.0	1.000	0.551	-0.824	**	0.442		Ssc71	4	2.4	4.0	1.000	0.595	-0.850	**	0.504
	KSs2	6	5.0	6.0	0.558	0.808	0.312	**	0.770		KSs2	6	3.2	6.0	0.417	0.694	0.402	**	0.654
	KSs3	9	6.3	9.0	0.635	0.850	0.255	**	0.822		KSs3	5	3.6	5.0	0.633	0.728	0.131	*	0.671
	KSs6	6	2.6	6.0	0.327	0.623	0.478	**	0.571		KSs6	3	2.5	3.0	0.133	0.611	0.709	**	0.537
	KSs7	6	3.8	6.0	0.596	0.741	0.223	n.s.	0.692		KSs7	6	3.5	5.9	0.550	0.719	0.236	n.s.	0.661
	KSs11	7	4.3	7.0	0.538	0.773	0.310	*	0.729		KSs11	6	4.5	6.0	0.450	0.785	0.429	**	0.744
	KSs12	4	1.8	4.0	0.038	0.440	0.913	**	0.403		KSs12	4	1.8	4.0	0.250	0.443	0.437	**	0.401
	KSs17	2	1.2	2.0	0.077	0.176	0.564	n.s.	0.159		KSs17	2	1.2	2.0	0.067	0.154	0.569	n.s.	0.141
	KSs18	2	1.0	2.0	0.000	0.038	1.000	n.s.	0.037		KSs18	2	1.1	2.0	0.000	0.065	1.000	**	0.062
	KSs20	2	1.1	2.0	0.000	0.110	1.000	**	0.103		KSs20	2	1.1	2.0	0.000	0.125	1.000	**	0.117
	KSs26	6	3.4	6.0	0.635	0.710	0.108	n.s.	0.673		KSs26	6	3.3	6.0	0.617	0.704	0.124	**	0.669
SSR	Average	5.2	3.3	5.2	0.414	0.592	0.398	-	-		Average	4.7	2.9	4.7	0.381	0.569	0.398	-	-
	Ssc12	10	5.2	8.9	0.633	0.815	0.270	**	0.786	SSNAC2	Ssc12	7	4.7	7.0	0.559	0.795	0.313	**	0.768
	Ssc23	10	6.0	9.7	0.767	0.840	0.088	n.s.	0.813		Ssc23	6	4.4	6.0	0.883	0.778	-0.141	**	0.740
	Ssc51	8	4.1	7.9	0.650	0.761	0.150	n.s.	0.719		Ssc51	9	6.1	9.0	0.500	0.842	0.426	**	0.814
	Ssc69	5	3.6	4.9	0.467	0.727	0.319	**	0.672		Ssc69	4	3.4	4.0	0.400	0.711	0.432	**	0.654
	Ssc71	2	2.0	2.0	1.000	0.504	-1.000	**	0.375		Ssc71	6	4.0	5.9	0.672	0.754	0.131	**	0.724
	KSs2	7	4.3	6.9	0.700	0.772	0.094	n.s.	0.734		KSs2	6	4.7	6.0	0.800	0.795	-0.016	n.s.	0.757
	KSs3	9	6.2	9.0	0.533	0.845	0.373	**	0.818		KSs3	7	4.6	7.0	0.729	0.791	0.059	n.s.	0.760
	KSs6	6	3.8	6.0	0.467	0.741	0.373	**	0.693		KSs6	7	4.3	7.0	0.667	0.774	0.122	*	0.740
	KSs7	8	3.6	8.0	0.717	0.728	0.016	n.s.	0.685		KSs7	6	4.6	6.0	0.638	0.789	0.212	**	0.766
	KSs11	8	6.8	8.0	0.783	0.859	0.089	n.s.	0.834		KSs11	7	4.6	6.9	0.678	0.789	0.137	n.s.	0.759
	KSs12	5	2.3	4.9	0.250	0.575	0.567	**	0.482		KSs12	2	2.0	2.0	0.050	0.504	0.902	**	0.375
	KSs17	2	1.1	2.0	0.000	0.096	1.000	**	0.090		KSs17	4	1.1	4.0	0.083	0.128	0.352	**	0.124
	KSs18	2	1.0	2.0	0.000	0.033	1.000	n.s.	0.032		KSs18	7	4.7	7.0	0.433	0.796	0.477	**	0.759
	KSs20	2	1.1	2.0	0.000	0.096	1.000	**	0.090		KSs20	4	2.4	4.0	0.600	0.592	0.008	n.s.	0.540
KSs26	6	3.3	6.0	0.617	0.703	0.124	n.s.	0.665		KSs26	4	2.2	4.0	0.967	0.544	-0.787	**	0.434	
Average	6.0	3.6	5.9	0.506	0.606	0.298	-	-		Average	5.7	3.9	5.7	0.577	0.692	0.175	-	-	

¹Data are quoted from Wang et al. 2012; * and ** denote Hardy-Weinberg disequilibrium, * denotes significance ($P \leq 0.05$), ** denotes high significance ($P \leq 0.01$); n.s. denotes Hardy-Weinberg equilibrium with non significant after Bonferroni correction; - denotes that the average value of the parameter cannot be calculated

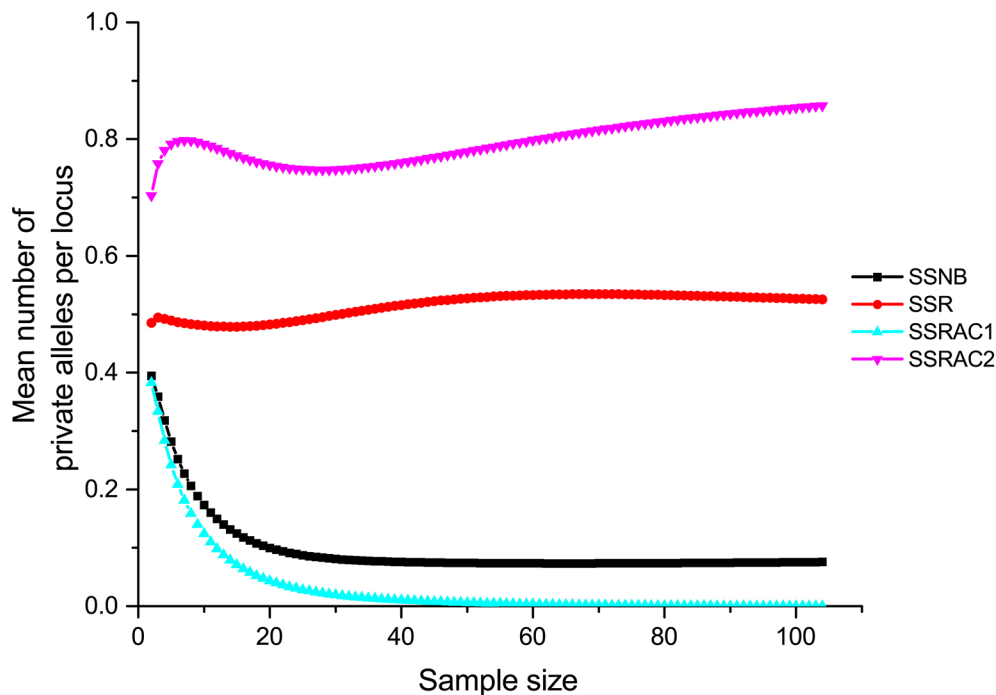


Fig. 2 The number of private alleles in the four *S. schlegelii* stock when the sample size is standardized

Table 2 Estimation of Nei's unbiased genetic distance (above the diagonal) and Nei's unbiased genetic identity (below the diagonal) of the four *S. schlegelii* stocks

	SSNB	SSR	SSNAC1	SSNAC2
SSNB		0.109	0.035	0.407
SSR	0.897		0.142	0.382
SSNAC1	0.966	0.868		0.426
SSNAC2	0.666	0.683	0.653	

The number of private alleles per stock when the sample size is standardized was different. The value in the SSR stock was 0.5, whereas the value of the SSNB stock was considerably lower at 0.07. Among the stocks examined, the SSRAC2 stock achieved the highest value of 0.8, whereas the SSRAC1 stock exhibited the lowest value of 0.01 (Fig. 2). The analyzed stocks contained a total of 23 private alleles, of which one was exclusively present in the SSNB stock, nine exclusively in the SSR stock, and thirteen exclusively in the SSNAC2 stock.

Population Structure and Gene Flow among Stocks

The results of the exact testing of the genetic differentiation between population pairs revealed that there was a clear genetic differentiation in these studied stocks (Table 2). The genetic differentiation between the SSNB and SSR was relatively low ($F_{ST} = 0.055$), and the lowest genetic divergence was observed between SSNAC1 and SSNB ($F_{ST} = 0.013$). However, the F_{ST} between SSNAC2 and SSNB ($F_{ST} = 0.152$) was much larger than that between SSNAC1 and

SSNB. The results also showed that the largest differentiation was observed between the two restored-natural stocks SSNAC1 and SSNAC2 ($F_{ST} = 0.167$). The G_{ST} values, genetic similarity, and genetic distance values presented similar results (Table 2; Fig. 3).

Furthermore, the scatter plots of PCA (Fig. 4) clearly revealed three main clusters (i.e. SSNB-SSNAC1, SSR, and SSNAC2). Similar to the results of the F_{ST} analysis, the PCA results showed that the SSNAC1 stock was not genetically distinct from the SSNB stock, and the two restored-natural stocks were genetically distinct from each other. According to ΔK , the optimal value for K was determined as $K=2$ (Fig. 5). This result was consistent across all replicate runs of the analysis. STRUCTURE estimated that the samples from the SSNB, SSR, and SSNAC1 stocks derived on about 97%, 96%, and 95% of their ancestry from the group one, respectively, while the SSNAC2 samples derived only 98% of their ancestry from the group two. Analysis of molecular variance (AMOVA) showed that most of the genetic variance occurred among individuals within stocks, providing 88.98% variation, while only 11.02% of the variance occurred between stocks.

The estimation of the effective population size (N_e) using the linkage disequilibrium method indicated that the N_e value was the highest in the SSNB, while of the values were lower in the SSNAC1 and SSNAC2. However, the N_e value in the SSR could not be calculated (Table 3). The estimates for all stocks were infinite by using heterozygote excess method.

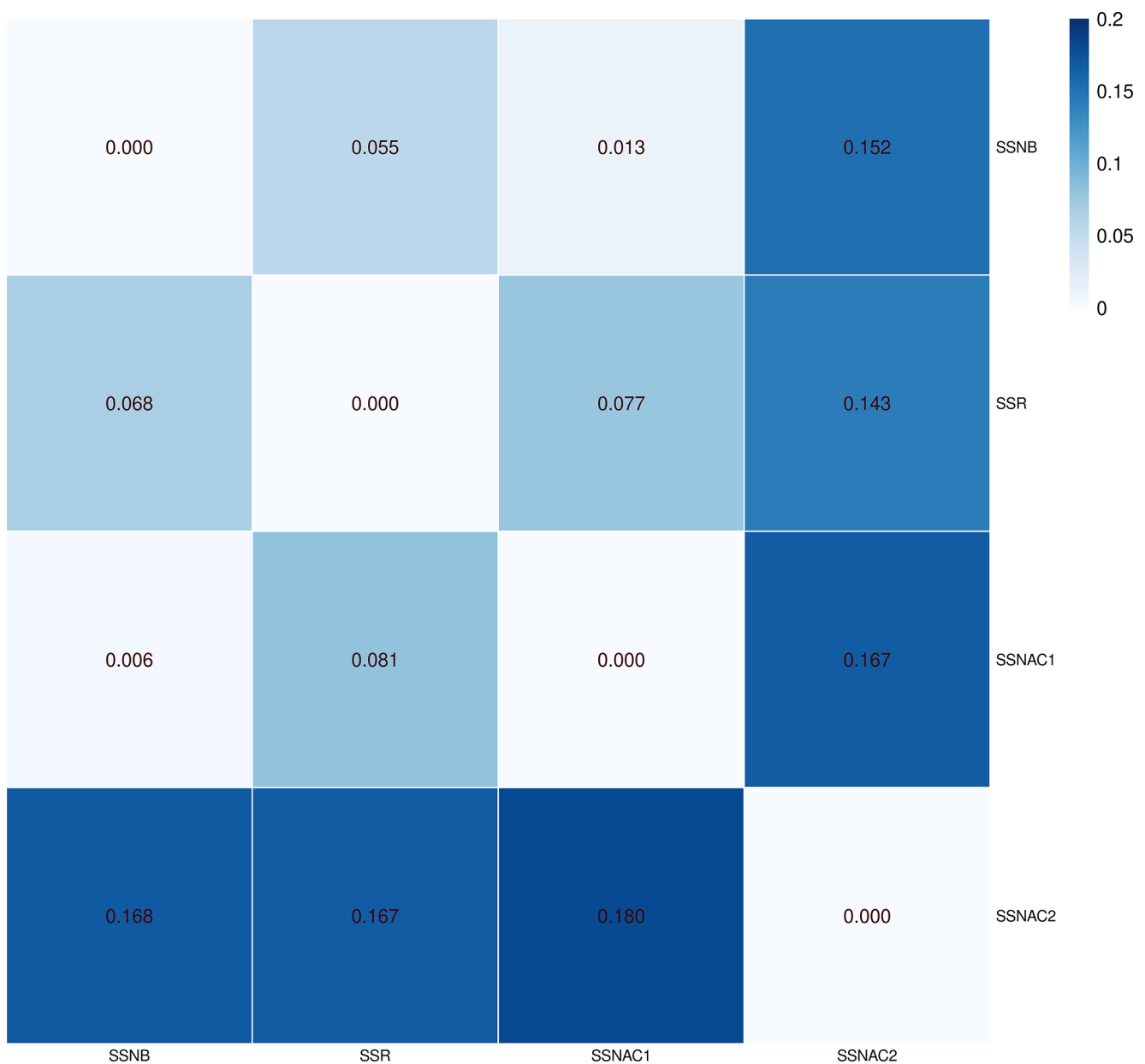


Fig. 3 Heatmap of pairwise genetic differentiation index (F_{ST}) (values above the diagonal) and G_{ST} (values below the diagonal) among the four *S. schlegelii* stocks

The IAM, TPM, and SMM models were applied to test for population bottlenecks. The results are shown in Table 4. The sign, standardized difference, and Wilcoxon tests all showed significant heterozygosity excess in all stocks under the IAM model ($P < 0.05$). The Wilcoxon test, which is considered more reliable than the sign test and the standardized difference test, showed significant results for population bottleneck under the IAM and TPM models in all stocks. But under the SMM model, only the SSNAC2 stock showed a significant result. Furthermore, a mode shift was detected in the frequency distribution of alleles, resulting in a normal L-shaped curve.

The results of estimating full-sib family pairs in the *S. schlegelii* stocks showed that the ratio of full-sib and half-sib pairs was the lowest between SSR and SSNAC, while it was the highest between SSNB and SSNAC. However, all the ratios were relatively low. There were 509 full-sib family pairs between the SSNAC and SSNB stocks, accounting for 8.2% of the total. Between the SSNAC and SSR stocks, there were 188 full-sib family pairs, representing 2.6% of the total (Tables 5 and 6).

Fig. 4 PCA of allele frequencies from 15 microsatellite loci typed in the four *S. schlegelii* stocks

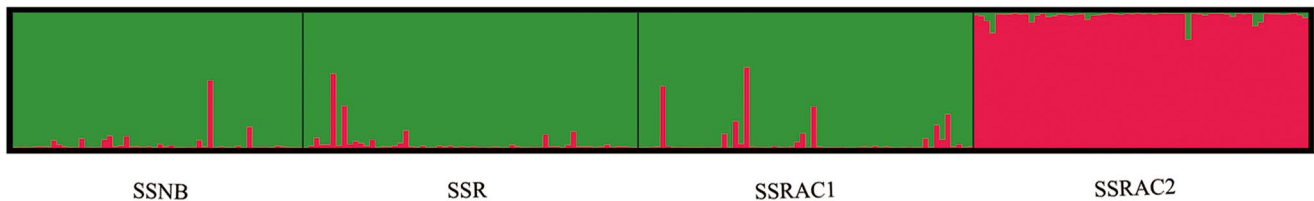
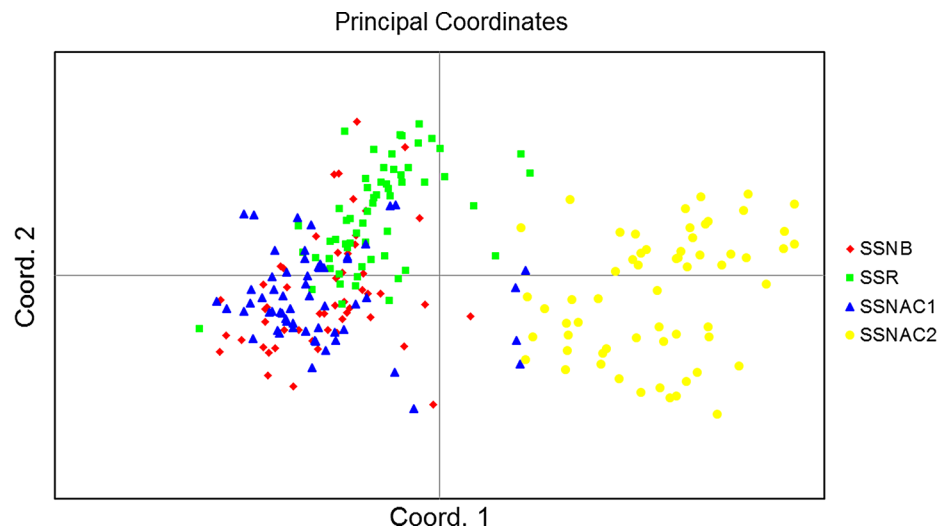


Fig. 5 Graphical representation of the results of the STRUCTURE analysis for the most probable model ($K=2$)

Table 3 Estimation of the effective population size in the four *S. schlegelii* stocks

Stock	Linkage Disequilibrium	Heterozygote Excess
SSNB	121.3 (84.5–202.9)	∞
SSR	-	∞
SSNAC1	37.9 (32.2–45.1)	∞
SSNAC2	35.9 (31.5–41.2)	∞

Discussion

Nowadays, the growing demand for seafood can no longer be met by natural captures alone. Consequently, many stock enhancement programs have been developed to augment the biomass of target species. It is well recognized that enhancement programs should be coupled with habitat restoration and should attempt to ensure the greatest possible genetic diversity (McCay and Rowe 2003). The maintenance of genetic diversity affects the ability of a population to survive (Schindler et al. 2010). Loss of genetic variation affects adaptive potential, i.e. the ability of wild populations to respond to ecological challenges. Ideally, these enhancement programs should be achieved with little or no negative impact on the local gene pool. Several strategies have been proposed to minimize genetic alterations and reduce the loss of genetic diversity. To prevent rapid genetic shifts in the hatchery population, it is recommended to use native individuals that are genetically compatible with the individuals

at the release site as broodstock (Grant et al. 2017). Small broodstock sizes can rapidly alter the genetic composition of the hatchery population. Therefore, using a large number of broodstock to generate hatchery populations is an effective approach for preserving genetic diversity (Kitada et al. 2009). In addition, brood stock must be derived from naturally produced individuals rather than hatchery-reared individuals to avoid the accumulation of domestication effects over generations (Willoughby and Christie 2019). Regrettably, most hatchery restocking results in a decline in combined wild and hatchery populations (Grant et al. 2017). The negative effects on the genetic diversity of recipient populations caused by large-scale releases have been documented in many plant and animal species (Laikre et al. 2010), such as *Acacia saligna lindleyi* (Millar and Byrne 2007), eucalypt (Barbour et al. 2008), red drum (*Sciaenops ocellatus*) (Gold et al. 2008), and steelhead trout (*Oncorhynchus mykiss*) (Araki et al. 2007). However, despite the genetic risks of stock enhancement programs have long been recognized, they have rarely been monitored appropriately and effectively (Laikre et al. 2010). Such is the case with the stock enhancement program of *S. schlegelii*. The program has been conducted for more than 30 years, but there is a lack of studies evaluating its genetic impact.

In this study, significant deviations from HWE were detected in all the *S. schlegelii* stocks analyzed, even after

Table 4 Results of the bottleneck test under three models for the four *S. schlegelii* stocks

Stock	Test	IAM	TPM	SMM
SSNB	Sign test: No. of loci with heterozygosity excess (probability)	0.040289	0.2804	0.485467
	Standardized differences test: T2 values (probability)	0.002133	0.033654	0.396424
	Wilcoxon test (Probability of heterozygote excess)	0.000839	0.02063	0.598022
SSR	Sign test: No. of loci with heterozygosity excess (probability)	0.035228	0.24881	0.343954
	Standardized differences test: T2 values (probability)	0.001217	0.029915	0.290651
	Wilcoxon test (Probability of heterozygote excess)	0.000839	0.03186	0.700256
SSNAC1	Sign test: No. of loci with heterozygosity excess (probability)	0.031569	0.121605	0.061215
	Standardized differences test: T2 values (probability)	0.001192	0.026605	0.480421
	Wilcoxon test (Probability of heterozygote excess)	0.000656	0.010773	0.532959
SSNAC2	Sign test: No. of loci with heterozygosity excess (probability)	0.002523	0.003353	0.018039
	Standardized differences test: T2 values (probability)	0.000005	0.000574	0.137397
	Wilcoxon test (Probability of heterozygote excess)	0.001678	0.004181	0.007538

Table 5 Estimation of full-sib family pairs in the *S. schlegelii* stocks

	SSNB	SSR	SSNAC
SSNB		210	509
SSR	6.7		188
SSNAC	8.2	2.6	

Full-sib family pairs, above the diagonal; the percentages in all individuals, below the diagonal

Table 6 Estimation of half-sib family pairs in the *S. schlegelii* stocks

	SSNB	SSR	SSNAC
SSNB		121	279
SSR	3.9		86
SSNAC	4.5	1.2	

Half-sib family pairs, above the diagonal; the percentages in all individuals, below the diagonal

sequential Bonferroni correction. These departures may be mainly caused by heterozygote deficiencies. Thirteen significant HWE deviation loci show heterozygote deficiencies, while only 1 shows heterozygote excess. In wild stocks, heterozygote deficiencies could have several causes, such as the decreases of wild population size, inbreeding, or possibly assortative mating due to repeated interbreeding between the natural population and mass-released hatchery strains (Marchant et al. 2009; An et al. 2014). As for hatchery stocks, non-random mating, the limited number of founders, inbreeding, or artificial selection forces during cultivation might result in heterozygote deficiency (Kohlmann et al. 2005; An et al. 2014). Null alleles are also likely to be an important explanation for the deviation observed in marine fish (Dick et al. 2014) and could potentially be responsible for heterozygote deficiency. In our study, the presence of null alleles was also observed at some loci. All the above mechanisms may have contributed to the heterozygote deficiencies observed in the *S. schlegelii* stocks.

Genetic evaluations have been recommended for both natural stocks and hatchery-released stocks before and after release to assess the genetic impact of the subsequent stock enhancement. This is necessary to describe the genetic structure of the natural stock to determine the most suitable broodstock to provide hatchery-reared juveniles for stock enhancement. In general, the number of broodstock used to produce hatchery-released juveniles is limited due to the constraints imposed by hatchery techniques. Maintenance of broodstock in hatcheries leading to hatchery progeny usually yields low genetic diversity compared to the natural population (Ryman and Laikre 1991; Alarcón et al. 2004). However, unlike other farmed fish species, no deep depression was observed in the hatchery stock of *S. schlegelii* in this study. The hatchery-released stock (SSR, $A=6.0$, $A_e=3.6$, $A_r=5.9$, $PIC=0.565$, $H_o=0.506$, $H_e=0.606$) displayed slightly higher genetic diversity and heterozygosity compared to the natural stock (SSNB, $A=5.2$, $A_e=3.3$, $A_r=5.2$, $PIC=0.552$, $H_o=0.414$, $H_e=0.592$). And the genetic differentiation between the natural and hatchery-released stocks is relatively low ($F_{ST}=0.055$). One possible reason for the high genetic diversity and limited genetic differentiation between the natural and SSR stocks was the relatively short domestication history of *S. schlegelii* in China. Another reason was that the broodstock amount in the artificial selective breeding of the black rockfish was large due to its viviparous reproduction. Overall, the genetic diversity indices of the *S. schlegelii* stocks in this study did not differ significantly (average allele A varied from 4.7 to 6.0, mean average H_e varied from 0.569 to 0.692) and are lower than those reported for the Korean coastal stocks (average allele $A=20.1$; average $H_e=0.81$) (An et al. 2012). The variations are also lower than other *Sebastes* species and other

marine fish species (average allele $A=19.96\pm 6.6$, average $He=0.77\pm 0.19$, 12 species) (DeWoody and Avise 2000). This may be due to the limited sample size ($n=60$ in each stock) and nondenaturing PAGE method, which reduced the possibility of detecting more novel and rare alleles.

After release, the contribution of the released individuals to the gene pool of the mixed stock and the changes in genetic structure should also be assessed. In this study, the genetic effects of stock enhancement for the natural stock were examined. The results showed that two mixed stocks after the release of *S. schlegelii* (SSNAC1 and SSNAC2) exhibited similar average genetic diversity, although slightly lower in SSNAC1 and slightly higher in SSNAC2, compared to the natural stock before the release (SSNB) (Table 1). No significant difference in average PIC (SSNB=0.553, SSNAC1=0.528) or average He (SSNB=0.592, SSNAC1=0.569) was observed when comparing the diversity level of the natural stock with that of the restored-natural stock SSNAC1. Both the exact tests of genetic differentiation (Table 2) and F_{ST} (0.013) indicate that the SSNB and SSNAC1 stocks are genetically closely related. AMOVA also indicated that most of the variation in microsatellite alleles of these two stocks is explained within stocks and not between stocks. Moreover, the results of phylogenetic tree clustering and PCA analyses showed weak genetic subdivision of the SSNB and SSNAC1 stocks. All the above results demonstrated that genetic variation in the natural stock did not decrease after the stock enhancement and that stock enhancement did not have a negative genetic effect on the natural population of *S. schlegelii* in Rongcheng Bay in a short time. This could be due to the relatively high genetic diversity of the hatchery-released stock. To reduce the difference between the released offspring and the wild populations, it is optimal to select the broodstock for the hatchery-released stock from the native population at the release site. However, it is not feasible (Ward 2006) and animals are usually transported from other areas for release. Previous studies have shown that there is no genetic differentiation among populations in wild stocks of *S. schlegelii* (Wang et al. 2012; Zhang et al. 2016; Gao et al. 2018), which could minimize the genetic effect of using non-native individuals as broodstock. It is worth noting that the mean F_{ST} values between the SSNAC2 samples and the other two stock samples are much larger compared with SSNAC1, and the largest differentiation was observed between the two restored-natural stocks. The results showed that there was no obvious genetic impact on the wild stock 1 year after stock enhancement, but genetic divergence was observed 1.5 years after stock enhancement. This suggests that the hatchery-released individuals may have a lasting impact on the genetic structure of the mixed stock as time goes on. In this study, differentiation was found between

the hatchery stock (SSR) and the wild stock (SSNB) ($F_{ST}=0.055$), suggesting that the hatchery-reared released population may influence the original population structure of the wild population. Differentiation between the hatchery-released and wild black rockfish stocks before and after enhancement were also reported in Lidao Bay (Wang et al. 2020) and Liaoning Peninsula (Liu et al. 2022), but their F_{ST} values were all low. Another possible reason for SSNAC2 differentiation is the behavior of *S. schlegelii*. Although it is a demersal rockfish that does not migrate over long distance, *S. schlegelii* can disperse with the complex oceanic current systems during its early developmental stage (Gao et al. 2018). Moreover, *S. schlegelii* moves seasonally over short distances. Therefore, the aggregation of rockfish populations may not be completely uniform in different years. Some new alleles could be introduced into the restored-natural stock. In addition, the Ne values of the SSNAC stocks after release in this study were much smaller than those of the wild stock in this study. In general, populations with smaller Ne values tend to be more inbred and impoverished in terms of local genetic variability due to strong genetic drift, limiting evolutionary potential (Waples et al. 2016). Further study is required to assess the influence of the long-term effects of the stock enhancement.

This study examined the genetic variability of the hatchery-released and natural *S. schlegelii* stocks after artificial release in Rongcheng Bay, Yellow Sea, China. Our results showed that there was no marked decrease of genetic diversity indices in the natural stock of *S. schlegelii* in Rongcheng Bay after stock enhancement. More detailed surveys for elucidating the long term genetic effects of *S. schlegelii* stock enhancement are still required. Nevertheless, the evaluation of the genetic variability of *S. schlegelii* in this study may provide valuable insights into the strategy for wild stock management and stock enhancement in Rongcheng Bay.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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