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Temporal Changes in Genetic Diversity of *Fenneropenaeus chinensis* Populations from Jinzhou Bay: Implications for Management

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ABSTRACT

Fenneropenaeus chinensis is an important fishery species in China. Hatchery-reared seeds have been released into the wild for improvement of shrimp productivity. Jinzhou bay is the major natural habitat and we used this location to temporally monitor the genetic effects of release of hatchery stocks on local *F. chinensis* populations across five years. A set of 13 microsatellite markers were used to evaluate genetic patterns across 2015, 2016 and 2019. We observed a significant Hardy-Weinberg disequilibrium across all stocks. The inbreeding coefficient (F_{is}) was positive (0.121–0.131) for all stocks. A loss of genetic diversity was detected in the 2019 stock and significant differences were observed for number of different alleles, number of effective alleles, allelic richness and unbiased expected heterozygosity in the 2019 stock ($P < 0.05$). The highest pairwise relatedness and the lowest observed heterozygosity were also observed in 2019 stock. Moreover, a small but significant genetic differentiation was detected between the 2019 stock and the stocks of the previous two years. Given the continuously large scale of artificial enhancements in this area, these data may indicate that releases of hatchery-reared *F. chinensis* individuals may be associated with inbreeding and potentially the reduction in genetic diversity of the *F. chinensis* population.

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QL and HY designed the research. WL, QZ, ZW, RG, WL, LZ and TT performed the research. QL and WL analyzed the data. QL wrote the paper. YL and HY revised the manuscript. All authors read and approved the final version of the manuscript.

Key words
***Fenneropenaeus chinensis*, Genetic diversity, Management, Stock enhancement, Microsatellite markers**

INTRODUCTION

Stock enhancement programs are widely applied to fishery, wildlife conservation and forestry sectors to: (i) replenish threatened stock species or increase resource yields (Taylor *et al*., 2017), (ii) mitigate environmental disturbances from anthropogenic stresses (i.e., fishery over-exploring) and (iii) maintain ecological system balance (Lorenzen *et al*., 2010). From 2011 to 2016, it was reported that vast numbers of marine juveniles from approximately 187 species were released into the wild in 20 countries and regions across the world (Kitada, 2018). In Japan, as part of the largest scale marine enhancement programs in the world, over nine million hatchery-reared red sea bream (*Pagrus major*) are released each year (Kitada, 2018). In China, approximately 63.66 billion seedlings of various aquatic species, including fish, crustaceans and shellfish, were released from 2007 to 2009 (China Association for Science and Technology, 2012). Large-scale marine stock enhancement programs have rapidly grown across China; e.g., an excess of 29 billion Chinese shrimp (*Fenneropenaeus chinensis*) larvae were released into the inshore area of the Liaodong peninsula, from 2012 to 2019, at a cost of 34.29 million US dollars (225 million RMB) (Fig. 1). A total of more than 2 billion *F. chinensis* were released around Chinese coastal waters in each year.

Due to economic requirements and benefits, the mass release of hatchery-reared juveniles into the marine environment has dramatically increased on a global scale. However, concomitant monitoring efforts and investigations have been weak and insufficient, when compared with the scale of enhancement (Gonzalez *et al*., 2015). Several researchers have voiced concerns that the artificial enhancement with hatchery-reared juveniles could exert ecological and genetic impacts on wild populations of salmonoid and other marine species because body size, adaption and genetic backgrounds are different between released individuals and their wild companions. Other complications include environmental

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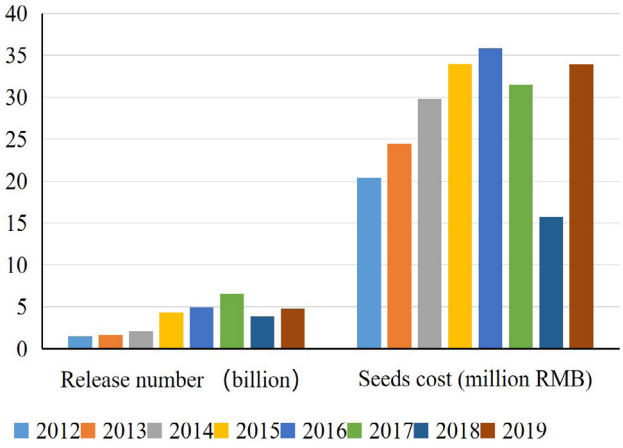

Fig. 1. Total *F. chinensis* release numbers and seed costs from 2012–2019 in Liaodong peninsula.

capability and food competition (Segovia-Viadero *et al*., 2016; Ozerov *et al*., 2016). For example, hatchery-reared Japanese Spanish mackerel (*Scomberomorus niphonius*) were larger in size than wild fish. After release, they reduced the growth rate of wild fish and could replace them if stock enhancement exceeded the carrying capacity of the environment (Nakajima *et al*., 2013). In contrast to wild populations, hatchery cultured Chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Oncorhynchus mykiss*) only showed half or reduced levels of relative reproductive success when released into the wild (Araki *et al*., 2007; Christie *et al*., 2014). Reductions in reproductive adaptation were also detected in hatchery-reared *P. major* (Kitada *et al*., 2019). Use of insufficient numbers of parental broodstocks leads to a limited proportion of the total gene pool of a species being represented. The domesticated populations usually originate from a small number of parental broodstock in stock enhancement programs. Hence, released seeds are likely to have a decreased effective population size, in contrast to the wild population (Hold *et al*., 2012; Liu *et al*., 2018). Consequently, Ryman-Laikre effects could initially decrease the effective population size of the local wild populations, then may reduce their genetic diversities or may change their genetic structure after release (Waples *et al*., 2016; Grant *et al*., 2017). Moreover, introgression (gene flow) from hatchery-reared individuals, caused by artificial release or escape from aquaculture facilities, may alter local population structures and composition, or even replace them (Jenkins *et al*., 2020). This is a serious concern for cultured Atlantic salmon (Glover *et al*., 2017), gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (Arechavala-Lopez *et al*., 2018). Hence, monitoring the population variations in their habitats and evaluating the potential genetic effects of stock enhancement on local wild populations are important for resource restoration and management.

F. chinensis is derived from the Penaeidae family. This species grows rapidly, has a relatively large body and is suitable for human consumption (Guo *et al*., 2014). Due to habitat destruction and diseases, the *F. chinensis* catch has dropped dramatically, from over 40000 tons in 1979 to around 1000 to 2000 tons landed annually after 2000 (Wang *et al*., 2006). To protect *F. chinensis* populations and improve production, hatchery-reared *F. chinensis* release programs were initiated.

Since 2012, large-scale *F. chinensis* stock enhancement programs have been conducted every year in the Liaodong peninsula (Liu *et al*., 2016). However, most of the genetic research into this species has focused on the spatial evaluation of the population's distribution (Liu *et al*., 2006; Cui *et al*., 2007; Meng *et al*., 2009). Thus, limited information is available on the genetic monitoring of specific enhancement programs and the temporal genetic assessment of these local wild populations. The genetic patterns of local populations can be influenced by anthropogenic factors, such as the offspring of hatchery-reared individuals comprising a large percentage of the overall local wild population in the next generation (Laikre *et al*., 2010; Kitada, 2018). Hence, genetic monitoring efforts are required to properly evaluate enhancement programs and their implications for management. The Jinzhou bay sea area is a major habitat of *F. chinensis* and an artificial enhancement area of the Liaodong peninsula. In recent years, around 0.5–0.7 billion shrimp seeds, with a total length about 1 cm, have been released each year. This species has a life span of approximately one year and lives and forages in the Jinzhou bay area since birth, from June to October. They usually swim out to deep water, based on water temperature, to overwinter, and swim back in April to May of the following year for reproduction.

In this study, although the individuals reared in hatcheries were not sampled, we used 13 microsatellite markers to evaluate temporal genetic variations in the local wild population of *F. chinensis* distributed throughout Jinzhou bay, from 2015 to 2019. Genetic changes in diversity and structure of wild populations can be influenced by stocking activities. Hence, our results will provide basic information for the assessment of potential genetic impacts on the local wild population from continuous enhancement activities.

MATERIALS AND METHODS

Ethics statement

All animal experiments and animal treatment protocols conformed to the regulations established by the

Fishery Resources Enhancement Laboratory at Dalian Ocean University.

Sampling

In total, 277 *F. chinensis* individuals at 3–4 months old, with a body length ranging from 91-130 cm, were sampled from Jinzhou bay in August 2015 (JZ2015), August 2016 (JZ2016) and August 2019 (JZ2019). All samples were collected by shrimp trawling, in gillnets. The sampling area is shown in Figure 2. In the laboratory, fresh muscle was clipped and DNA was isolated using the TIANamp Marine Animal DNA Kit (Tiangen, China). The quality of extracted DNA was assessed by 1% agarose gel electrophoresis. Thirteen highly polymorphic microsatellite loci that had been used previously were chosen for this study (Supplementary Table S1).

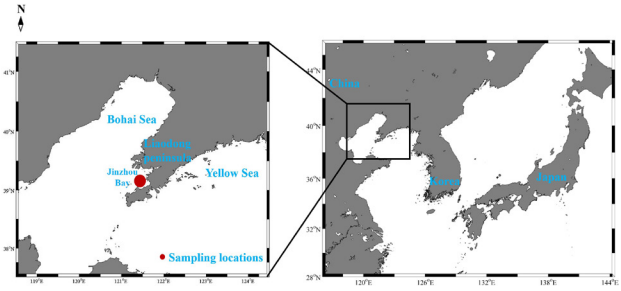

Fig. 2. Map of sampling locations. Red dot indicated the location of Jinzhou bay where *F. chinensis* were sampled.

The PCR reactions were performed in 15 μl volume reactions. These contained 10 ng genomic DNA, 1.6 μl 10 × PCR buffer, 1.2 μl dNTPs (2.5 mM), 1.2 μl $MgCl_2$ (2.5 mM), 0.6 μL forward and reverse primers (10 μM), 0.12 μl Taq polymerase Tap (5 U/μl) (Takara, Japan) and sterile water. The forward primers were labeled with the FAM, HEX or TAMRA fluorescent dyes at the 5' end. The PCR was performed on an Applied Biosystems 2720 Thermal Cycler system (Thermo Fisher Scientific, USA), using an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 1 min at a specific annealing temperature, 72 °C for 1 min and 5 min at 72 °C for a final extension. Amplified PCR products were analyzed on an ABI Prism 3,730XL DNA Analyzer (Thermo Fisher Scientific, USA), with a ROX 500 size standard. Raw genotyping data were scored using GeneMarker 3.2 (SoftGenetics, USA).

Estimation of genetic diversity

MicroChecker software (Van Oosterhout *et al*., 2004) was used to check for null alleles. For microsatellite loci and stocks, deviation from Hardy-Weinberg equilibrium (HWE) was assessed before sequential Bonferroni correction (Gaetano, 2018), based on Arlequin 3.5 (Excoffier and Lischer, 2010). Genetic diversity parameters, including number of different alleles (Na), number of effective alleles (ne), allele frequencies, observed heterozygosity (Ho), unbiased expected heterozygosity (uHe) and expected heterozygosity (He), were estimated by GenAlEx 6.503 (Peakall and Smouse, 2006). Allelic richness (Ar) was calculated by HP Rare 1.0 (Kalinowski *et al*., 2006), in terms of sample size. The Wright's inbreeding coefficient (F_{is}) was analyzed using Genepop 4.7 (Rousset, 2008). The mean polymorphism information content and null allele frequency (Null) were calculated by Cervus 3.0.7 (Kalinowski *et al*., 2007). The individual relatedness within each population was estimated using ML-Relate (Kalinowski *et al*., 2006). Mean pairwise relatedness, with 95% confidence intervals, were performed in SPSS 24.0 (IBM, USA).

Significance was adjusted by sequential Bonferroni correction under multiple comparisons (Gaetano, 2018). To understand genetic deviation across years, differences in ne, Ar, uHe and F_{is} between each year were compared. Shapiro-Wilk tests were initially computed for normality, before paired *t*-test or Wilcoxon signed-rank tests were conducted. All statistical analyses were performed on SPSS 24.0 (IBM, USA).

Genetic differentiation evaluations

To analyze the genetic subdivisions of *F. chinensis* populations in their natural habitat over a given period, molecular variance (AMOVA) and the fixation index (F_{ST}) between each pair of sampling stock were assessed using Arlequin 3.5.2 (Excoffier and Lischer, 2010). Gene flow (Nm) was also calculated in Arlequin 3.5.2. The software structure 2.3.4 (Falush *et al*., 2003; Pritchard *et al*., 2000) was used to identify the number of clusters (K) for our microsatellite genotypes. Ten independent runs were performed for different numbers of clusters, each with a burn-in period of 100,000, followed by 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. Structure Harvester Web (Earl and Vonholdt, 2012; Pritchard *et al*., 2000) was applied to determine K values based on mean values of lnP(K). To investigate whether any *F. chinensis* stocks experienced a genetic bottleneck, sign tests and 2-tailed Wilcoxon tests for heterozygosity excess were computed under the two-phased model of mutation (TPM), using Bottleneck 1.2.02 software (Cornuet and Luikart, 1996). In our analysis, a variance among multiple steps of 30 and a proportion of single-step mutations of 70% was set. To calculate the probability of the distribution, 1000 simulation replicates were conducted. Qualitative model-shift tests were also applied to compute allele frequency distribution.

RESULTS

Estimation of genetic diversity

Our data were analyzed across a five year period in Jinzhou bay (Table I, Supplementary Tables SII, SIII and SIV). We detected no evidence of genotypic errors induced by allele dropout or stuttering. In total, 510 different alleles were observed for all loci and samples. All loci exhibited high polymorphic levels, except locus FC22, which had five alleles. The highest polymorphic level was observed at locus EN0033, which had 78 alleles. The average number of Na, ne and Ar ranged from 20.923–30.308, 10.529–15.869 and 19.953–29.350, respectively. For most loci (29 out of 39), the uHe was larger than Ho, which showed obvious homozygote excess. This result was in agreement with the F_{is} analysis, in which positive values were observed for all stocks. For all individuals collected in 2015, 2016 and 2019, from Jinzhou bay, a deviation from HWE was detected after sequential Bonferroni correction (Table I), especially for the EN0033, FC06, FCKR009, Hd3169 and Rs0676 loci (five out of 13), which exhibited Hardy-Weinberg disequilibrium in all the stocks. In general, the smallest mean values of Na, ne, Ar, Ho, He and uHe were observed in JZ2019 stocks. Moreover, although there was no significant difference in F_{is}, significant differences appeared for Na, ne, Ar and uHe between JZ2019 and the two stocks from previous years, following sequential Bonferroni correction ($P < 0.05$) (Tables II, III). In addition, the mean relatedness value in JZ2019 was higher than in JZ2015 and JZ2016 (Table I).

Population genetic differentiation

Weak but significant genetic differentiation between JZ2019 and the other stocks (for JZ2015 and JZ2019, pairwise $F_{st} = 0.03291$; for JZ2016 and JZ2019, pairwise $F_{st} = 0.02875$) was observed ($P < 0.05$), even though samples were taken from the same locality. Gene flow (Nm) results agreed with F_{st} evidence, which suggested that limited Nm occurred between JZ2019 and the two stocks of previous years (JZ2015 and JZ2016) (Table IV). An AMOVA hierarchical analysis showed that 89.13% of genetic variation could be attributed to within individuals. However, the variance was significant when considering among stocks, among individuals within stocks and within individuals (Table V). Structure analyses revealed the highest support for two clusters, based on the delta K value (Supplementary Fig. S1). Our results showed that almost all the individuals from JZ2019 were assigned to the first cluster, while the individuals from JZ2015 or JZ2016 stock were evenly assigned to the first and second cluster (Supplementary Fig. S2). Both sign and Wilcoxon tests were not significant in estimating the probability of

Table I. Genetic diversity information for studied *F. chinensis* stocks distributed in Jinzhou Bay.

Stocks	Sample locations	Date	Sample numbers (N)	No. of different alleles (Na)	No. of effective alleles (ne)	Allelic richness (Ar)	Observed heterozygosity (Ho)	Expected heterozygosity (He)	Unbiased expected heterozygosity (uHe)	Wright's inbreeding coefficient (Fis)	Polymorphism information content (PIC)	Hardy-weinberg equilibrium deviation test (HWE)	Mean relatedness ± 95% CI
JZ2015	Jinzhou Bay	2015.8	91	30.308	15.869	28.506	0.788	0.893	0.898	0.131	0.881	***	0.0372 ± 0.002
JZ2016	Jinzhou Bay	2016.8	92	31.154	14.889	29.35	0.793	0.893	0.898	0.126	0.882	***	0.0349 ± 0.002
JZ2019	Jinzhou Bay	2019.8	94	20.923	10.529	19.953	0.738	0.841	0.846	0.121	0.827	***	0.040 ± 0.002

Significant levels of HWE deviation test are presented by asterisks as ns: conform to HWE, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$.

Table II. Paired *t*-test of Wright's inbreeding coefficient (F_{is}), the number of different alleles (Na) and allelic richness (Ar) between each year of studied *F. chinensis* stocks in Jinzhou Bay.

	Paired differences				t	df	P (2-tailed)	P'
	Mean	standard deviation	95% CI of difference					
			Upper	Lower				
Wright's inbreeding coefficient (F_{is})								
JZ2015-JZ2016	0.005	0.022	-0.045	0.055	0.223	12	0.827	1
JZ2015-JZ2019	0.01	0.046	-0.089	0.11	0.226	12	0.825	1
JZ2016-JZ2019	0.005	0.039	-0.079	0.09	0.135	12	0.895	1
No. of different alleles (Na)								
JZ2015-JZ2016	-0.846	1.067	-3.171	1.479	-0.793	12	0.443	0.443
JZ2015-JZ2019	9.384	2.229	4.528	14.241	4.21	12	0.001	0.002
JZ2016-JZ2019	10.23	1.772	6.368	14.093	5.771	12	0	0
Allelic richness (Ar)								
JZ2015-JZ2016	-0.844	0.84	-2.675	0.986	-1.005	12	0.335	0.335
JZ2015-JZ2019	8.552	1.973	4.253	12.852	4.334	12	0.001	0.002
JZ2016-JZ2019	9.397	1.625	5.855	12.939	5.78	12	0	0

Significance without (*P*) or with sequential Bonferroni correction (*P'*) are shown in bold.

Table III. Wilcoxon signed-rank test of no. of effective alleles (ne) and unbiased expected heterozygosity (uHe) between each year of studied *F. chinensis* stocks in Jinzhou Bay.

	JZ2015-JZ2016	JZ2015-JZ2019	JZ2016-JZ2019
No. of effective alleles (ne)			
Z	-1.013	-3.11	-3.18
P	0.311	0.002	0.001
P'	0.311	0.004	0.003
Unbiased expected heterozygosity (uHe)			
Z	-0.245	-3.112	-3.185
P	0.806	0.002	0.001
P'	0.806	0.004	0.003

Significance without (*P*) or with sequential Bonferroni correction (*P'*) are shown in bold.

Table IV. Estimation of F_{ST} (below the diagonal) and Nm (gene flow, above the diagonal) of studied *F. chinensis* stocks distributed in Jinzhou Bay.

Stocks	JZ2015	JZ2016	JZ2019
JZ2015	/	486.84	7.35
JZ2016	0.00051	/	8.45
JZ2019	0.03291	0.02875	/

Significance with sequential Bonferroni correction (*P*) are shown in bold.

Table V. The AMOVA analysis of studied *F. chinensis* stocks distributed in Jinzhou Bay.

Source of variation	df	Sum of square	Variance composition	Percentage of variation	P
Among populations	2	51.08	0.1078 Va	2.04	0
Among individuals within populations	274	1544.077	0.46567 Vb	8.82	0
Within individuals	277	1303	4.70397 Vc	89.13	0
Total	553	2898.157	5.27744		0

Significance with sequential Bonferroni correction (*P*) are shown in bold.

heterozygote excess under the TPM model, which suggested that all stocks were at mutation-drift equilibrium (Supplementary Table SV). These statistical data also supported the L-shaped allele frequency curves and therefore indicated no genetic bottleneck in recent years. The above results indicated that weak temporal genetic differentiation had appeared in Jinzhou bay after continued stock enhancement programs over the study years.

DISCUSSION

Genetic diversity

The JZ2019 population had substantially lower Na, ne, Ar and uHe values than other years, following sequential Bonferroni correction ($P < 0.05$). Although the significant

test was not compared within populations, the highest pairwise relatedness value and the lowest Ho were also observed for JZ2019. These analyses suggested that long-term, large-scale stock enhancement may be related to the reduction of genetic diversity in local *F. chinensis* populations. Similar results were also observed for other high intensity enhancement programs, i.e., black sea bream (*Acanthopagrus schlegelii*), *P. major* and salmonid fish (Utter, 1998; Christie *et al*., 2012; Kitada *et al*., 2019; Shan *et al*., 2020). We observed significant deviations from HWE in all of our collected stocks. Meanwhile, five out of 13 microsatellite markers showed consistently significant Hardy-Weinberg disequilibrium across JZ2015, JZ2016 and JZ2019 stocks. This HWE deviation phenomenon also existed in other farmed populations, such as *P. major,* the swimming crab (*Portunus trituberculatus*) (Perez-Enriquez *et al*., 2001; Liu *et al*., 2018) and recently in *F. chinensis* wild populations in China (Wang *et al*., 2016; Song *et al*., 2018). Indeed, null alleles may cause heterozygote deficits and Hardy-Weinberg disequilibrium (Dharmarajan *et al*., 2013). In our study, in most cases, our microsatellite markers exhibited low null allele frequencies and only seven out of 39 cases showed relatively large allele frequencies (null > 0.2). Moreover, F_{is} values were positive for all of the stocks and ranged from 0.121–0.131, which inferred a heterozygote deficit in this study. The above results suggested that *F. chinensis* population inbreeding behaviors may have been widespread over the study period (Dakin and Avise, 2004). Due to a lack of genetic monitoring in *F. chinensis* enhancement programs, there is practically no regulation on how many parent shrimps are required to make up a broodstock suitable to produce one million seeds for release. At present, the number of parental spawners is determined by enhancement scale rather than genetic conservation assessment. Each female parent *F. chinensis* can spawn approximately 700,000 eggs and in some enhancement programs not all female broodstocks spawn. This can lead to parental reproduction bias (Dong *et al*., 2006) in this species as well in other stock enhancement species, i.e., jungle perch (*Kuhlia rupestris*) (Hoskin *et al*., 2015). These scenarios may aggravate the inbreeding behaviors of hatchery-reared seeds in enhancement programs (Wang *et al*., 2016; Song *et al*., 2018). In our study, it was reasonable to infer that the large-scale release of the seeds over a given period may contribute to the positive F_{is}, Hardy-Weinberg disequilibrium and declining genetic diversity in local *F. chinensis* populations in Jinzhou bay, although the precise mixing proportions of hatchery shrimp populations was not estimated here. Previous research has also shown that fitness could decrease with increasing inbreeding coefficients, such as in the banner-tailed kangaroo rat (*Dipodomys spectabilis*) (Willoughby *et al*., 2019), and inbreeding behaviors of shrimp appeared to reduce their survival and growth performances (Moss *et al*., 2007; Goyard *et al*., 2008). Another factor that cannot be ignored is that shrimp catch may also affect genetic diversities (Pinsky and Palumbi, 2014), despite the lack of real evidence in the present study.

Genetic differentiation

Our pairwise F_{st} data demonstrated that small but statistically significant levels of genetic differentiation were identified between JZ2019 and the two stocks of previous years. This finding was consistent with our genetic diversity comparisons. In addition, the value of Nm between JZ2015 and JZ2016 was larger than the values between JZ2019 and the two stocks of previous years. In considering the short life span of this species, the genetic structure of wild populations may be influenced by temporal genetic drift from hatchery release individuals replacing or mating with wild populations in the present study (Willoughby and Christie, 2019). The structure analysis showed two clusters, which suggested that JZ2019 may share a weak genetic structure with the two stocks of previous years. Our bottleneck data demonstrated that populations in Jinzhou bay did not undergo a recent genetic bottleneck.

CONCLUSIONS

Overall, our results indicated that long-term stock enhancement may be related to inbreeding and potentially the reduction of genetic diversity in *F. chinensis* populations. More sampling locations and more evaluation periods are required in the future to better understand the impacts of artificial stock enhancement on local populations.

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Statement of conflict of interest

The authors have declared no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20211104141137

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