Chapter

The Research of Population Genetic Differentiation for Marine Fishes (*Hyporthodus septemfasciatus*) Based on Fluorescent AFLP Markers

Yongshuang Xiao, Zhizhong Xiao, Jing Liu, Daoyuan Ma, Qinghua Liu and Jun Li

Abstract

Hyporthodus septemfasciatus is a commercially important proliferation fish which is distributed in the coastal waters of Japan, Korea, and China. We used the fluorescent AFLP technique to check the genetic differentiations between broodstock and offspring populations. A total of 422 polymorphic bands (70.10%) were detected from the 602 amplified bands. A total of 308 polymorphic loci were checked for broodstock I ($P_{\text{broodstock I}}$ = 55.50%) coupled with 356 and 294 for broodstock II ($P_{\text{broodstock II}}$ = 63.12%) and offspring ($P_{\text{offspring}}$ = 52.88%), respectively. The levels of population genetic diversities for broodstock were higher than those for offspring. Both AMOVA and F_{st} analyses showed that significant genetic differentiation existed among populations, and limited fishery recruitment to the offspring was detected. STRUCTURE and PCoA analyses indicated that two management units existed and most offspring individuals (95.0%) only originated from 44.0% of the individuals of broodstock I, which may have negative effects on sustainable fry production.

Keywords: genetic diversity, population structure, fluorescent AFLP technology, *Hyporthodus septemfasciatus*, sustainable management

1. Introduction

Genetic diversity is one of the most important natural properties for commercially interesting species, because it can influence the species' adaptive capacity to environmental change, and loss of genetic variation is destructive to domestication of cultured stocks [1, 2]. Limited effective population size and the effects of artificial selection on hatchery progeny may lead to population genetic drift, which will cause offspring to differentiate from broodstock [3]. Monitoring population genetic diversity and genetic structure, therefore, has become a key aspect and long-term mission for the success of commercial breeding programs [4].

Seven-band grouper, *H. septemfasciatus* (Thunberg, 1793), belongs to the family Serranidae and is distributed in waters of Korea, Japan, and China. It is a sedentary, reef-associated species and lives in shallow water (5–30 m) [4]. *H. septemfasciatus* is a commercially important marine fish with rapid growth and low-temperature tolerance characteristics, which make the species as proliferation fish for cage culture and marine ranching in the coastal waters of Japan, Korea, and China [4–6]. Although the artificial breeding processes for *H. septemfasciatus* have been established, crucial technique problems of artificial breeding with female-fish sex reverse, artificial induction of natural spawning, and treatment of virus diseases for hatched larvae are needed to be solved urgently [4, 5]. Seven-band grouper is a protogynous hermaphrodite fish, which begins life as a female before becoming a male. Until now, almost all of seven-band grouper hatcheries maintain wild-caught sources of *H. septemfasciatus* as broodstock for artificial reproduction [4]. The limited male broodstock (given the extended maturation age) may limit the genetic diversity in breeding programs [3]. The excellent traits for species were always derived from rich genetic variations. In order to avoid the detrimental loss of genetic variation during the artificial propagation process, it is necessary to frequently monitor the genetic diversity and population structure for *H. septemfasciatus*.

Research reports of genetic diversity and population structure between wild and hatchery populations of *H. septemfasciatus* have been carried out based on the microsatellite DNA loci [3, 4]. These genetic studies revealed reduced genetic variability in hatchery populations compared with wild populations, based on microsatellite analyses. Significant population genetic differentiation was also examined among the hatchery population in South Korea [3, 4]. AFLP is a PCR-based, multi-locus fingerprinting technique that can detect genetic variations effectively. It combines the strengths and overcomes the weaknesses of the RFLP and RAPD methods. Until now, AFLP has been proven to be successful in studying population genetic structure and differentiation of plants, animals, and some fish species such as *Carassius auratus*, *Pleuronectes yokohamae*, and *Synechogobius ommaturus*. Until now, research of the population diversity and genetic differentiation between broodstock and offspring from Japan was not reported. In the present research, different fluorescent AFLP markers were used to estimate population genetic variabilities of broodstock and offspring from Japan and Korea. The results will provide a useful genetic basis for future planning of sustainable culture and management of *H. septemfasciatus* in fisheries.

2. Population genetic differentiation for marine fishes based on fluorescent AFLP markers

2.1 Materials and methods

2.1.1 Material information

Sixty-two specimens with two broodstock and one offspring populations were gathered from Korea (broodstock II), Japan (broodstock I), and China (offspring—Rizhao) during May 2014 to September 2014 (**Figure 1**, **Table 1**). We used the *Epinephelus moara* as an outgroup with six individuals sampled from Guangzhou. The taxonomic status of 62 specimens was confirmed based on the measures and segment features, and the fin-clip tissue was collected from each specimen and preserved in 100% ethanol.

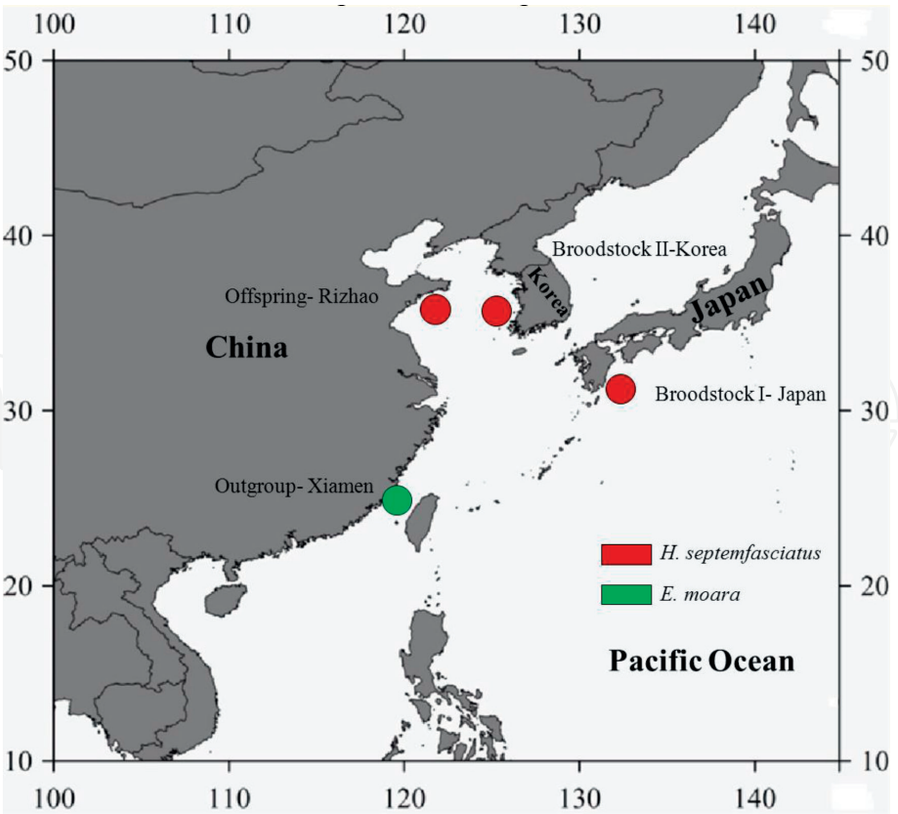

Figure 1.
Sampling location of H. septemfasciatus.

2.1.2 Genomic DNA extraction and AFLP processes

We used a standard phenol-chloroform method to extract the genomic DNA from the fin-clip tissue with proteinase K digestion. The process of AFLP experiment was followed with the procedures developed by Vos et al. [7] and Wang et al. [8]. The genomic DNA with about 100 ng was digested using 1 unit of *Eco*RI and *Mse* I (New England Biolabs Inc., UK) at 37°C for 6 h. We used the ligation system with 1 μL 10× ligation buffer, 5 pmol *EcoR*I adapter (*EcoR*I-1/*Eco*RI-2; **Table 1**), 50 pmol *Mse*I adapter (*Mse*I-1/*Mse*I-2; **Table 1**), and 0.3 unit of T4 DNA ligase (Takara Bio Inc., China) to ligate double-stranded adapters and the restriction fragments together at 20°C overnight. We used TaKaRa Thermal Cycler to conduct the pre-amplification PCR for AFLP with a pair of primers including a single selective nucleotide. The annealing temperature for amplification was 53°C with length of 30 s. Then, the PCR product was diluted tenfold and used as templates for the subsequent selective PCR amplification. We used the FAM fluorescent to label the primer *EcoR*I (FAM-E-AAC, FAM-E-ACA, FAM-E-ACC) (**Table 1**). We used the PCR system with 1 μL pre-amplification product, 1× PCR buffer, 150 μM of each dNTP, 30 ng of each selective primer, and 0.5 unit of *Taq* DNA polymerase to conduct the selective amplifications. The gradient thermocycler with a touchdown cycling procedure of nine cycles of 30 s at 94°C, 30 s at 65°C (−1°C at each cycle), and 30 s at 72°C followed by the cycling procedure of 28 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C was used to PCR. The final step was a prolonged extension at 72°C with the length of 7 min. The quality of selective amplification product was checked by electrophoresis in a 4% denaturing polyacrylamide gel (DPG) at 50 W (maximum to 3,000 V) for 2.4 h in ABI Prism 377 DNA Sequencer (Applied Biosystems, USA). Sequences of AFLP adapters and primers are listed in **Table 1**.

Primer pairs	Population	*I*	*He*	*Ne*	*Hw*/*Ht*	G_{st}
FAM-E-AAC/ M-CTC	Rizhao	0.190 ± 0.029	0.125 ± 0.020	1.211 ± 0.037	0.200/0.227	0.116
	Japan	0.199 ± 0.030	0.131 ± 0.021	1.223 ± 0.038		
	Korea	0.266 ± 0.031	0.176 ± 0.022	1.297 ± 0.040		
	Average	0.218 ± 0.017	0.144 ± 0.012	1.244 ± 0.022		
	E. moara	0.152 ± 0.027	0.098 ± 0.018	1.162 ± 0.034		
FAM-E-AAC/ M-CTG	Rizhao	0.234 ± 0.027	0.153 ± 0.018	1.249 ± 0.032	0.229/0.256	0.107
	Japan	0.232 ± 0.027	0.153 ± 0.019	1.260 ± 0.036		
	Korea	0.272 ± 0.027	0.177 ± 0.019	1.290 ± 0.034		
	Average	0.246 ± 0.016	0.161 ± 0.011	1.266 ± 0.019		
	E. moara	0.195 ± 0.027	0.130 ± 0.019	1.225 ± 0.036		
FAM-E-ACA/ M-CAT	Rizhao	0.196 ± 0.024	0.129 ± 0.017	1.214 ± 0.030	0.199/0.249	0.197
	Japan	0.242 ± 0.025	0.159 ± 0.017	1.265 ± 0.032		
	Korea	0.237 ± 0.025	0.155 ± 0.017	1.260 ± 0.032		
	Average	0.225 ± 0.014	0.148 ± 0.010	1.246 ± 0.018		
	E. moara	0.157 ± 0.023	0.104 ± 0.016	1.176 ± 0.029		
FAM-E-ACA/ M-CTT	Rizhao	0.254 ± 0.024	0.168 ± 0.017	1.284 ± 0.030	0.234/0.266	0.119
	Japan	0.227 ± 0.023	0.148 ± 0.016	1.250 ± 0.030		
	Korea	0.306 ± 0.024	0.201 ± 0.017	1.338 ± 0.031		
	Average	0.262 ± 0.014	0.173 ± 0.010	1.291 ± 0.018		
	E. moara	0.150 ± 0.022	0.102 ± 0.015	1.180 ± 0.029		
FAM-E-ACC/ M-ACT	Rizhao	0.298 ± 0.022	0.199 ± 0.016	1.343 ± 0.029	0.257/0.285	0.099
	Japan	0.287 ± 0.022	0.191 ± 0.016	1.328 ± 0.029		
	Korea	0.305 ± 0.021	0.201 ± 0.015	1.340 ± 0.029		
	Average	0.297 ± 0.013	0.197 ± 0.009	1.337 ± 0.017		
	E. moara	0.234 ± 0.022	0.157 ± 0.016	1.274 ± 0.029		
Total	Rizhao	0.243 ± 0.011	0.161 ± 0.008	1.271 ± 0.014	0.229/0.261	0.124
	Japan	0.244 ± 0.011	0.161 ± 0.008	1.272 ± 0.015		
	Korea	0.281 ± 0.011	0.185 ± 0.008	1.310 ± 0.015		
	Average	0.256 ± 0.007	0.169 ± 0.005	1.284 ± 0.008		
	E. moara	0.182 ± 0.011	0.121 ± 0.008	1.210 ± 0.014		

I, Shannon diversity index; He, expected heterozygosity; Ne, no. of effective alleles; Hw, gene diversity within populations; Ht, population genetic structure.

Table 1.
Parameters of genetic diversity for three populations of H. septemfasciatus.

Similarity indices were calculated using the formula S = 2Nab/(Na + Nb), where Na and Nb were the number of bands in individuals a and b, respectively, and Nab was the number of sharing bands. Genetic distances between individuals were computed using the formula D = −ln S. Genetic relationship among populations was constructed based on unweighted pair-group method analysis (UPGMA) by TFPGA [9].

2.2 Data analysis

2.2.1 AFLP data analysis

We used the ABI 3730xl Genetic Analyzer (Applied Biosystems) to check the PCR products, which were genotyped by using the internal size standard LIZ 500 (Applied Biosystems) and scored using the GeneScan3.1 software (Applied Biosystems) in Shanghai Personal Biotechnology Co., Ltd.

2.2.2 Genetic variability parameter analyses

The fragment size with length ranging from 70 to 1000 bp was used for further analyses. Firstly, the clear and unambiguous AFLP bands were scored with 1 or 0, and then we transformed the bands into 0/1 binary matrix. The genetic variability parameters with polymorphic bands, effective number of alleles per loci (*Ne*), expected heterozygosity (*H*), Shannon's information index (*I*), and Nei's genetic distances were calculated by GenALEx 6.501 [10]. The parameters of genetic structure (*Ht*) among populations were also evaluated by AFLP-SURV [11].

Genetic differentiation between population pairs was evaluated by fixation indices F_{st} and Nei's genetic distance using Arlequin [12] and GenALEx 6.501 [10] software. When multiple comparisons were performed, *P* values were adjusted using the sequential Bonferroni procedure. Analysis of molecular variance (AMOVA) [13] was employed to further examine hierarchical population structure as well as the geographical pattern of population subdivision [13]. We used the software STRUCTURE version 2.3.3 with a Bayesian model to evaluate genetic structures of the *H. septemfasciatus* populations [14]. The admixture model and independent allelic frequencies were used to analyze the data set and the length of the burn-in period and a number of MCMC reps after burn-in were set to 25,000 and 100,000, respectively. These steps were used to determine the ancestry value, which estimated the proportion of an individual's genome that originated from a given genetic group. The algorithm was run 10 times for each K value, from 1 to 4.

2.3 Results

2.3.1 Polymorphic information of different AFLP primers

To remove false-positive bands, a total of 602 clear and unambiguous bands were detected from two broodstock and one offspring populations by using five-pair selective primers. A total of 422 polymorphic bands (70.10%) were detected from the 602 amplified bands (**Table 1**). The average bands of polymorphic sites for five-pair primers were 84.4, which were ranged from 52 to 129 (**Table 1**). The maximum of polymorphic bands was detected in the FAM-E-ACC/M-ACT primer pair (129 bands), and the minimum of polymorphic bands was found in the FAM-E-AAC/M-CTC primer pair (52 bands). Three hundred and eight polymorphic loci were checked for broodstock I ($P_{\text{broodstock I}}$ = 55.50%) coupled with 356 and 294 for broodstock II ($P_{\text{broodstock II}}$ = 63.12%) and offspring ($P_{\text{offspring}}$ = 52.88%), respectively. Different patterns of polymorphic sites were detected between broodstock and offspring populations of *H. septemfasciatus* from the same primer pair. Two hundred and eighty-five bands were found from the individuals of *E. moara*, 74.74% of which (213) were polymorphic (**Table 1**). Special bands were also detected based on the five-pair selective primers, which ranged from 1 to 11 for broodstock I (Japan,

n = 1), broodstock II (Korea, n = 11), and offspring (n = 8) populations (**Table 1**, **Figure 2**). The number of special bands was 317 for *E. moara* compared with *H. septemfasciatus*.

2.3.2 Genetic variability of H. septemfasciatus

The average values of *Ne* for broodstock I (Japan), broodstock II (Korea), and offspring were 1.272 ± 0.015, 1.310 ± 0.015, and 1.271 ± 0.014, respectively. The effective alleles of broodstock II (Korea) were higher than that of broodstock I (Japan). And the average value of *Ne* for both broodstock and offspring populations was 1.284 ± 0.008. The average value of expected heterozygosity for broodstock II population was higher than those of broodstock I (*He* = 0.161 ± 0.008) and offspring (*He* = 0.161 ± 0.008) populations. The homogeneous trend was also detected from the parameter of Shannon diversity index (*I*) in broodstock ($I_{\text{broodstock II}}$ = 0.281 ± 0.011 and $I_{\text{broodstock I}}$ = 0.244 ± 0.011) and offspring ($I_{\text{offspring}}$ = 0.243 ± 0.011) populations. The total *He* and *I* for the three populations of *H. septemfasciatus* were 0.169 ± 0.005 and 0.256 ± 0.007, respectively. The parameter of genetic diversity within population (*Hw*) ranged from 0.199 to 0.257, and the average value of *Hw* was 0.229 for the three populations (**Table 1**). According to the results of *Ne*, *He*, and *I* parameters, although different primer pairs showed different levels of genetic diversity, the broodstock II showed the highest level of genetic diversity among the three populations (**Table 1**).

Though the five primer pairs showed different genetic differentiations among populations, the values of genetic structure (*Ht*) parameter ranged from 0.227 to 0.285, which showed significant genetic structure existed among populations

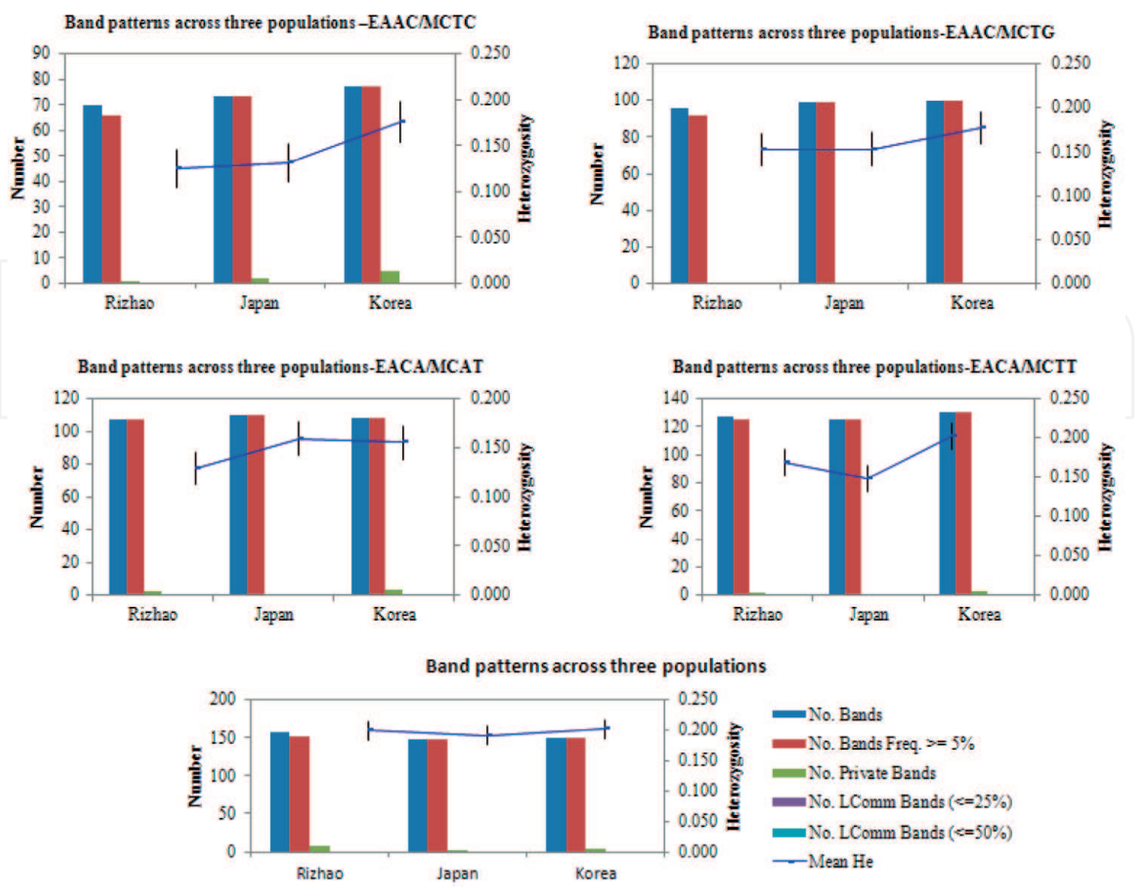

Figure 2.
Number of bands based on different AFLP primers for H. septemfasciatus.

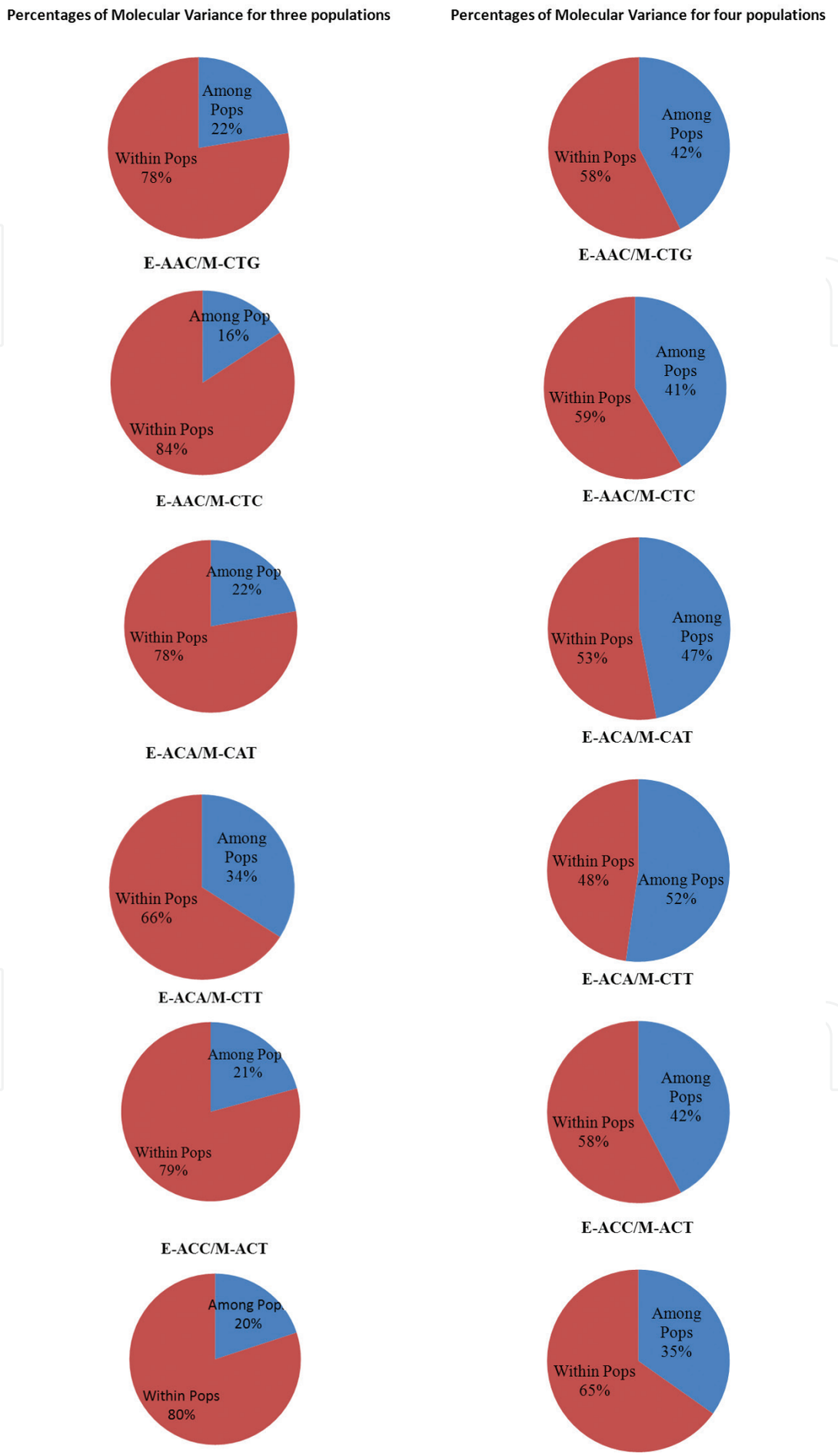

Figure 3.
AMOVAs based on different AFLP primers for H. septemfasciatus.

(**Table 1**). The AMOVA showed that 77.63% of variation was derived within populations and 22.37% of genetic variation happened among populations. The genetic variations were further estimated by each of the five primer pairs, which indicated more than 15.83% genetic variation originated from the variation among populations. In addition to the AMOVA and *Ht*, the values of genetic differentiation ranged from 0.099 to 0.197 based on the G_{st} parameters (**Figure 3**). The genetic structure was also detected by the pairwise F_{st} analysis, which showed the F_{st} values ranging from 0.169 to 0.292 among broodstock and offspring populations (**Figure 4**). And the largest value was detected between Korea and offspring (F_{st} = 0.292, $P < 0.01$) populations, whereas the lowest value was found between Japan and Korea (F_{st} = 0.169, $P < 0.01$) populations. The Nei's genetic distances among populations were ranged from 0.048 to 0.083. The result was similar with the pairwise F_{st} analysis, and the largest value was found between Korea and offspring (D = 0.083) populations, whereas the lowest value was checked between Japan and Korea (D = 0.048) populations (**Figure 5**).

We used the software of STRUCTURE to estimate the ancestor composition based on the coalescent theory. Three clusters were checked between broodstock and offspring populations based on a clear maximum for ΔK at K = 3 calculated by the MCMC method (**Figures 6** and **7**). Cluster 1 was mainly composed of offspring (F = 95.0%) and broodstock I (Japan, F = 44.0%) populations, and the contribution of broodstock II (Korea) to the offspring was only 9.1%. Cluster 3 was mainly originated from the broodstock I (F = 55.9%) and broodstock II (F = 90.3%), and the contribution of them to the offspring was only 4.9%. Cluster 2 was occupied by all the individuals of *E. moara* (F = 98.1%). Three groups of the specimens were also verified by principal coordinates analysis (PCoA), which showed the individuals of offspring population being derived from the broodstock I and broodstock II, respectively. The individuals of *E. moara* formed the independent group (**Figure 8**).

The relationship of individuals was further illustrated by a dendrogram using the UPGMA algorithm based on Nei's genetic distance (**Figure 5**). Significant genealogical structure was detected in *H. septemfasciatus*. The dendrogram showed that three significant genealogical branches (Clade A, Clade B, and outgroup) corresponding to sampling localities of Japan and Korea existed in the species, respectively (**Figure 9**). Clade A was composed of Korea population. Most individuals of

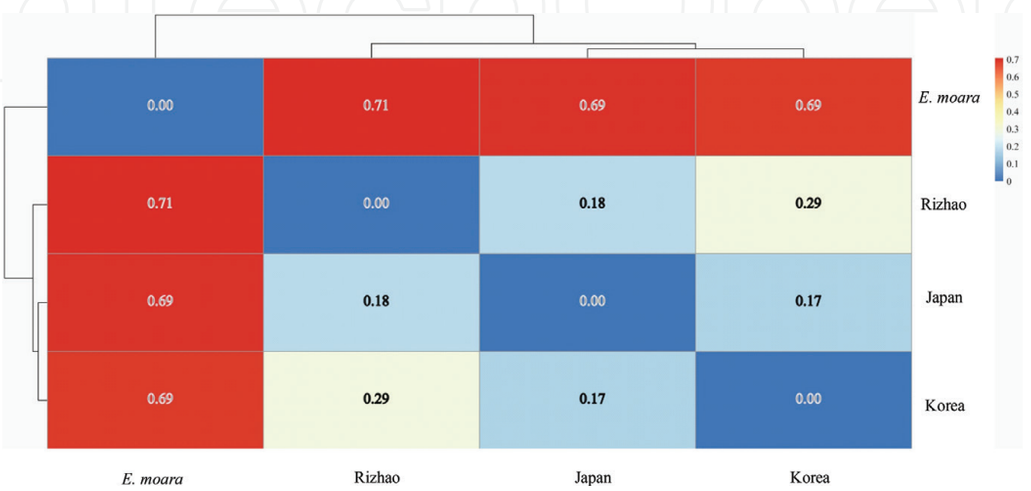

Figure 4.
Pairwise F_{st} among populations of H. septemfasciatus. All of the values were significant ($p < 0.01$).

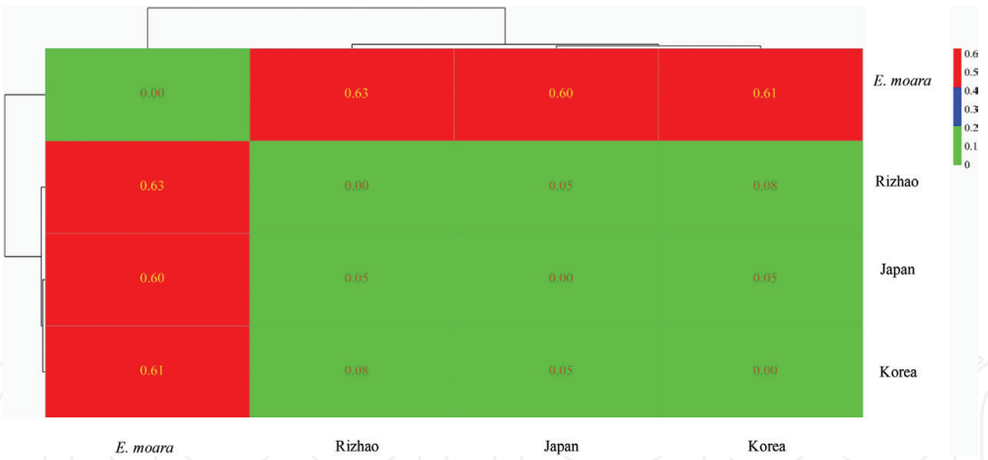

Figure 5.
Nei's genetic distance among populations of H. septemfasciatus.

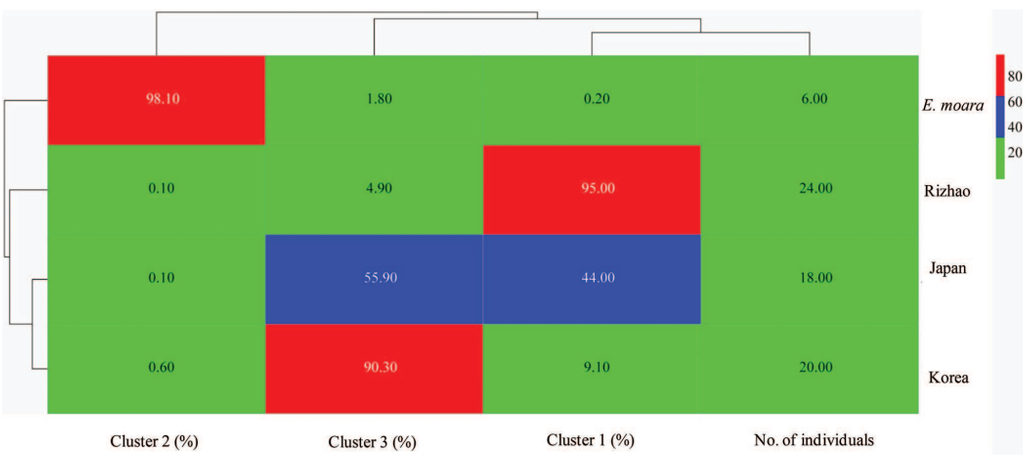

Figure 6.
The distribution of individuals of three populations in two genetic groups inferred by STRUCTURE.

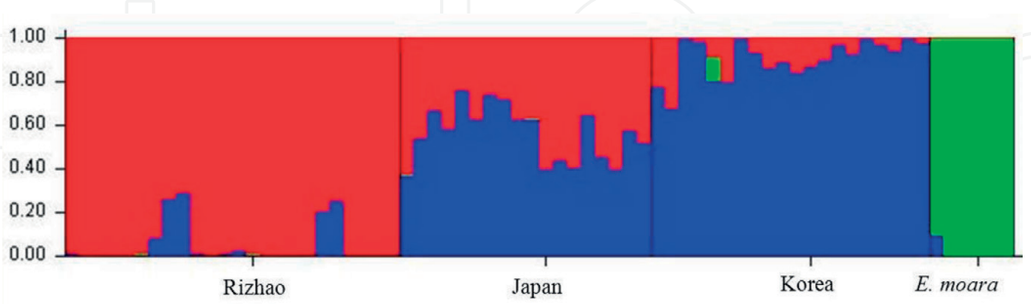

Figure 7.
Clustering of three populations for H. septemfasciatus. Each individual is shown as a vertical line divided into segments representing the estimated membership proportion in the three ancestral genetic clusters inferred from STRUCTURE.

Japan population and whole individuals of Rizhao population formed Clade B. The relationship among populations was also carried out based on Nei's genetic distance by TFPGA, and near relationship between Japan and Rizhao populations was checked.

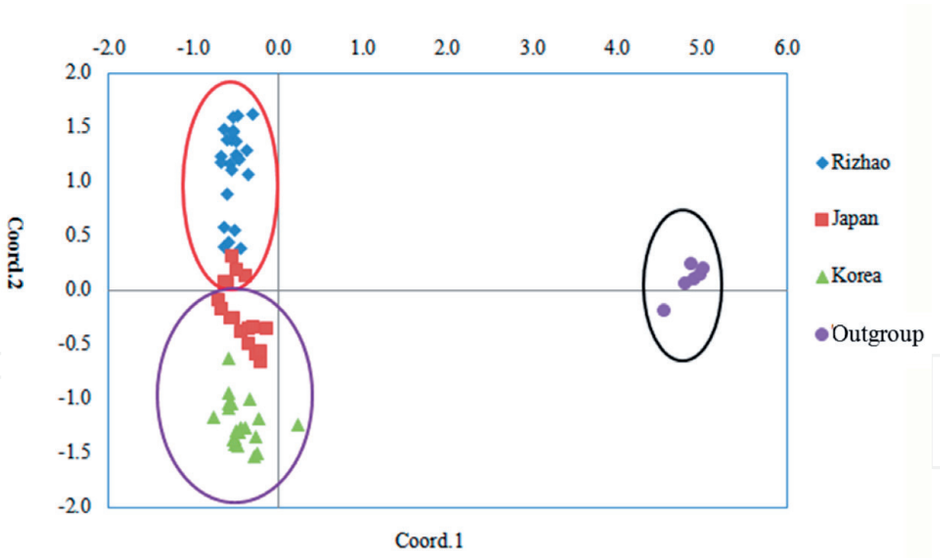

Figure 8.
Principal coordinates analysis (PCoA) of three populations of H. septemfasciatus.

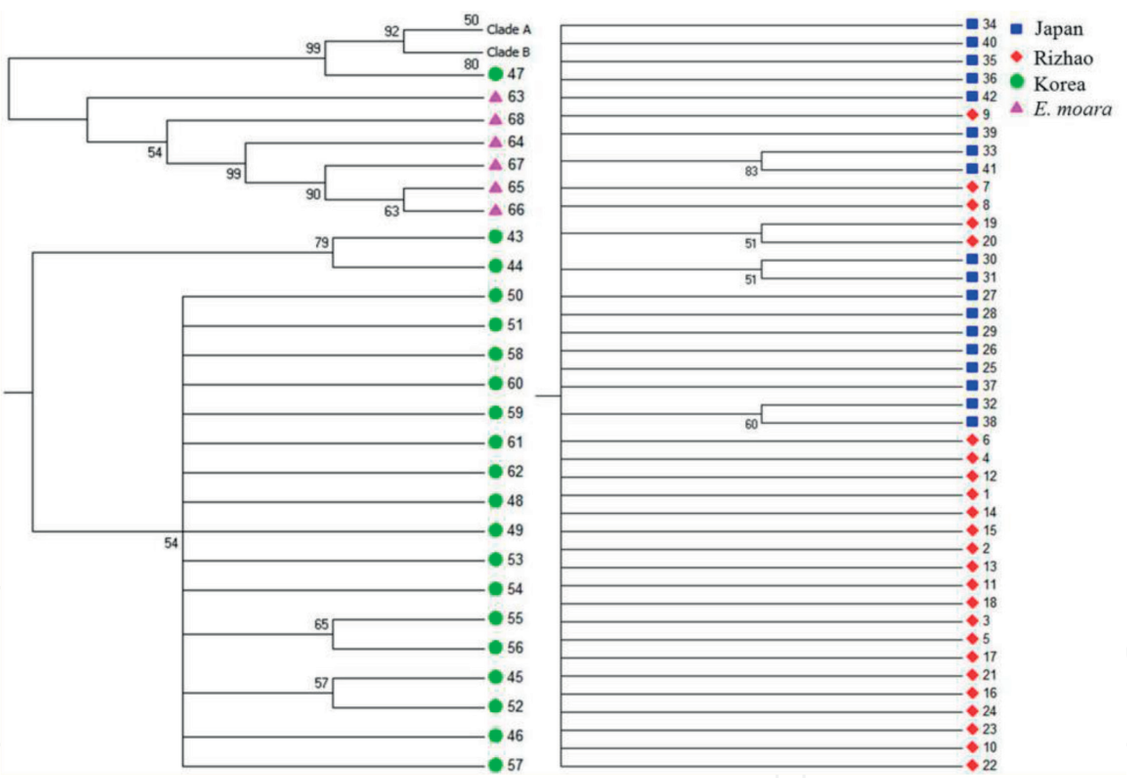

Figure 9.
UPGMA tree of individuals of H. septemfasciatus based on Nei's genetic distance.

3. Conclusions

In this study, significant genetic differentiations were checked among the broodstock and offspring populations of *H. septemfasciatus* by using F_{st}, AMOVA, and STRUCTURE analyses. The broodstock I population from Japan was a significant divergent from the broodstock II population from Korea. The contribution of broodstock I (Japan, F = 44.0%) to the offspring population reached up to 95.0%; on the contrary, 55.9% of broodstock I individuals and 90.3% of broodstock II individuals only have contributed 4.9% of individuals to the offspring population.

According to the STRUCTURE, PCoA, and UPGMA algorithm, two management units were checked from the broodstock and offspring populations based on the five fluorescent AFLP primer pairs. The differentiation among populations was not only originated from the genetic variabilities between broodstock I population (Japan) and broodstock II populations (Korea) but also originated from the low number of founding individuals in the hatcheries, especially for the broodstock II (Korea). The passive founder effect could lead to serious genetic drift and significant genetic differentiation, which has been well documented in other fish species [15].

High level of polymorphic sites (70.10%) was detected for *H. septemfasciatus* based on the fluorescent AFLP technique, which was higher than those of other fishes using general AFLP technique (18.6–55.8%) [16, 17]. We checked significant degradation of genetic diversity in the two broodstock and one offspring populations of *H. septemfasciatus* (P = 70.10%, I = 0.256, H_e = 0.169, N_e = 1.284) compared with wild populations (P = 98.73%, I = 0.288, H_e = 0.189, N_e = 1.319) of other rock reef fish species using fluorescent AFLP with similar primer pairs [18]. The parameters of genetic diversity in the wild-caught broodstock II population (I = 0.281, H_e = 0.185, N_e = 1.310) from Korea was higher than that of wild-caught broodstock I population (I = 0.244, H_e = 0.161, N_e = 1.272) from Japan. The level of genetic diversity of offspring population was similar with that of broodstock I population from Japan.

The genetic variability is one of the most crucial foundations for the conservation of marine species from long-time objective. Although significant degradation of genetic variations has been previously reported between broodstock and offspring in other marine fishes, no significant reduction of genetic diversity was checked between broodstock I (Japan, I = 0.244, H_e = 0.161, N_e = 1.272) and offspring (I = 0.243, H_e = 0.161, N_e = 1.271) populations in this study [3, 4, 19]. High levels of genetic diversity in wild populations of fish can be related to large effective population size, environmental heterogeneity, life history traits, and genetic divergence [18]. The value of heterozygosity derived from significant geographic difference for *Mugil cephalus* in coastal waters of China was higher than that in Florida based on AFLP analysis [20, 21]. The offspring population of *H. septemfasciatus* may have experienced passive founder effect derived from small population size of broodstock and protogynous hermaphrodite characteristics for grouper fish. Significant reduction of genetic diversity for offspring population may occur compared with broodstock population. On the contrary, the offspring of *H. septemfasciatus* harbored a high level of genetic variability compared with broodstock I population in the present study. Therefore, we believed that significant heterozygosity might exist in the offspring to maintain the high level of genetic diversity.

Significant genetic differentiations were detected between broodstock I population from Japan and broodstock II population from Korea for *H. septemfasciatus* based on AMOVA, F_{st}, STRUCTURE, PCoA, and UPGMA tree analyses. All the results showed that the achieved hatchery progeny of *H. septemfasciatus* might be a composite population originated from broodstock I and broodstock II populations. It was confirmed that the offspring of *H. septemfasciatus* was a composite population, and 95.0% of offspring individuals was derived from 44.0% of broodstock I individuals and 9.1% of broodstock II individuals. The left individuals of offspring (5.0%) were originated from 55.9% of broodstock I individuals and 90.3% of broodstock II individuals. Limited effective population size of the broodstock was always responsible for the pattern of genetic differentiation [3, 4]. Hence, genetic drift has probably played an important role in causing offspring to differentiate from broodstock [4]. In fact, no significant reduction of genetic diversity was checked in the offspring originated from limited population size of broodstock. The offspring of *H. septemfasciatus* was a composite population, which supported the

finding for *H. septemfasciatus* with a high level of genetic diversity compared with broodstock I. The present results indicated that broodstock with different genetic backgrounds could make contributions to the maintenance of genetic diversity for compounded offspring. The results of the present study also supported the suggestion that the effective broodstock management procedure be carried out to maintain the genetic diversity of hatchery stocks. It was also reported that added effective population size of broodstock with genetic differentiation backgrounds might be responsible for the reproductive success of grouper [22]. In relation to the management of hatcheries, the geographic sources of different broodstock should be labeled to avoid inbreeding [3, 4]. Hence, continued genetic monitoring of broodstock is necessary for hatchery progeny [3, 4].

Understanding of population genetic diversity and genetic structure has become a key aspect and long-standing issue in speciation and biological conservation. Uncovering the situation of marine population genetic diversity and gene flow is critical for the decision about sustainable exploitation. Evaluating population genetic diversity and structure also can be a vital tool for managing and maintaining a productive fishery [23]. Severe population size declines can also result in the loss of genetic diversity [24]. These results also indicate that genetic drift has led to negative effects on the reproductive capacity of the stock, which may have resulted in significant genetic differentiation between broodstock and offspring populations. The present study of *H. septemfasciatus* indicated at least two management units existed in the wild populations from Japan and Korea, which has supported crucial genetic information to ensure the success of ongoing breeding and stock enhancement programs. Thus, the monitor and management with fixed frequency to hatchery populations will be necessary to ensure the success of artificial seed production [3, 4]. Our data provide a useful genetic basis for future planning of sustainable culture and management of *H. septemfasciatus* in fisheries.

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Conflict of interest

The authors declare that they have no competing interests.

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Author details

Yongshuang Xiao[1,2,3*†], Zhizhong Xiao[1,2,3†], Jing Liu[1,2,3*], Daoyuan Ma[1,2,3], Qinghua Liu[1,2,3] and Jun Li[1,2,3*]

1 Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China

2 Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

3 Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China

*Address all correspondence to: dahaishuang1982@163.com, jliu@qdio.ac.cn and junli@qdio.ac.cn

† These authors equally contributed to this work.

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