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Genetic variability of mass-selected and wild populations of Iwagaki oyster (*Crassostrea nippona*) revealed by microsatellites and mitochondrial COI sequences

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ABSTRACT

Crassostrea nippona has recently been identified as a potential aquaculture species for its high glycogen content and delicate flavor in summer when *Crassostrea gigas* suffer from low meat quality. In 2014, we initiated the selective breeding program to improve growth traits of *C. nippona* through successive generations of mass selection, yet the genetic impact of an intense artificial selection on the genetic variability of *C. nippona* was not fully understood. In this study, the genetic diversity and genetic structure of three generations of mass-selected lines (G1-G3) and three wild populations were investigated using both 15 microsatellite loci and mitochondrial COI sequences (mtCOI). The selected lines exhibited no significant decrease in the average number of alleles (N_a: 7.53–9.87), observed heterozygosity (H_o: 0.58–0.64), expected heterozygosity (H_e: 0.67–0.71) and alleles richness (A_r: 6.35–7.75), compared with those of the wild populations. The abundant genetic diversity of selected lines was successfully maintained during three generations of mass selection due to no detectable loss. The effective population sizes (N_{e-lin}) estimated by linkage disequilibrium methods for G1, G2 and G3 were 64.1, 25.3 and 47.4, respectively. Moreover, little genetic differentiation within the selected lines was observed in AMOVA analysis (global F_{ST}: 0.005, $P > 0.05$) and significant genetic differentiation among the wild populations was revealed by global F_{ST} (0.15, $P < 0.01$), pairwise F_{ST} (0.095–0.211), *Nei's D* (0.260–0.730) and clustering results, which might suggest genetically isolated populations occurring in these sampling locations. To maximize future selective breeding efforts, a larger scale of broodstock and multi-line breeding strategy along with alleviating selection intensity is recommended against the reduction in the genetic diversity and effective population size in subsequent breeding practices. This study contributes to an increasing understanding of the efficiency of current breeding procedures in maintaining genetic variation and provides insight into future genetic improvement programs of *C. nippona*.

1. Introduction

Advances through selective breeding and the genetic improvement of newly domesticated or captively bred species are considered promising options to elevate aquaculture production (Gjedrem et al., 2012; Vu et al., 2021). However, a challenging problem in the aquaculture sector is how to effectively prevent the loss of genetic diversity over subsequent breeding generations, as the reductive processes of genetic drift (e.g. founder effects) and non-random mating are intensified in closed populations, especially when high selection intensity is applied to obtain the desired trait. (Lind et al., 2009). In many cases, the potential threat to genetic variation of cultured populations is generally exacerbated by overlooking the significance of genetic variability or improper breeding practices. If appropriate precautions aren't implemented the genetic diversity from natural populations accumulated over thousands of years can be even eroded in only one cross-generational transmission (Jackson et al., 2003; Porta et al., 2007). Genetic homogenization within the breeding populations can in turn hinder further improvements in stock performance and important production traits as well as limit the ability to withstand environmental disturbances and disease outbreaks due to the random fixation of deleterious alleles or inbreeding depression (Bentsen and Olesen, 2002; Lind et al., 2009; Zhang et al., 2010). Consequently, it is a major priority for breeding operations to maintain high levels of genetic diversity in domesticated

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populations.

The potential for genetic improvement of desired traits through mass selection is well documented in shellfish species, particularly due to the short generation intervals and the possibility of stringent selection as a consequence of their great fecundity (Gjedrem and Baranski, 2010). Although the risk of unintentional inbreeding may be further increased in the selection scheme due to non-random mating and the absence of pedigree records (Bentsen and Olesen, 2002; Boudry et al., 2002), mass selection with a high selection intensity has been widely used in shellfish species to enhance growth or disease resistance (Li et al., 2011; Dégremont et al., 2015). What must be highlighted, however, is that minimization of inbreeding and control of the genetic structure are key issues for the selected lines to obtain sustained improvement. To preserve genetic diversity during mass selection, various effective measures to prevent inbreeding accumulation in mass-selected populations have been proposed. For instance, some cost-effective and easy-to-operate preventive measures have been applied to the oyster breeding process, including the implementation of abundant broodstock and a balanced sex ratio, and successfully maintained the genetic diversity in two shell color lines of the Pacific oyster for growth (Han et al., 2019; Xu et al., 2019b). The other breeding strategies such as subdividing the breeding nucleus and keeping independent sublines to store rare alleles, cross-breeding with the wild animals, and alleviating selection intensity were demonstrated to be valid as precautionary methods (In et al., 2016; Chen et al., 2017). Genetic tools can be used to enable a better understanding of how efficient current breeding practices are at maintaining genetic variation without direct information about the parents, which can open the possibility for timely modification of pertinent schemes in subsequent selective breeding (Hillen et al., 2017).

The Iwagaki oyster (*Crassostrea nippona*) is naturally distributed in coastal areas of East Asia such as China, Japan and South Korea (Itoh et al., 2004; Lu et al., 2017). The biological properties and its market price have recently prompted widespread interest in initializing the aquaculture industry of *C. nippona*, because *C. nippona* can maintain a high glycogen content and delicate flavor and thus has the potential of substitution for the edibility-restricted Pacific oyster (*C. gigas*) during the summer (Okumura et al., 2005; Masahiro et al., 2018). However, due to unimproved growth performance, the industry of *C. nippona* has never matured into a large-scale aquaculture producer during long-term domestication (Xu et al., 2019a). Therefore, we initiated the successive three-generation mass selection to improve the growth performance of *C. nippona*. A sustained genetic gain of approximately 10% per generation for shell height had been obtained at harvest, yet the genetic impact of an intense artificial selection on the genetic variability of *C. nippona* was not fully understood and rarely monitored. This study is the first to report genetic variation in *C. nippona* using both 15 microsatellite loci and mitochondrial COI sequences (mtCOI), and aims at estimating the stability of genetic variability of *C. nippona* during continuous selection by assessing the genetic diversity level and genetic structure of the mass-selected lines and wild populations. We hypothesize that the overall genetic diversity of the selected lines will be maintained at a high level and will not decline significantly compared to the wild population during successive three-generation mass selection.

2. Materials and methods

2.1. Breeding procedures, sample collections and DNA extraction

In 2014, 200 adult *C. nippona* (over 5-year-old) were collected from the wild population in Niigata Prefecture, Japan. From 2015 to 2019, three consecutive generations of mass selection by truncation selections for shell height were conducted to improve the growth performance of *C. nippona*. Breeding candidates were ranked by their phenotypic value on shell height before each selection, and the individual with the best phenotype was selected for breeding of the next generation. The intensity of selection was calculated as the difference in mean shell height between the selected parents and the base stock divided by the standard deviation of the stock. In theory, when 10% of breeding candidates are selected, the corresponding selection intensity is about 1.7.

In August 2015, 60 wild individuals from the top end of the size distribution were selected to establish the first-generation selected line (G1) with a selection intensity of 1.33 and truncation point of 103 mm. Similarly, truncation selections for the growth trait were implemented for ensuring two successive generations of mass selection (G2 and G3) in 2017 and 2019, respectively. Selection intensity for each generation was 1.64 for G2 with a truncation point of 50 mm in shell height, and 1.71 for G3 with a truncation point of 49 mm in shell height. The number of parents in three selected generations is shown in Table 1. Mature broodstock was induced to spawn using a sudden increase in water temperature from 23 °C to 27 °C. When 60% of the fertilized eggs were observed to release the first polar body, embryos were transferred to the new 20 m^3 tank for hatching at 27 °C, and subsequent culture procedures as described by Li et al. (2011). In brief, larvae were fed a mixed diet of *Isochrysis galbana* and *Chaetoceros calcitrans* three times a day. When 40% of the larvae appeared with eyespots, strings of scallop shells were hung in the tanks for larvae to attach. Finally, the spats were transported to Sanggou Bay (Rongcheng, Shandong province, China) and put into 10-layer lantern nets hanging on long-lines for farming.

Samples of the G1, G2 and G3 were randomly collected at harvest on day 720 and three wild populations were collected from Zhoushan, Zhejiang, China (CZ), Niigata Prefecture, Japan (JN) and Geoje Island, Korea (KG) respectively (Fig. 1). The sample size and sample time of each experimental population are shown in Table 1. All oysters from six populations were sampled by collecting the adductor muscle and storing it at −30 °C. Samples for mtCOI analysis were collected from the same individuals used for microsatellite analysis. The individual genomic DNA was extracted from muscle tissue according to the phenol-chloroform method as described in Li et al. (2006). Then, DNA samples were preserved in 1× TE buffer and diluted to 100 ng/μl for polymerase chain reaction (PCR).

2.2. Microsatellite analysis

Five multiplex PCR sets containing 15 microsatellite loci (MK078352, MK078350, MK078355, MK078356, MK078334, MK078340, MK078347, MK078342, MK078324, MK078343, MK078328, MK078320, MK078344, MK078351 and MK078341) developed by Liu et al. (2020) were amplified to obtain PCR products on all 240 individuals from six populations, and the amplified fragments were electrophoresed on ABI 3130 Automated DNA Sequencer (Applied Biosystems) for genotyping.

Alleles sizes were calculated by utilizing GeneMapper software v.4.0 (Applied Biosystems) based on the genotyping results. Micro-Checker (Van Oosterhout et al., 2004) was first used to examine PCR errors due to allele dropout, stuttering and null alleles. Then, FREENA software (Chapuis and Estoup, 2007) was used to estimate the null alleles frequencies for each locus following the EM algorithm. Exact probability tests of deviation from Hardy-Weinberg equilibrium at each locus were performed in each population with GENEPOP v.4.7 (Raymond, 1995).

The average number of alleles (N_a), inbreeding coefficient (F_{is}), observed heterozygosity (H_o), expected heterozygosity (H_e) and Shannon Wiener index (I) were calculated to assess the genetic diversity using GenALEx v.6.501 (Peakall and Smouse, 2012). The allelic richness (A_r) was estimated by FSTAT v.2.9.3.2 (Goudet, 1995) and the polymorphism information content (PIC) was assessed using the software CERVUS v.3.0 (Kalinowski et al., 2007). The non-parametric test (Kruskal-Wallis test) was performed to analyze the difference among populations when the obtained data deviated from a normal distribution, and then P values for multiple comparisons were adjusted by Bonferroni correction.

Effective population size (N_{e-lin}) in each mass-selected generation was computed in NeEstimator v.2.01 with linkage equilibrium method

Table 1
List of sample information of *C. nippona* and effective population size in the selected lines.

Populations	Number of parents		Selection intensity	Sample time	Sample size		N_e-linkage disequilibrium	
	Female	Male			Microsatellite	mtCOI	N_e-lin	95% CI (lower-upper)
Selected lines								
G1	34	26	1.33	2017.07	48	20	64.1	54.4–77.1
G2	42	48	1.64	2019.07	48	20	25.3	22.9–28.0
G3	43	45	1.71	2021.07	48	20	47.4	39.5–59.0
Wild popualtions								
CZ	–	–	–	2020.10	20	20	–	–
JN	–	–	–	2016.08	28	20	–	–
KG	–	–	–	2021.07	48	20	–	–

G1, first-generation mass selected line; G2, second-generation mass selected line; G3, third-generation mass selected line; CZ, wild population from Zhoushan, China; JN, wild population from Niigata Prefecture, Japan; KG, wild population from Geoje Island, Korea.

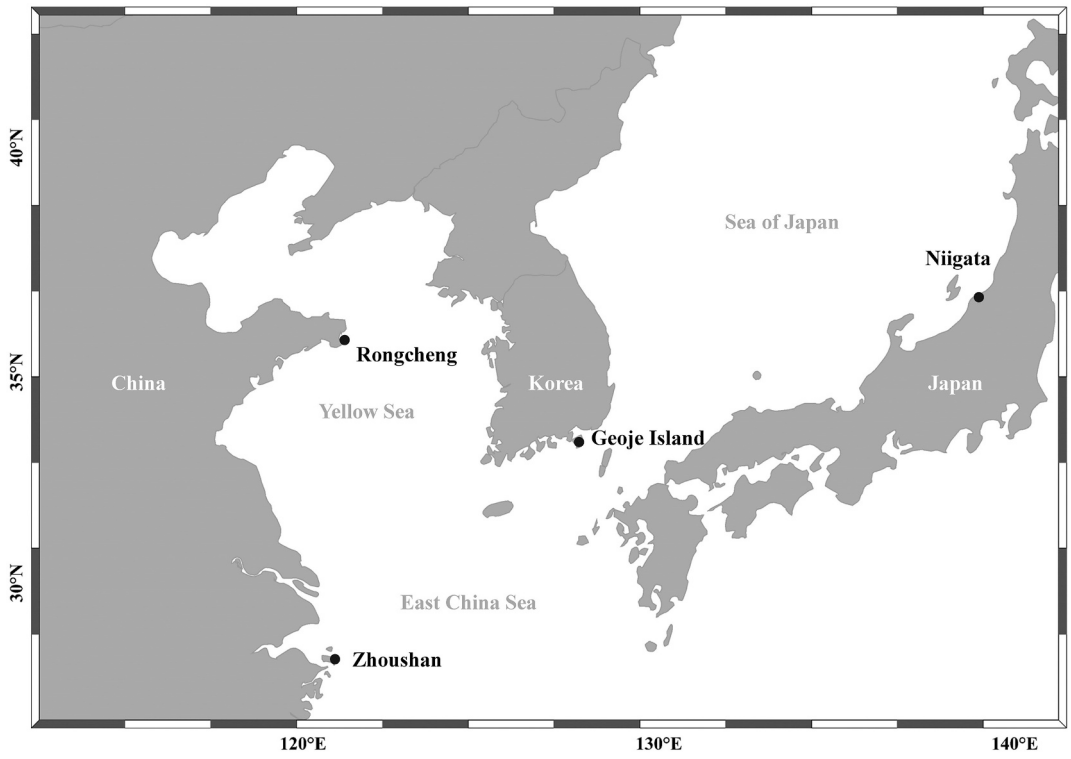

Fig. 1. Map of sampling sites of selected and wild populations of *C. nippona*.

(Do et al., 2014), which implements the bias-correction and reads genotypic data in standard formats, and N_{e-lin} values with 95% confidence intervals (95% CI) were given. Pairwise F_{ST} values and analysis of molecular variance (AMOVA) were utilized to assess population differentiation and partition the genetic variance within/among populations using ARLEQUIN v.3.5 (Excoffier and Lischer, 2010). Genetic relationship among populations was estimated in a bidimensional space by performing principal coordinates analysis (PCoA) implemented in GenAlEx 6.5 (Peakall and Smouse, 2012). A pairwise matrix assessing allele frequency heterogeneity with six populations was computed using GenAlEx 6.5 based on the method of *Nei's* unbiased genetic distance (*Nei's D*) (Hedrick, 2009), and a neighbor-joining tree was displayed in MEGA v.5.0 (Tamura et al., 2011).

2.3. Mitochondrial DNA sequencing and analysis

The primers originally developed by Liu and Li (2018) were used to amplify the mtCOI of *C. nippona*, and the amplified fragment was sequenced by Personal Company (Shanghai, China). Sequencing results of each population were first edited and assembled by SeqMan software from DNASTAR and then the aligned sequences in MEGA v.5.0 were used to calculate the number of haplotypes (N_h), haplotype diversity (H_d) and nucleotide diversity (P_i) by DNASP v.5.10 (Librado and Rozas, 2009).

3. Results

3.1. Genetic diversity

No evidence of genotypic errors due to large allele dropout and stuttering was discovered in all loci by Micro-Checker. The frequencies of null alleles above 0.2 were detected at only nine locus-population combinations: MK078347 and MK078341 in the G1 and the remaining combinations in three wild populations. Remarkably, neither inclusion nor deletion of these loci had a qualitative impact on the experimental outcome. Meanwhile, the *PIC* of each marker was above 0.25 ranging from 0.386 to 0.861 with an average of 0.655 which exhibited a good ability to reveal polymorphism among populations. Therefore, the

subsequent analysis was performed based on all 15 microsatellite loci in six populations.

No significant loss of genetic diversity in N_a (7.20–10.07 across six populations), I (1.34–1.83), H_o (0.46–0.64), H_e (0.63–0.75) and A_r (6.35–10.07) was found from three wild populations to the selected lines (Table 2). Meanwhile, the abundant genetic diversity of selected lines was successfully maintained during three generations of mass selection due to no detectable loss and the mean values of N_a (7.35–9.87 across three selected lines), H_o (0.58–0.64), H_e (0.67–0.71) and A_r (6.35–7.72) were at relatively high levels. The reduction in N_a (23.71%), I (11.11%), H_e (5.63%), A_r (17.75%) and *PIC* (6.63%) observed from G1 to G3 might indicate the potential influence of an intense mass selection on the genetic variability of the selected lines. The *PIC* ranging from 0.5914 in JN to 0.7265 in CZ were all >0.5, indicating high polymorphism in six populations, and the positive F_{is} value suggested heterozygote deficiency could be prevalent in all analyzed populations. More locus-population combinations deviate from the Hardy-Weinberg equilibrium (*dHW*) after Bonferroni correction was observed in the mass-selected lines (9–13) than in the wild populations (4–8).

A total of ten mtCOI haplotypes were found by analyzing the sequencing results of COI fragments from 120 oysters (Table 3). Besides the conspicuous haplotype (1), which was detected in all populations and had a frequency of 68.33% (82/120) in all analyzed samples, remaining haplotypes were not shared between the selected lines and wild populations. Three of the ten haplotypes were shown in the selected lines and the other one to three private haplotypes (haplotypes present in a single population) were distributed in three wild populations. The mean H_d of six populations ranged from 0.100 in KG to 0.721 in G1, while the mean P_i of six populations ranged from 0.018% in KG to 0.197% in G1.

3.2. Effective population size

The effective population size and 95% confidence intervals of G1-G3 were measured based on linkage equilibrium methods (Table 1). Estimated values in each selected line were as follows: G1 (N_{e-lin}: 64.1, 95% CI: 54.4–77.1), G2 (N_{e-lin}: 25.3, 95% CI: 22.9–28.0) and G3 (N_{e-lin}: 47.4, 95% CI: 39.5–59.0). Except for G1, the N_{e-lin} value in the other two generations was lower than the actual number of broodstock. The lowest N_{e-lin} in G2 could be related to the unbalanced parental contributions and insufficient broodstock and then a higher N_{e-lin} value was found in G3 (47.4) compared with G2.

3.3. Genetic differentiation and population structure

Analysis of molecular variance (AMOVA) revealed that most of the variation in the selected and wild populations was observed within population (84.93–99.49%) (Table 4). The global F_{ST} of 0.151 in the wild populations was recorded ($P < 0.01$), suggesting large genetic differentiation among populations, while that of the selective breeding lines was 0.005. Similarly, the values of pairwise F_{ST} (0.006–0.010) and *Nei's D* (0.003–0.023) in G1-G3 were relatively low and gradually increased between adjacent breeding generations (Table 5). In this study, the highest pairwise F_{ST} (0.211) and *Nei's D* (0.730) were found in a pairwise comparison between JN and KG. All pairwise F_{ST} values were

Table 3
Genetic diversity of the selected and wild populations of *C. nippona* at mtDNA COI region.

Haplotype	Selected lines			Wild populations			Total
	G1	G2	G3	CZ	JN	KG	
1	7	5	17	16	18	19	82
2				2			2
3				1			1
4				1			1
5	4	2	1				7
6	9	13	1				23
7					1		1
8			1				1
9					1		1
10						1	1
N_h	3	3	4	4	3	2	
H_d	0.721 ± 0.065	0.532 ± 0.10	0.284 ± 0.128	0.363 ± 0.131	0.195 ± 0.115	0.100 ± 0.088	
P_i (%)	0.197 ± 0.026	0.126 ± 0.029	0.057 ± 0.027	0.141 ± 0.080	0.124 ± 0.107	0.018 ± 0.016	

N_h, number of haplotypes; H_d, haplotype diversity; P_i, percent nucleotide diversity.

Table 4
Analysis of molecular variance (AMOVA) for the selected and wild populations based on 15 microsatellite loci.

Source of variance	d.*f.*	Variance components	Percentage of variation	*F*-statistic
Among selected lines				
Among populations	2	0.02708	0.50778	F_{ST} = 0.00508
Among individuals/ within population	141	0.70534	13.22569	F_{IS} = 0.13293*
Within individuals	144	4.60069	86.26653	F_{IT} = 0.13733*
Total	287	5.33312		
Among wild populations				
Among populations	2	0.91433	15.07175	F_{ST} = 0.15072*
Among individuals/ within population	93	1.43867	23.71484	F_{IS} = 0.27923*
Within individuals	96	3.71354	61.21342	F_{IT} = 0.38787*
Total	191	6.06655		

* Significant at $P < 0.01$.

Table 2
Genetic parameters within the selected and wild populations based on 15 microsatellite loci.

Population	N_a	I	H_o	H_e	A_r	*PIC*	F_{is}	*dHW*
Selected lines								
G1	9.87 ± 5.99^a	1.62 ± 0.60^{ab}	0.58 ± 0.25^a	0.71 ± 0.14^a	7.72 ± 4.17^{ab}	0.6745	0.18	12
G2	9.60 ± 5.59^a	1.60 ± 0.58^{ab}	0.64 ± 0.21^a	0.71 ± 0.14^a	7.51 ± 4.01^{ab}	0.6735	0.10	13
G3	7.53 ± 4.90^a	1.44 ± 0.58^{ab}	0.62 ± 0.21^a	0.67 ± 0.15^a	6.35 ± 3.68^a	0.6298	0.07	9
Wild populations								
CZ	10.07 ± 5.06^a	1.83 ± 0.63^b	0.58 ± 0.21^a	0.75 ± 0.15^a	10.07 ± 5.06^b	0.7265	0.23	4
JN	7.20 ± 2.60^a	1.34 ± 0.40^a	0.46 ± 0.22^a	0.63 ± 0.13^a	6.48 ± 2.23^a	0.5914	0.27	8
KG	8.93 ± 5.16^a	1.52 ± 0.60^{ab}	0.48 ± 0.30^a	0.67 ± 0.17^a	7.28 ± 3.74^a	0.6355	0.28	7

N_a: average number of alleles; I, Shannon's information index; H_o, observed heterozygosity; H_e, expected heterozygosity; A_r, alleles richness; *PIC*, polymorphic information content; F_{is}, inbreeding coefficient; *dHW*, number of loci deviating from Hardy-Weinberg equilibrium. Different letters in the same column indicate significant differences among populations ($P < 0.05$).

Table 5
Estimated pairwise F_{ST} (lower diagonal) and *Nei's D* (upper diagonal) values of *C. nippona* based on 15 microsatellite loci.

	G1	G2	G3	ZC	JN	KG
G1		0.003	0.017	0.315	0.580	0.707
G2	0.001		0.023	0.281	0.528	0.720
G3	0.008*	0.010*		0.251	0.527	0.616
ZC	0.087*	0.080*	0.083*		0.260	0.332
JN	0.170*	0.161*	0.172*	0.095*		0.730
KG	0.182*	0.184*	0.180*	0.105*	0.211*	

The significance of population pairwise F_{ST} tested by 1000 permutations.
* Significantly at $P < 0.05$.

significantly different at the $P < 0.05$ level except between G1 and G2.

To further understand the relationship among different populations, the neighbor-joining tree was constructed based on *Nei's* unbiased genetic distance (Fig. 2). The tree topology showed that all experimental populations were clustered into two main branches. Wild population KG was placed on a separate branch of a cluster dendrogram and the other populations clustered on another branch which was further classified into three subgroups according to JN, CZ and the selected lines. Meanwhile, there was increasing differentiation between adjacent generations in the subcluster of G1-G3. Similar conclusions were confirmed by the results presented in PCoA that three principal components accounted for 30.51% of the total molecular variation, and the Coordinate axis 1 and 2 explained 16.73% and 8.90%, respectively (Fig. 3).

4. Discussion

In selective breeding programs, a fundamental knowledge of how to maintain the maximum level of genetic variability in the successive breeding process is requisite to obtain a sustained response from long-term selection (Lind et al., 2009). It is generally believed that the genetic diversity of mass-reared populations is inadvertently eroded in highly fecund shellfish due to genetic drift and unintentional inbreeding (Xu et al., 2019b; Rhode et al., 2020). In addition, the intense selection for best-performing individuals to achieve faster gains might further decrease the effective population size. It therefore should be necessary systematically to evaluate the genetic diversity and population structure of currently selected lines to inform decision-making for stock management and slow down consanguineous-derived adverse effects.

The complementary combination of microsatellites with mitochondrial COI sequences has been extensively applied to investigate the genetic variability, population genetic structure and demographic history in many bivalves (In et al., 2016; Cordero et al., 2017; Xu et al., 2019b). Here, this investigation revealed that no significant differences in genetic diversity, such as N_a, I, H_o, H_e and A_r, was observed in the mass-selected lines when compared to wild populations, demonstrating that current breeding procedures for *C. nippona* appeared efficient at maintaining available genetic variability. In the analysis results of mtCOI, relatively few haplotypes (2–4) were observed in analyzed populations

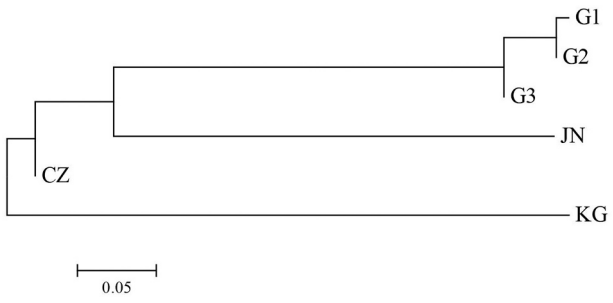

Fig. 2. Neighbor-joining tree of the selected and wild populations using *Nei's* unbiased genetic distance based on 15 microsatellite loci.

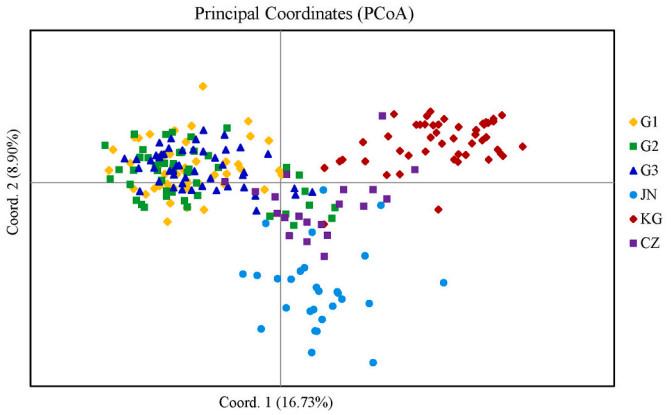

Fig. 3. Principal coordinates analysis (PCoA) of the selected and wild populations of *C. nippona* based on 15 microsatellite loci. Coordinate axis 1 explains 16.73% of the variation, coordinate axis 2 explains 8.90% of the variation, and Coordinate axis 3 (not shown) explains 4.88%.

including three wild populations compared to other oyster species, and the inference was that the low number of haplotypes cannot simply be considered evidence of inbreeding but might reflect the overall level of haplotype diversity in *C. nippona*, as the similar result was also been shown in other studies (In et al., 2016; Xu et al., 2019b). Nevertheless, the number of private haplotypes and haplotype diversity were reduced in the selected lines implying the potential influence of mass selection on the genetic variability of the fast-growing lines, which might be consistent with the observed slight reductions in N_a, I, H_o, H_e, A_r and *PIC*. Similar conclusions were reported in the study of Sydney rock oysters (*Saccosstrea glomerata*), and In et al. (2016) found that the line with the fewest DNA microsatellite alleles also had the fewest haplotype. Losses of microsatellite alleles variation at the population level could reduce the potentially important functional genetic variation in the genome and affect the fitness of subsequent generations with the loss of rare alleles (Lind et al., 2009). In addition, the potential loss of genetic diversity during farming seems unavoidable which has been demonstrated both theoretically and empirically. In this study, a decreasing trend was found in N_a (23.71%) during three generations of mass selection suggesting that the breeding strategy might require prompt intervention to avoid the inadvertent loss of N_a in subsequent selective breeding. A multi-line breeding program may be feasible to store the rare alleles by subdividing the breeding nucleus into multiple independent sublines, and In et al. (2016) suggested that interline crossing can help restore the genetic diversity among selected lines even after many generations of selection. Although the expected heterozygosity higher than the observed heterozygosity was found in each of the selected lines which might be indicative of some level of inbreeding, the closed line of *C. nippona* showed limited evidence for an increase in a homozygous state. Analogous pattern was shown in hatchery-cultured *Pinctada maxima* (Lind et al., 2009) and genetically improved *C. gigas* (Han et al., 2019), further confirming the conclusion that alleles are more susceptible to change than heterozygosity in the immediate term. The reason for this phenomenon might be that the low-frequency alleles contribute little to overall heterozygosity (Lundrigan et al., 2005). What must be highlighted, finally, is that indications of a slight decline in overall genetic diversity are detected in the selected lines, suggesting that regular genetic monitoring is indispensable for avoiding potential problems associated with inbreeding depression in long-term selection.

In this study, 53 of 90 population-locus combinations that deviated from Hardy-Weinberg equilibrium were detected in total, and only 9 combinations showed heterozygote excess, 6 of which were in selective breeding lines. The high prevalence of heterozygote deficiency has been reported previously in studies of mollusk species (Li et al., 2007; Chen et al., 2017), which could be caused by null alleles, non-random mating,

a commixture of independent populations, and artificial and natural selection during seed production and cultivation (Chen et al., 2017). As expected, the *dHW* of the selected lines (44) was much higher than those of the wild populations (19), exhibiting obvious signatures of artificial selection, and the maximum value of *dHW* in G2 (13) might be associated with the lowest effective population size which was also recorded in the mass-selected lines of Pacific abalone (*Haliotis discus hannai*) (Chen et al., 2017). In addition, both the relatively low genetic diversity and high *dHW* observed in JN indicated non-random mating in the wild population, probably because the continuous input of low-variability oysters from hatcheries in the location had diluted the natural genetic resources of *C. nippona* or sampling effects (Zhang et al., 2010; Cordero et al., 2017). This might also be the reason why the inbreeding coefficient of the selected lines in this study is lower than that of the wild populations.

The fluctuation of effective population size (N_e) is generally conditioned by farming constraints limiting the contribution of selected parents to future generations (Lallias et al., 2010). In aquaculture practice, N_e can be diminished by insufficient broodstocks, biased sex ratio, the unequal contribution of gametes, and different viability of gametes (Li et al., 2007). Simultaneously, selected parents based on the best performance of important commercial traits can lead to further reduction of N_e in mass-farmed populations due to broodstocks just from a few outstanding families (Bentsen and Olesen, 2002). In the present study, the linkage disequilibrium method was applied to predict the effective population size (N_{e-lin}), and the results showed that N_{e-lin} for G1, G2 and G3 was 64.1, 25.3 and 47.4, respectively (Table 1). The lowest N_{e-lin} in G2 might be related to unbalanced parental contributions, insufficient broodstocks and high selection pressure. Specifically, asynchronous gonadal development among broodstocks could further exacerbate the differences in reproductive success and the breeder population size (90) of G2 generations was relatively low compared to the other mass-selected programs of *C. gigas* (Han et al., 2019; Chen et al., 2022). In addition, the selection intensity of G2 generation was as high as 1.64 which might intensify sibship among broodstocks. A reasonable inference therefore was that the subsequent observed loss of genetic diversity in G3 was mainly a consequence of small N_{e-lin} in G2 leading to allele loss due to genetic drift (Sukmanomon et al., 2012). Maintaining a sufficiently large N_e is essential to minimize the effect of inbreeding and loss of genetic variability while selecting for better productive performance (Hillen et al., 2017). Encouragingly, the increase of N_{e-lin} was found in G3 with a selection intensity of 1.71 which might be primarily benefitted by well-developed gonads providing opportunities for equal parental contributions. In addition to using a larger scale of broodstock, the practice of spawning animals in isolated small groups is also a promising option to overcome potential inequality in parental contribution (Robinson et al., 2010).

F_{ST} is an important indicator widely applied to evaluate genetic differentiation between/among populations. In this study, little genetic differentiation ($F_{ST} < 0.05$) among the selected lines was detected using the AMOVA analysis and no statistically significant was observed, which could be attributed to the lack of significant genetic structure or population subdivision in the selected lines (Diyie et al., 2021). Similar conclusions were also revealed by pairwise F_{ST} (0.001–0.010) and *Nei's D* (0.003–0.023) between three different generations, which was also reported in the selective breeding or cultured populations of *C. gigas* (Appleyard and Ward, 2006) and Nile tilapia (*Oreochromis niloticus*) (Sukmanomon et al., 2012; Diyie et al., 2021). Although a general increment in genetic differentiation within G1-G3 was detected based on pairwise F_{ST} and *Nei's D*, the relatively small value might imply that the genetic variance of selected lines was stabilized under the selection pressure (Diyie et al., 2021). Within the wild populations, a large genetic differentiation among different geographical localities was observed by significant F_{ST} of 0.15 ($P < 0.05$), and then the highest pairwise F_{ST} (0.211) and *Nei's D* (0.730) were found in pairwise comparison between JN and KG which might be attributed to geographic isolation, different natural selection stresses and low gene flow between them. In addition, the genetic relationships and clustering patterns among six populations were further demonstrated in the neighbor-joining tree topology and PCoA plots. Interestingly, although both the PCoA and tree topology clearly separate three wild populations, the overlap between few wild individuals from KG and JN was visualized in CZ based on PCoA, suggesting that CZ might share similar genetic backgrounds with the wild populations from Japan and Korea. The oyster transportation and the relatively long floating time of the larval stage (20–30 d) may provide opportunities for facilitating intra-regional gene flow and inter-population genetic mixing of *C. nippona*, and similar conclusions also appeared in population genetics studies of Manila clam (*Ruditapes philippinarum*) (Cordero et al., 2017; Tan et al., 2020).

5. Conclusions

In conclusion, no significant loss of genetic diversity was observed over three successive mass-selected generations of *C. nippona* for fast growth based on microsatellites and mtCOI. However, a decreasing trend in the number of alleles indicated the potential influence of an intense mass selection on the genetic variability of the selected lines. A larger scale of broodstock and the multi-line breeding strategy along with lower selection pressure would be implemented to increase effective population size and maintain genetic diversity in subsequent breeding practices. In addition, little genetic differentiation among the selected lines might indicate a stable genetic background. However, significant genetic differentiation as well as cluster analysis showed a clear separation between the wild populations implying genetically isolated populations occurring in these sampling locations. This study further confirms the importance of monitoring genetic diversity in long-term selective breeding of the mass-selected lines and provides improved breeding procedures for future genetic improvement of *C. nippona*.

CRediT authorship contribution statement

Yiming Hu: Investigation, Methodology, Resources, Data curation, Writing – original draft. **Qi Li:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Chengxun Xu:** Software, Supervision. **Shikai Liu:** Software. **Lingfeng Kong:** Resources. **Hong Yu:** Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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