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Short communication

Heritability estimate for mantle edge pigmentation and correlation with shell pigmentation in the white-shell strain of Pacific oyster, *Crassostrea gigas*

De Xing[a], Qi Li[a,b,*], Lingfeng Kong[a], Hong Yu[a]

[a] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, China

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ABSTRACT

The pacific oyster (*Crassostrea gigas*) is one of the most widely farmed aquatic animal species and of great economic importance. Mantle edge color in *C. gigas* is highly variable and considered as a new potential trait for a better commercial value. In this study, heritability for mantle edge pigmentation and its correlation with shell pigmentation were explored in the white-shell strain of *C. gigas* with mixed-family method. A total of 460 offspring raised under communal conditions were successfully assigned to their parents using four multiplex PCR protocols based on 11 microsatellite loci. Mantle edge pigmentation was measured through assay of melanin content using spectrophotometric analysis. Animal model heritability estimate were 0.215 ± 0.092 for mantle edge pigmentation. The genetic and phenotypic correlations between two pigment traits were 0.980 ± 0.094 and 0.423 ± 0.042, respectively. Pearson analyses for average mantle edge and shell pigmentation of family also showed strong and significant correlation ($r = 0.729$, $P = 0.000$, n = 29). Chi-square analyses showed that mantle edge pigmentation of family 7 segregated into "lighter" and "darker" groups in a 3:1 ratio ($P = 0.833$), suggesting that a major locus might control mantle edge pigment trait. The results obtained in this study will benefit selective breeding of *C. gigas* with desired mantle and shell pigmentation.

1. Introduction

The pigmentation in seafood is known to affect consumer preferences and willingness to pay, because it interferes with judgments of flavor intensity and quality identification (Clydesdale, 1993). For example, consumers are willing to choose rich red salmon fillets with premium price (Alfnes et al., 2006). Similarly, the shell and mantle color of the Pacific oyster (*Crassostrea gigas*), which is usually sold live-in-shell and served on the half-shell in the restaurants and markets, also influence consumer preferences. The Pacific oyster with golden shell and golden mantle coloration are rarely seen in the market and are sold at much higher prices than other common oysters (Nell, 2001). In Korea, the Pacific oyster with black mantle are favored by consumer and traded at about 20% higher price (Kang et al., 2013). It is obvious that shell and mantle colors have been regarded as new high potential traits for a better commercial value. In our selective breeding practice of Pacific oyster, a white-shell oyster strain has been obtained after four generations family-based selection. Mantle edge in most of white-shell oysters shows light coloration, only a few occurred deeper coloration which vary from yellow to black.

The Pacific oyster shell pigmentation distributes continuously from near-white, pigment-free shells to near-black, fully pigmented shells, which led most researchers to consider Pacific oyster shell pigmentation as a quantitative trait under polygenic controlled (Brake et al., 2004; Imai and Sakai, 1961), and further study demonstrates that this genetic variation is largely additive and both broad-sense and narrow-sense heritability of left-shell pigmentation were very high (Evans et al., 2009). Results from the recent studies support the hypothesis that left-shell coloration may have different patterns in which a dominant major gene may exist (Evans et al., 2009; Ge et al., 2015). The physiological evidence that the bivalve shell are secreted by mantle edge (Marin and Luquet, 2004), and the morphological observation that mantle edge color and shell color are positively correlated (Brake et al., 2004; Kang et al., 2013), suggest that a high degree genetic relationship exists between mantle edge and shell pigmentation, and mantle pigmentation is under genetic control in *C. gigas*. To date, several studies on genetic basis of shell color traits have been carried out in *C. gigas* (Ge et al., 2015; Feng et al., 2015), but there is no major gene experimentally confirmed to dominate the mantle pigmentation.

Mantle edge pigmentation in *C. gigas* was regarded as continuously distributed, and divided into four or ten levels by eyes (Brake et al., 2004; Kang et al., 2013), but the systems with mantle pigmentation

* Corresponding author at: Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China.
E-mail address: qili66@ouc.edu.cn (Q. Li).

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categories are vulnerable considering of subjective assessments and inaccuracy. Therefore, a new method is demanded urgently to measure mantle edge pigmentation. The extracted black particles from the shells and mantles of oysters were confirmed to be melanin (Yu et al., 2015), indicating that it possible to evaluate mantle edge pigmentation by melanin content for a truly continuous measurement.

In this study, heritability for mantle edge pigmentation applying spectrophotometric analysis of melanin content was estimated with mixed-family approach, and correlation between mantle edge and shell pigmentation was analyzed in the white-shell strain of *C. gigas*, which can provide important information for efficient selective breeding.

2. Materials and methods

2.1. Spawning and nursery protocol

In 2010, white-shell-color *C. gigas* were selected from wild population in Rushan, Shandong province, China, and used to establish the first-generation selective families for white shell color and rapid growth. In 2011–2013, the second-generation, third-generation and fourth-generation of family selection were constructed, using the same selection intensity of 10%. In each generation, the oysters were cultured on ropes suspended from rafts along the coastal regions of Rushan.

Samples of adult *C. gigas* descended from the fourth generation of white-shell families were collected from Rushan, and brought to the hatchery at Laizhou, China. Ten males and 30 females were chosen at random to produce 30 families following a nested half-sib mating design (each male was mated with three different females). Eggs and sperm of each parental pairs were individually collected and fertilized separately. Adductor muscle samples from all parental animals were collected and stored in 70% ethanol for DNA analysis. Fertilized eggs were incubated for 24 h at temperature of 24 °C and salinity of 30 psu, then approximately equal number of D-shaped larvae of all families were mixed and pooled into a 500 L plastic bucket providing a common rearing environment. Before mixing, larval density of every family was counted. Larvae were reared as described by Kong et al. (2015). Briefly, initial larval density was adjusted to 15 larvae/mL, and reduced to 5 larvae/mL and 1 larvae/mL on day 7 and 14 after fertilization, respectively. Half volume of water was changed twice per day with fresh filtered seawater held at 25 °C. Veliger larvae were supplied with daily rations of *Isochrysis galbana* until the larvae had reached 120 μm shell length, then supplied with Platymonas helgolandica and Chaetoceros calcitrans at later stage. When eye spots occurred, strings of scallop shells were placed into culture tank as substrates. After a week, successfully metamorphosed spat were transferred to outdoor nursery tank. After rearing over a 30 day period, all spat were inserted into nylon ropes randomly and transported to grow-out areas in Rushan, China (36.89° N, 121.52° E).

2.2. Shell and mantle edge pigmentation measurement

All offspring were collected at 24 months of age, then each of which was shucked, adductor muscle was retained in 70% ethanol for DNA analysis and a sample of 0.100 g mantle edge was obtained for assay of melanin content. The mantle was sampled from the same location where left and right mantle edge are connected. As described by Chakraborty et al. (1998), samples of mantle edge were placed in centrifugal tubes containing 1 mL of 1.0 M NaOH. Suspensions of mantle samples were prepared by homogenizing for 5 min with an IKA T10 basic homogenizer and heated in a boiling water bath for 30 min. After cooling, suspensions were cleared by centrifugation at 10,000 rpm for 10 min. Supernatants were transferred for spectrophotometric analysis of total melanin content at 500 nm absorbance (A_{500}). In detail, spectrophotometric reading were made in a Shanghai Spectrum Model SP-721 UV–visible spectrophotometer, which had a grid monochromator, and the band pass was set at 1 nm. The wavelength setting was 500 nm. Absorbance values of extracts in pathlength cuvettes were read to three decimal places. The A_{500} values are referred to as total melanin, i.e. mantle edge pigmentation values.

The shells of all offspring were brushed with freshwater, soaked in a 6% sodium hypochlorite solution for 2 h to remove biotic and abiotic fouling (Sturm et al., 2006), and then brushed again and dried in shade. We photographed shells according to Evans et al. (2009). A digital camera (Nikon D80) was used to acquire images with two bulbs mounted on both sides. Bulbs provided uniform and consistent illumination for photographing. Images were taken on a matte black background to avoid reflections. All images were dealt with and the optical density of each shell was analyzed by Image-Pro Plus image analysis software 6.0 (Media Cybernetics Inc., Silver Springs, MD, USA). Measure/Count/size/Scale Adjuster was set to outline oyster shells automatically, creating the area of interest (AOI); intensity calibration was set at Std.optical Density, gray scale value was converted to optical density value, on a scale ranging from 0 (completely white) to 2.4 (completely black). The optical density (OD) values of all images were measured by using macros as values of pigmentation of shells.

2.3. Parentage assignment

DNA of both parent and progeny samples was extracted following Li et al. (2006). Four multiplex PCR protocols based on 11 microsatellite loci (ucdCg-117, ucdCg-120 and ucdCg-198; ucdCg-146, Crgi3 and uscCgi-210; otgfa0_0129_E11, otgfa0_0007_B07 and Crgi4; ucdCg-200 and otgfa0_408293) (Liu et al., 2017) were used to genotyped the parents and progeny. Parental assignment was performed using VITASSIGN V8–5.1 (Vandeputte et al., 2006). This package achieved fast reassignment of offspring to their putative parents by exclusion (incompatible genotypes excluded), with the optional possibility to accept up to n mismatches (= n incompatible alleles for each sire-dam-offspring triplet) in order to limit the impact of genotyping errors (Vandeputte et al., 2006). To minimize parental allocation error, less than three mismatched alleles were allowed and at least 95% confidence were accepted.

2.4. Data analysis

The software package ASReml 3.0 (Gilmour et al., 2009) was used to estimate the heritability (h^2) and phenotypic and genetic correlations ($r_{P/G}$). All data of two color traits (shell and mantle edge pigmentation) were tested for normally distributed. In matrix notation the mixed model can be written as:

$$y = Xb + Z_1a + Z_2c + Z_3d + e \quad \text{(Model 1)}$$

where y is the vector of observations on all individuals, b is the vector of the fixed effects, a is vector of the random animal additive effects, c is the vector of common environmental/maternal effects for dam half-sib other than additive genetics, d is the vector of dominance effects, and e is vector of residual effects.

The statistical significance of including additional random effects for each trait was assessed via a likelihood ration (log-LR) test (Gilmour et al., 2009) by comparing the log of the restricted likelihood (Log$L$). In this analysis, c and d were not significant and were removed from Model 1. The reduced animal model was applied as follows:

$$y = Xb + Za + e \quad \text{(Model 2)}$$

Under the Model 2, estimates heritability were calculated as $h^2 = \sigma_a{}^2/(\sigma_a{}^2 + \sigma_e{}^2)$, where $\sigma_a{}^2$ is the additive genetic variance and $\sigma_e{}^2$ is the residul variance. Genetic and phenotypic correlations between two traits were calculated as $r_{P/G} = \sigma_{12}/\sqrt{\sigma_1^2}.\sqrt{\sigma_2^2}$, where σ_{12} is estimated additive genetic or phenotypic covariance between the two traits, and $\sigma_1{}^2$ and $\sigma_2{}^2$ are the additive genetic or phenotypic variances of traits 1 and 2, respectively.

Application of histograms and Kolmogorov-Smirnov analyses were

Table 1
Number of offspring assigned to each of the 30 families, based on microsatellite genotyping, and mean value of mantle edge and shell pigmentation.

Sire	Dam	Number	Mean mantle edge pigmentation value (A_{500} value) ± sd	Mean shell pigmentation value (OD value) ± sd
1	1	11	0.427 ± 0.109	0.669 ± 0.054
	2	4	0.546 ± 0.154	0.674 ± 0.017
	3	11	0.399 ± 0.115	0.659 ± 0.033
2	4	22	0.603 ± 0.208	0.782 ± 0.102
	5	0	–	–
	6	8	0.603 ± 0.159	0.797 ± 0.116
3	7	30	0.425 ± 0.177	0.708 ± 0.081
	8	23	0.439 ± 0.127	0.738 ± 0.121
	9	12	0.624 ± 0.179	0.718 ± 0.109
4	10	6	0.520 ± 0.136	0.674 ± 0.030
	11	6	0.605 ± 0.275	0.728 ± 0.087
	12	2	0.504 ± 0.199	0.756 ± 0.090
5	13	15	0.621 ± 0.178	0.782 ± 0.107
	14	2	0.440 ± 0.138	0.702 ± 0.008
	15	17	0.632 ± 0.270	0.810 ± 0.144
6	16	18	0.516 ± 0.193	0.705 ± 0.102
	17	1	0.255	0.619
	18	11	0.513 ± 0.208	0.760 ± 0.086
7	19	22	0.427 ± 0.120	0.709 ± 0.099
	20	7	0.400 ± 0.123	0.686 ± 0.049
	21	7	0.562 ± 0.145	0.811 ± 0.114
8	22	4	0.529 ± 0.412	0.691 ± 0.099
	23	46	0.513 ± 0.179	0.762 ± 0.120
	24	35	0.469 ± 0.173	0.699 ± 0.091
9	25	46	0.539 ± 0.159	0.737 ± 0.102
	26	20	0.635 ± 0.229	0.807 ± 0.131
	27	12	0.565 ± 0.161	0.748 ± 0.136
10	28	12	0.427 ± 0.105	0.722 ± 0.125
	29	5	0.523 ± 0.175	0.776 ± 0.121
	30	45	0.562 ± 0.188	0.726 ± 0.129
Total		460	0.520 ± 0.189	0.737 ± 0.112

used to test frequency distributions of mantle edge pigmentation within each family. When these distributions showed discrete phenotypic classes, chi-square analysis was used to determine whether phenotypic frequencies segregation ratios differed from Mendelian segregation ratios on a per family basis.

3. Results

3.1. Parentage assignment and summary statistic in families

A total of 528 individual were available for parentage assignment, of which 460 could be successfully assigned to 29 of the 30 putative families (Table 1). For 68 unassigned progeny, poor allelic amplification or more than three genotyping errors of loci resulted in insufficient assignment. A large disparity in family contributions was found, with a range of 0–46 per family. Mean and sd of both mantle and shell pigmentation value data were summarized in Table 1.

3.2. Heritability and correlation

Genetic parameters estimates using the final animal model (Model 2) are listed in Table 2. Moderate heritability estimates were observed in the two traits. Heritability of mantle edge pigmentation (0.215 ± 0.092) was slightly higher than that of shell pigmentation (0.156 ± 0.078). The genetic correlation between mantle edge and shell pigmentation was very high and positive (0.980 ± 0.094), and phenotypic correlation was moderate (0.423 ± 0.042). Pearson analyses for average mantle edge and shell pigmentation of the families also showed strong and significant correlation ($r = 0.729$, $P = 0.000$, $n = 29$) (Fig. 1).

The histograms of the frequency distributions of mantle edge pigmentation were only constructed within 18 families (Fig. 2), in which offspring were more than ten. Mantle edge pigmentation was expected to be continuously and normally distributed within most of the families.

Table 2
Analysis separating components of variance and genetic parameter (heritability, genetic correlation and phenotypic correlation) estimates for mantle edge and shell pigmentation in the white-shell strain of pacific oyster, based on Model 2.

	Mantle edge pigmentation	Shell pigmentation
Animal variance (Va)	0.00777	0.00196
Residual variance (ve)	0.0284	0.0106
Heritability ± SE	0.215 ± 0.092	0.156 ± 0.078
Additive covariance (cov(a_1,a_2))	0.00383	
Residual covariance (cov(e_1,e_2))	0.00521	
Genetic correlation ± SE	0.980 ± 0.094	
Phenotypic correlation ± SE	0.423 ± 0.042	

However, Kolmogorov-Smirnov analyses showed that three families had significantly non-normal distributions ($P < 0.05$). Family 15 and family 30 showed skewed distribution, while family 7 showed bimodal distributions and were therefore amenable to chi-square analyses based on phenotypically recognizable groups (“lighter” and “darker”). Chi-square analyses showed that the ratios of “lighter” and “darker” mantle edge groups did not differ significantly from 3:1 ($\chi^2 = 0.044$, $n = 30$, $P = 0.833$).

4. Discussion

In agreement with previous studies, mantle edge pigmentation has been considered as a quantitative and continuous trait that is under a high degree of genetic control (Brake et al., 2004; Kang et al., 2013). Traditionally, mantle edge pigmentation were classified into different levels by naked eyes. But this method is not truly continuous measurement. Spectrophotometric analysis of melanin content is widely used to evaluate color in various pigmented tissues obtained from humans, mice and other animals (Chakraborty et al., 1998; Ito et al., 2011; Lamoreux et al., 2001; Ozeki et al., 1995). In the present study, we

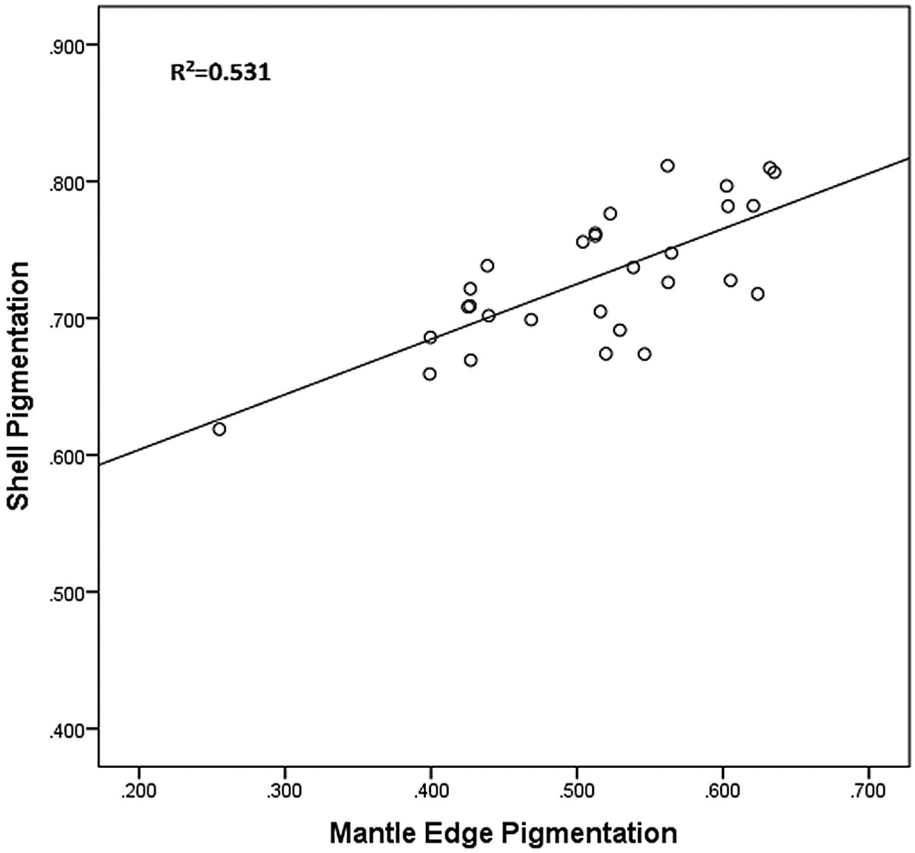

Fig. 1. Correlation of average family mantle edge pigmentation (A_{500} values) and shell pigmentation (OD values) for 29 families (r = 0.729, P = 0.000, n = 29).

firstly estimated mantle edge pigmentation in *C. gigas* by spectrophotometric analysis of melanin content, which achieved objective and continuous measurement of mantle pigmentation. Most of families were continuous and normally distributed, which was essential for estimates of genetic parameters in ASReml 3.0 software (Gilmour et al., 2009).

The animal model, a form of mixed model, is expected to provide estimates with higher precision by taking into account all relationships in pedigree, and reduce bias due to shared environment effects such as maternal or brood effects (Kruuk and Hadfield, 2007). It is nowadays broadly adopted to estimate quantitative genetic parameters with restricted maximum likelihood (Åkesson et al., 2008; Hung et al., 2013; Kruuk, 2004; Norris and Cunningham, 2004). The estimated common environmental/maternal effects on pigment traits were not significant in this study, being consistent with the previous report by Evans et al. (2009). No significant maternal effects was also observed in juvenile common carp (Vandeputte et al., 2004) and grass carp (Fu et al., 2016). Common environmental/maternal effects are known to occur primarily during the early life stages (correlated with egg size), and dissipate quickly after growing for a few months (Fu et al., 2016). Pigmentation measurements were at 24 months post-fertilization, which may result in minimal common environmental/maternal effects in this study. This is a little surprising that dominance effects were minor in this study, as the existence of dominance variation contributes to heterosis for crossbreeding (Hedgecock and Davis, 2007). However, the precision of the estimation of dominance variation might be low in this study due to the disequilibrium in family sizes and the small size of full-sib families, which generated a lot of missing values (see Table 1). Nevertheless, we found that the heritability estimates based on the reduced animal model for mantle edge and shell pigmentation traits were moderate, 0.215 and 0.156, respectively, suggesting that it is potentially feasible to obtain genetic improvement through selective breeding for both pigment traits in *C. gigas*. The heritability estimate of mantle edge pigmentation was similar to the results of Kang et al. (2013) (0.27 and 0.08 in two generations), but the heritability estimate of shell pigmentation was lower than the results of Evans et al. (2009) (0.59 for narrow heritability) and Kang et al. (2013) (0.41 and 0.77 in two generations). In this study, the parents from the fourth generation of selective breeding strain were used to create families for analysis. Reduced genetic variation observed within the strain (Xing et al., 2017) might result in less heritability estimate than wild population. In addition, all offspring from different families were raised under communal conditions to minimize environmental effects with microsatellite-based pedigree methods in our current work, which lead to more precise estimation of heritability than previous studies (Fu et al., 2016). Furthermore, it is important to acknowledge that heritability will change according to the broodstock population, the growing environment, age and estimated methods (Åkesson et al., 2008; Falconer and Mackay, 1996; Fu et al., 2016).

The study gives the first estimates of genetic correlation between mantle edge and shell pigmentation in the Pacific oyster. The results reveal a high genetic correlation between two pigment traits, suggesting that the underlying genetic variation between two traits is in a consistent way (Norris and Cunningham, 2004), phenotypic correlation are moderate, and relationships between the average value for both traits of family are of high magnitude (Fig. 1), which also has been found by Brake et al. (2004) and Kang et al. (2013). The positive and high correlations indicate that pleiotropic effects could exist (Brake et al., 2004) and a large number of the same genes may be involved in expression of two pigment traits (Falconer and Mackay, 1996). In molluscs, melanin is crucial to shell pigmentation, and formed in the secretory cells of the mantle edge (Comfort, 1951). It seems that the quantity of the secretory cells plays a role as a connection between mantle edge and shell pigmentation. Furthermore, the 3:1 segregation ratio (three lighter individuals for every one darker individual) of mantle edge pigmentation is found in family 7, which is explained by two alleles at a single locus with “lighter” allele being dominant over

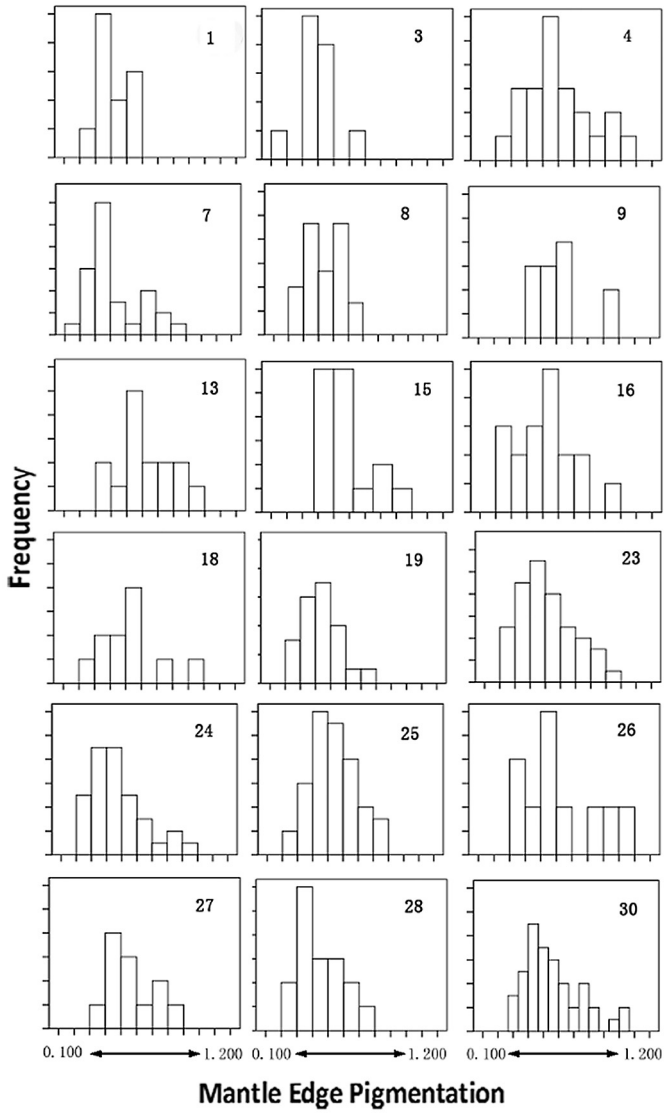

Fig. 2. Distribution of mantle edge pigmentation among individuals within each of 18 full-sib families (n = 11 to 46 per family). Mantle edge pigmentation values along the x-axis of histogram range from A_{500} = 0.100 (lightest mantle edge) to A_{500} = 1.200 (darkest mantle edge).

the "dark" allele. This result is consistent with the mode of inheritance of shell pigmentation that a dominant allele coded for the lighter shell and a recessive allele coded for the darker shell (Evans et al., 2009). Further work is needed to confirm the existence of common genes affecting two pigment traits in *C. gigas*.

5. Conclusion

This study estimated genetic parameters for mantle edge pigmentation in *C. gigas* by spectrophotometric analysis of total melanin content. Moderate heritability and high magnitude of genetic correlation between mantle edge and shell pigmentation indicate that it should be effective to obtain genetic improvement through selective breeding, and it is likely that improvement in shell pigmentation could be achieved by selecting for mantle edge pigmentation and vice versa. Our study provides useful information on selective breeding of *C. gigas* with desired mantle and shell pigmentation.

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