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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*

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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

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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