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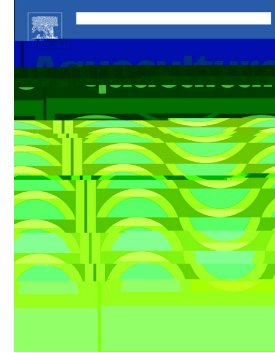
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Heritability estimates for shell color-related traits in the golden shell strain of Pacific oyster (*Crassostrea gigas*) using a molecular pedigree

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Abstract The Pacific oyster (*Crassostrea gigas*) is an important aquatic animal species with enormous production and economic benefits, but heritability estimation for shell color applying mixed-family approach and computer vision system has not been performed. In this study, heritability for shell color-related traits was estimated in the golden shell strain of Pacific oyster by fostering a single cohort of 30 families in a nested mating design consisting of 10 sires and 30 dams. After parentage assignment inference based on six microsatellite markers, 690 offspring were included in the subsequent analyses. Using an animal model, at 16 months of age, heritability estimates were 0.10 ± 0.12 for L^* , 0.41 ± 0.16 for a^* , 0.23 ± 0.15 for b^* and $0.33 \pm$ E . Genetic correlation between shell color-related traits and growth-related traits were generally inconspicuous ranging from -0.02 to 0.11. Conclusions can be obtained from our study that favourable improvement in aquatic selective breeding would be predictable for the genetic potential of the golden shell color in this strain of Pacific oyster, whereas independent selection conducted at the same time rather than correlative selection would be feasible in the improvement of both types of traits.

Keywords *Crassostrea gigas*; shell color-related traits; parentage assignment; microsatellite marker; genetic parameters

1. Introduction

The Pacific oyster *Crassostrea gigas* is the globally dominant farmed oyster in the world (FAO, 2012). In China it is the most widespread specie of farmed oyster (Li et al., 2006) with the production reaching 1083 thousand metric tons in 2015 (BOF, 2016), suggesting the considerable economic potential in selective breeding. In addition, as the main cultivating approach, raft-string cultivation facilitates the family segregation and selective breeding.

□

(Dekkers and Hospital, 2002), which is based on the variation of traits. As a essential phenotypic trait of bivalve species, shell coloration is highly

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preference can be affected by the visual perception of food products (Kahn and Wansink, 2004). For instance, consumers prefer redder salmon flesh (Sylvia et al., 1995), and when choosing seafood in the market, consumers consider their colors as a evaluation factors affecting price, which indicates economic value of the coloration (Alfnes et al., 2006). In *C. gigas*, shell pigmentation continuously varies from near-white, pigment-free shells to

near-black, fully pigmented shells, offering adequate foundation for selective breeding which indicates a possibility of enormous economic benefits of interest to the whole oyster industry (Brake et al., 2004). The coloration referred to above is considered as a polygenic and quantitative trait controlled by many small-effect genes (Evans et al., 2009). In contrast, in some cases, shell coloration was determined to be controlled by only a small quantity of major genes (Evans et al., 2009; Hedgecock et al., 2006). In recent studies, a study claimed the hypothesis that in the golden shell strain of the Pacific oyster the presence of golden background color is dominant over its absence (white) and has an epistatic effect on its black foreground pigmentation (Ge et al., 2015), which indicates the fixation of major genes of golden shell strain, and the gradual and sustained selection on polygenes are in need (Evans et al., 2009).

In bivalves, genetic parameters including heritability and correlations has been estimated by a number of studies, which reveal a part of hereditary character and suggest the potential of genetic improvement in shell pigmentation. For instance, researchers acquired heritability estimates for inner shell color in *Hyriopsis cumingii*: L^* (0.31 ± 0.22), a^* (0.11 ± 0.08), b^* E (0.29 ± 0.19) (Wang et al., 2014). Investigations using selected families and pedigreed oysters afford evidence for genetic control of shell pigmentation and mantle edge (Brake et al., 2004), which implicates the manipulation of pigmentation as a objective trait. Heritability for shell pigmentation in *C. gigas* was estimated with twenty-six and twenty-five full-sib families respectively (Evans et al., 2009 ; Wang et al., 2016).

The narrow-sense heritability and genetic and phenotypic correlations in terms of specific quantitative traits can be estimated to predict the flexibility of improving traits through selective breeding (Falconer and Mackay, 2000), which can provide useful information for making reasonable aquatic breeding schemes and estimating breeding value of candidate traits (Wang et al., 2006). Additionally, in the practice of fostering offspring, communal cultivation can avoid shortcoming of traditional approach introducing additional environmental variation and leading to imprecise heritability estimates and encumbrance in genetic improvement (Wang et al., 2008). The computer vision system (CVS) (Yam and Papadakis, 2004) including digital camera, computer and graphics software has been applied in *Pinctada martensii* (Gu, 2009), *H. cumingii* (Wang et al., 2014) and *C. gigas* (Wang et al., 2016) eyes (Evans et al., 2009).

In this study, we estimated genetic parameters (heritability and correlations) for *C. gigas* at 16 months of age by applying mixed-family approach combined with a nested mating

design and CVS imaging system.

2. Materials and methods

2.1 Broodstock and spawning

Targeting the traits of golden shell color and rapid growth, two generations of family selection was performed in 2010 and 2011, in which golden-shell-color individuals originated from wild population. In 2012 and 2013, mass selection was implemented to breed the F₃ and F₄ generation. In our experiment, F₄ generation was taken as the brood stock population of selective golden color strain of *C. gigas* and grew up in sea area of Rushan, and maintained in Laizhou breeding base, Shandong Province, China. In 2014 a nested mating design of ten sires and thirty dams was employed to reproduce progeny, in which three dams were nested within one sires respectively.

After stripping from every broodstock, sperm suspension of each sire was divided into three equal portions and fertilized respectively with a corresponding dam, then eggs were rinsed and kept in a communal pool to culture larvae. In the process, the sperm was kept in appropriate concentration with respect to the number of eggs and the suspensions was being stirred to ensure sufficient fertilizations. Adductor muscle of all the parents was separately collected and stored in 100% ethanol for subsequent DNA analysis.

2.2 Fostering of progeny oyster

All the practices carried out followed the standard and conventional routine of rearing *C. gigas*. Eggs and sperm were obtained by stripping from each broodstock including thirty dams and ten sires to produce progeny. A suspension of sperm from each sire was divided into three equal portions and fertilized eggs from 30 dams respectively.

After fertilization, eggs of 30 full-sib families were hatched in containers (100 L polyethylene plastic bucket) respectively for 22 h, in which fertilized eggs developed to D-shaped larvae. Then equal and approximate numbers of D-shaped larvae were mixed in two 400 L buckets in same conditions air inflation. Along with the growth, larvae were stocked in the adjusted densities decreasing from 10 individuals ml⁻¹, and the water temperature was maintained at 23-24°C and changed 50% twice every day in the morning and night, with

salinity at 30 psu. At early stage when shell length is less than 120 μm , the veligers were supplied with daily rations of *Isochrysis galbana*, at later stage *Platymonas sp.* and *Chlorella vulgaris* instead. When 30% larvae developed eye spots, settlement substrates made of scallop shells were hung in the buckets. Until about 15% larvae settled and developed to spats on each piece, spats were transferred to sedimentation tank for temporary nursery, and about a month later when wild oyster larvae completed settlement, spats were inserted into nylon ropes randomly and deployed to grow-out sea area in Rushan, Shandong Province, China. Scheduled examination, separation and reloading of oysters in culturing lanterns was conducted to warrant appropriate density of oysters in order to avoid clustering effect of high density on shell shape and sway effect of water flow on growth rate in low density.

2.3 Sampling and traits measurement

After cultivated in the same depth for 16 months, all 810 offspring were harvested and respectively measured with electronic vernier calliper (0.01 mm accuracy) and electronic balance (0.1 g accuracy) for the shell height, shell length, shell width and wet weight. The adductor muscle of all offspring was respectively collected and kept in 100% ethanol for subsequent DNA analysis. For the visualization and acquiring of the digitalized images, the computer vision system was applied to quantify standard color of oyster shells (Mendoza et al., 2006). All the left shells of offspring were cleaned with caution and placed on the black background matte. The color digital camera Nikon D80 was located vertically over the background at a distance of 30 cm. Samples were illuminated with two 20 W natural daylight parallel lamps with a color temperature of 6500 K and a color-rendering index close to 95%. Both lamps were situated 35 cm above and at an angle of 45° to the sample. The camera settings were used as manual mode: manual mode (M), iso 200, the lens aperture at $f = 5.6$, time of exposures at speed 1/160 s, automatic zoom, no flash, and storage in JPEG format. The lamps and camera were covered with a black cloth to avoid the external light and reflections. The camera was connected to the USB port of a PC provided with a remote capture software to visualize and acquire the digitalized images directly from the computer, in which Photoshop CS6 (Adobe System Incorporated) was used to analyze images for L , a and b values.

2.4 Parentage assignment

DNA of each parent and offspring was extracted from the adductor muscle for PCR amplification (Li et al., 2006), in which two panels of microsatellite multiplex PCR markers (Panel 1: Crgi3, ucdCg-146, and uscCgi-210; Panel 2: ucdCg-120, ucdCg-198, and ucdCg-117) were used for genotyping (Liu et al., 2016). Amplification products were resolved via ABI3130 with LIZ500 (Applied Biosystems) as internal size standard. The Gene Mapper v4.0 software was applied for assessment of fragment lengths. Based on the likelihood-based approach and the heterozygosity and genotyping reliability of microsatellite markers, parentage assignment was performed with CERVUS 3.0 (Kalinowski et al., 2007). 690 of 810 sampled offspring were successfully assigned to the parentage, and other individuals with unclear parentage were not included in the subsequent analyses.

2.5 Data analyses

L^* , a^* , b^* and E were acquired from L , a and b value with the following modules (Chakraborty et al., 2014):

$$L^* = \frac{L}{255} \times 100$$

$$a^* = \frac{240a}{255} - 120$$

$$b^* = \frac{240b}{255} - 120$$

$$\Delta E = \sqrt{(L^* - L_1^*)^2 + (a^* - a_1^*)^2 + (b^* - b_1^*)^2}$$

In the formulas, L , a and b are obtained from the Histogram Window of Photoshop CS6 measuring color components of every shell, but they are not standard color values and should be converted to standard color components including L^* , a^* and b^* . L^* represents the lightness component ranging from 0(darkest) to 100(brightest). Both a^* and b^* are chromatic components and range from -120 to +120, which means from green to red and from blue to yellow, respectively. L_1^* , a_1^* , and b_1^* was the values of the typical golden shell color individual and the typical one.

The polymorphic information content (PIC), combined non-exclusion probability of first parent and combined non-exclusion probability of second parent of every microsatellite loci

were calculated with CERVUS 3.0. Using the likelihood-based approach and simulation module, the critical score was calculated, which suggests the difference in log likelihood ratios between the two most-likely parents and was applied to assign the offspring to the most-likely candidate parent.

To perform the stimulation module as needed, the preferences were as follows: 10000 replication cycles, 30 candidate mothers and 10 candidate fathers, 100% of the candidate parents sampled and genotyped and a default typing error rate of 1% was used. Success rates of cumulative assignment was calculated according to the genotypes of every brood and offspring, in which locus were added from the most informative to the least one that was judged by the PIC values. The allele number, expected heterozygosity (H_e), observed heterozygosity (H_o), probability test for Hardy-Weinberg equilibrium (HWE) and null allele frequency were estimated with CERVUS 3.0 to reflect the genetic variation of the parent and progeny populations, and the presence of null alleles at microsatellite loci can be safely accommodated in this program. Significance with Bonferroni correction was also tested with it. Preliminary analysis of data was performed with SPSS 16.0 software, thereby testing normality and homogeneity of variances.

The heritability estimates for L^* , a^* , b^* and E were obtained from univariate animal model using ASREML 3.0 in the R Programming Language (Gilmour et al., 2009). As the random common environment effect can be simplified, the variance components was evaluated with the following animal model:

$$Y_{ijk} = \mu + I_{ij} + e_{ijk}$$

In the model, Y is the observation from sire i, dam j and the individual k

I_{ij} represents the random genetic effects for the individual, e_{ijk} shows the the residual error. I_{ij} is the interaction of sire i and dam j.

Genetic and phenotypic correlations were calculated among shell color-related traits and growth-related traits using bivariate model in ASREML 3.0.

3. Results

3.1 Descriptive statistics and polymorphism information of microsatellite makers

The descriptive statistics consisted of the mean values, standard deviations, skewness, kurtosis and coefficients of variation (CV) for color-related and growth-related traits are listed

in Table 1. The coefficient of variation was highest (60.66%) for shell height, whereas it lower for other traits ranging from 7.74 to 47.78%.

Recapitulative statistics for the six microsatellite markers applied are listed in Table 2. The allele number of six loci ranged from 5 to 12 with an average of 8.2, and PIC changed from 0.522 to 0.853 with a mean value of 0.685. The average probability that the locus will not exclude an unrelated candidate parent from parentage of an arbitrary offspring when the genotype of the other parent is unknown (NE-1P) and known (NE-2P) showed the lowest value 0.425 and 0.268 in ucdCg-117 separately. The observed heterozygosity (H_O) and expected heterozygosity (H_E) ranged from 0.596 to 0.852, and from 0.555 to 0.868, respectively.

3.2 Parentage assignment and summary statistic in families

Cumulative assignment success of the six loci in the simulated offspring to the hypothetical candidate parents and real sampled individuals to their parents was calculated by adding loci from the most to the least informative one (Fig. 1). In the strict level of 95% confidence interval, 10000 simulated offspring were assigned to the hypothetical parent pairs and the success rate is beyond 90% with five loci. In addition, in the real sampled populations, when combined with nested mating design and the number of loci is up to 6, 690 of 810 progeny were unambiguously assigned to the broodstock with the success rate of 85.19% (Table 3). After family reconstruction, we evaluated the color-related traits of each family in terms of L^* , a^* , b^* and E . The full-sib family produced by dam 22 and sire 8 has the largest average b^* of 32.14, whereas the family produced by dam 24 and sire 8 has the lowest E of 5.26 (Fig. 2).

3.3. Genetic parameters

The heritabilities for L^* , a^* , b^* and E and the phenotypic and genetic correlations among them are given in Table 4. Estimates of L^* gave the low heritability (0.10), whereas the a^* , b^* and E were moderately to highly heritable ranged from 0.23 to 0.41. The estimates of correlations among color traits had large difference. L^* showed inconspicuous genetic correlations with other traits. Only both estimates between L^* and a^* were negative correlation (-0.08, -0.11). The genetic correlations among a^* , b^* and E showed moderate values ranging from 0.21 to 0.28. The highest estimates of phenotypic correlations was between b^* and E

(0.65).

The phenotypic and genetic correlation parameters between the shell color and growth traits are shown in Table 5. Both correlation parameters between a^* and E and b^* and E was relatively higher than other color parameters. In addition, the correlation between L^* and shell length, L^* and shell width, b^* and total weight showed relatively low negative values. In terms of genetic correlations, the value between a^* and shell width was highest (0.11 ± 0.16), and between L^* and shell height, L^* and shell length, b^* and shell width, they were lowest. On the phenotypic correlation side, the estimate was the highest between a^* and shell width (0.18 ± 0.06), and it is the lowest between L^* and shell length (0.02 ± 0.05).

4. Discussion

4.1 Measurement and analysis of shell color traits

The traditional approach is observing with naked eyes to contrast the colors, which decide which grades they are classified to. The method is subjective and its standards may be different from people considerably, which was used in some aquatic species like Banana shrimp (*Fenneropenaeus merguensis*) (Nguyen et al., 2014). It was also seen in some studies about shell pigmentation in *C. gigas* (Ge et al., 2015), in which the whole left shell color of each individual was generally divided into four or six levels depending on magnitude of darkness and all colors different from white were regarded as darkness. With electronic instruments like the colorimeter or colour photometer, hazy subjective standards can be quantified precisely, which is useful to evaluate the color variation. In Australian snapper *Pagrus auratus* (Doolan et al., 2008), sea urchin *Strongylocentrotus intermedius* (Chang, 2010) and large yellow croaker *Larimichthys croceus* (Yi et al., 2014), the CIE color scale (L^* , black-white; a^* , green-red; b^* , blue-yellow) were used. However, boundedness and unreliability is obvious when it is used in measuring shells of oysters for the great shape variation, bumpiness and uneven distribution of different colors in the surface.

In this study, a computer vision system (CVS) (Mendoza et al., 2006) was applied to quantify the CIE color scale of oyster shell colors. With this system, data can be collected from the whole image in a non-contact way, which can avoid effects of environmental and human factors and has been widely used in professions like food technology (Yam and Papadakis, 2004), especially in aquatic food products (Alçiçek and Balaban, 2015).

4.2 Family reconstruction

The correct inference of genealogy is essential for genetic parameter estimates (Dodds et al., 2005). The parentage assignment methods based on the microsatellite markers have been proved accurate and efficient in tracing the pedigree information of many aquatic species (Fu et al., 2016) including *C. gigas* (Kong et al., 2015), which enable the target population to be reared communally to avoid environmental effects resulting from separate rearing of full- or half-sib families. The reliability and accuracy of relationship inference and parentage assignment depends heavily on the informative degree of the microsatellite markers in the research group (Kristjánsson and Arnason, 2016). The factors affecting the resolution power also include the number and polymorphisms of microsatellite markers, null allele frequency, number of parents, the increase of homozygosity along with select breeding and genotyping errors (Navarro et al., 2009). In nature, effects of null alleles would probably not alter the overall outcome of assignment testing (Carlsson, 2008) and the discriminatory power of a

4.3 Genetic parameters

Some studies about shell pigmentation have been performed in *C. gigas*, which reported markers and loci linked to shell pigmentation and the Mendelian inheritance of golden shell color (Ge et al., 2014; Ge et al., 2015). In addition, heritability estimates for the shell color of *C. gigas* were performed (Evans et al., 2009; Kang, Kang et al., 2013; Wang et al., 2016). In our study, the heritability for the shell color in *C. gigas* was estimated with the combination of mixed families reconstructed with molecular markers, and CVS color quantification system.

To a lesser extent, our estimates for L^* (0.10), a^* (0.41), b^* (0.23), and E (0.33) are lower than those acquired in the previous studies, which may reflect the elimination of the random common environment effect. It suggests the comparatively high genetic potential of color trait in *C. gigas* and favorable improvement can be acquired through continuous selection. In other shellfish species, using the similar method, only the heritability of *H. cumingii* was estimated for inner shell color, whose results also indicates the genetic potential for shell color (Wang et al., 2014). Generally, in the four color traits, the differences between the phenotypic correlations and genetic correlations indicates that only L^* is not correlative to other three parameters, whereas a^* , b^* and E have relatively higher phenotypic correlations and genetic correlations ranging from 0.35 to 0.65 and 0.21 to 0.28 respectively.

In contrast, no apparent correlations were found between color traits and growth traits, suggesting that in most cases there are no apparent correlations among different types of traits. This is consistent with that concluded in recent studies in *C. gigas* (Wang et al., 2016) and *H. cumingii* (Wang et al., 2014). Though all progenies were cultivated in a single environment consulting in the difficulty in the evaluation of $G \times E$ interactions in our work, Evans and Langdon (Evans and Langdon, 2006) concluded that $G \times E$ interactions were significant but not large enough to prevent selection from requiring favourable gains when environments changed from a limited number of environments to others. In addition, the low number of families and sampled progeny may interfere the estimates.

In conclusion, our studies further confirmed the feasibility of the posteriori family reconstruction based on molecular markers in communal rearing environment. In the golden shell color strain of *C. gigas*, shell pigmentation is under relatively high genetic control with the moderate-to-high narrow-sense heritability value. This indicates there is potential for genetic improvement, and suggests that good improving efforts would be predictable and it would be a ideal candidate for selective breeding programme in aquaculture. In contrast, there

are no significant correlations between the golden shell color and growth traits, suggesting that reciprocal selection would not work. Therefore, it would be feasible to take both two types of traits as target traits in order to improve them at the same time. Our study offers an important reference in the process of selective breeding in golden shell color stain of *C. gigas*.

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Figure legends

Fig. 1 Cumulative assignment success rate vs. number of assignments. BDC BT1 0 0 1 217.97 718.9 Tm()JTJ

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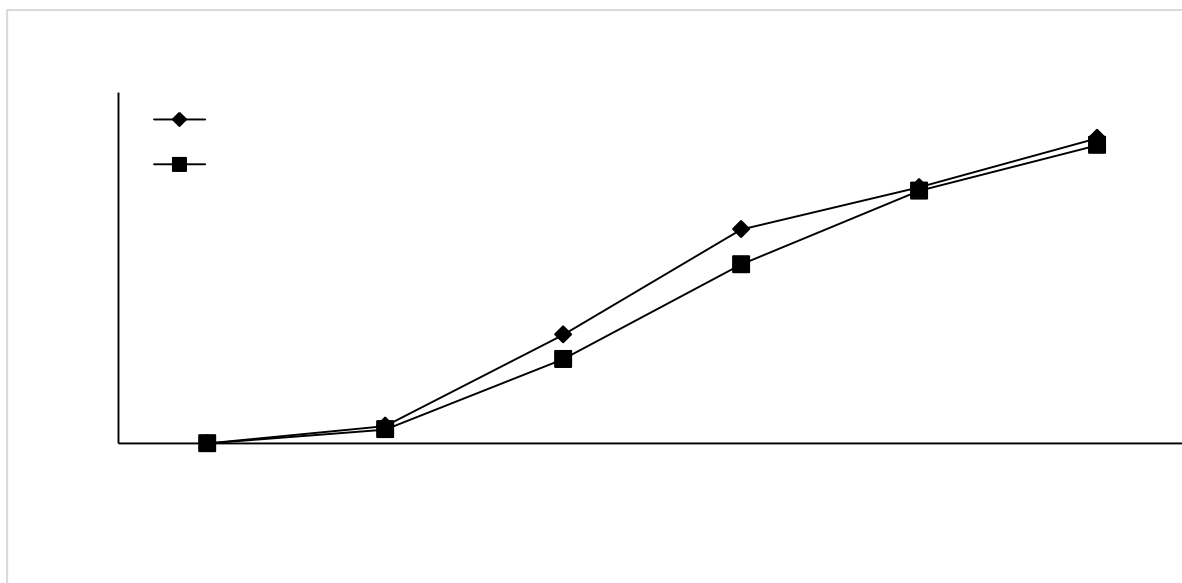


Fig. 1. Cumulative assignment success rates by CERVUS 3.0 of 10000 simulated offspring to their 40 hypothetical candidate parents and real genotype data sampled in a strict level of 95% confidence interval, adding loci from the most to the least polymorphic information content one

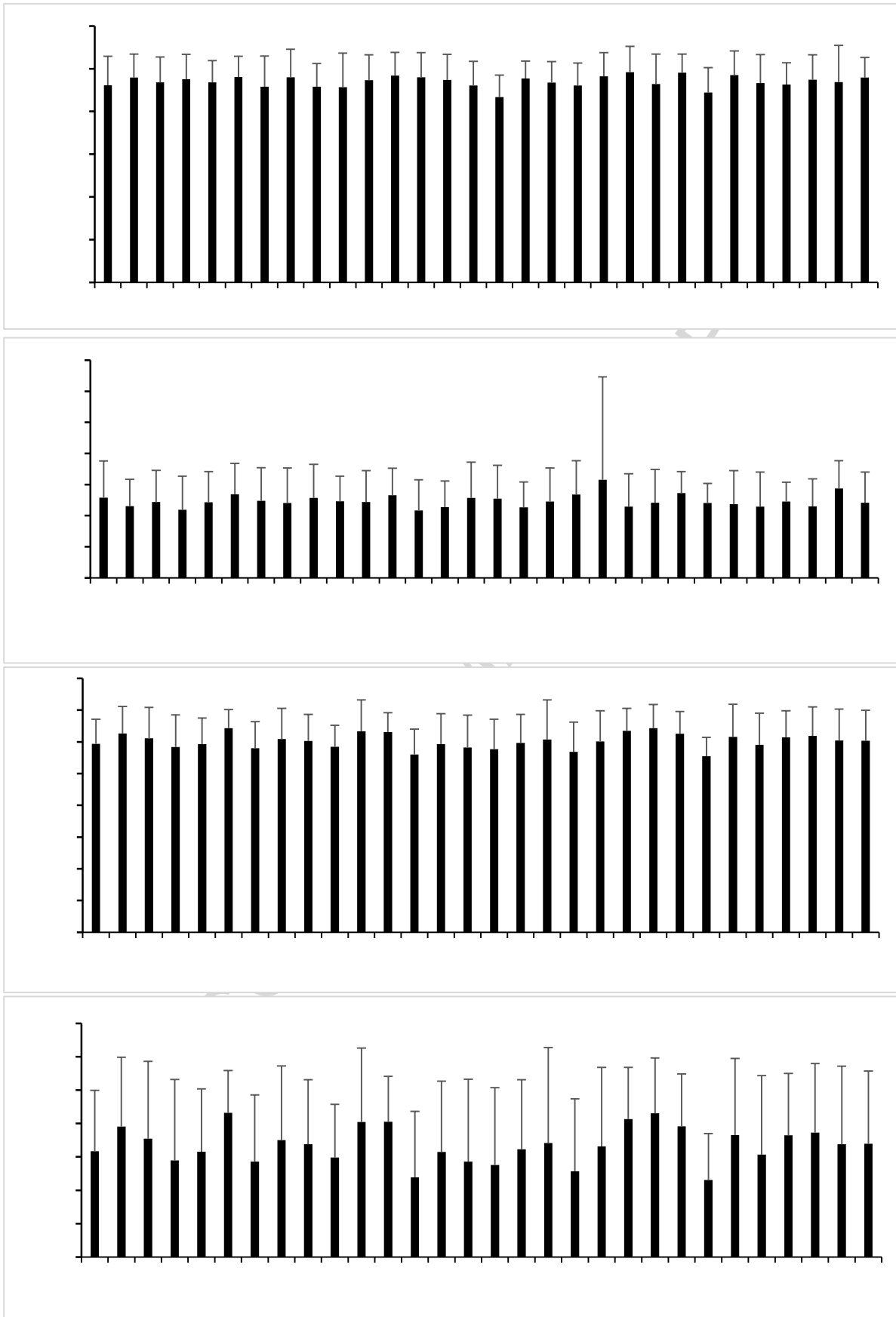


Fig. 2. Thirty full-sib family-related trait performance in terms of L^* , a^* , b and E at 16 months of age. Each family is coded by its dam number

Table 1 Descriptive statistic of the golden shell color-related parameters (L^* , a^* , b^* , E) and the growth-related traits of *C. gigas*

Trait	Mean value	Standard deviation	Skewness	Kurtosis	CV (%)
L^*	47.09	5.90	-0.28	0.11	12.54
a^*	4.92	2.21	1.86	20.04	44.92
b^*	30.16	4.55	-0.29	0.41	15.09
ΔE	5.48	0.42	-0.59	0.97	7.74
Shell height (mm)	83.78	50.81	18.42	381.22	60.66
Shell length (mm)	46.48	22.04	23.02	609.26	47.43
Shell width (mm)	27.12	6.33	1.50	4.85	23.34
Total weight (g)	50.63	24.18	8.84	163.59	47.78

Table 2 Numbers of alleles (k), polymorphic information content (PIC) , probabilities of non-exclusion either on genotype of no parent known (NE-1P) or one parent known (NE-2P), observed heterozygosity (H_O) and expected heterozygosity (H_E) for the six microsatellite loci analysed in this study.

Locus	k	PIC	NE-1P	NE-2P	H_O	H_E
Crgi3	7	0.522	0.828	0.657	0.645	0.555

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Table 3 Number of progeny assigned to each of the 30 full-sib families based on microsatellite genotyping.

Sire	1		2		3		4		5		6		7		8		9		10		Total										
Dam	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
Number	33	50	36	40	28	15	48	46	63	4	17	20	18	22	24	10	15	24	18	13	10	5	14	3	39	17	6	32	15	5	690

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Table 4 Genetic correlations (above diagonal) and phenotypic (below diagonal) with heritabilities in the diagonal

Parameter	L^*	a^*	b^*	E
L^*	0.10 ± 0.12	-0.11 ± 0.09	0.01 ± 0.09	0.05 ± 0.15
a^*	-0.08 ± 0.05	0.41 ± 0.16	0.25 ± 0.09	0.21 ± 0.08
b^*	0.35 ± 0.07	0.38 ± 0.08	0.23 ± 0.15	0.28 ± 0.35
E	0.47 ± 0.07	0.35 ± 0.03	0.65 ± 0.02	0.33 ± 0.19

Table 5 Phenotypic and genetic correlation parameters between the shell color and growth traits of 16-month-old golden shell color *C. gigas*.

Trait	L^*		a^*		b^*		E	
	r_g	r_p	r_g	r_p	r_g	r_p	r_g	r_p
Shell height	$0.01 \pm$	$0.03 \pm$	$0.02 \pm$					
(cm)	0.08	0.09	0.08					

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Highlights

1. Genetic parameters(heritability, phenotypic and genetic correlations) for shell color in the golden shell color strain of *C. gigas* was estimated.
2. The shell color was quantified with a Computer Vision System (CVS).
3. The families of oysters were fostered communally and reconstructed with six microsatellite makers.

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