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Mendelian inheritance of orange shell color in the Pacific oyster *Crassostrea gigas*

Ziqiang Han[a], Qi Li[a,b,*]

[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, Qingdao, 266237, China

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ABSTRACT

The oyster shell color has become an important factor influencing consumer choice. Understanding the genetic basis of shell colors is important in oyster genetic breeding. In this study, we investigated the inheritance of the orange shell color trait in the Pacific oyster. The oysters with orange shell color were generated in our breeding studies that has never been observed in natural population. It is a novel mutant trait in the Pacific oyster. In this study, we investigated the inheritance of the orange shell color trait crossing the orange variant with two other color variants (white and black). The shell color morph was determined for each in 27 first- and 12 s-generation crosses as well as 13 test crosses. We found that the orange shell color is recessive trait compared to black and white, and is determined by two independent recessive genes. In addition, our data revealed that the genetic loci controlling orange shell have no effects on the formation of black foreground pigment. The information obtained in this study could guide the breeding program for desired shell color in pacific oyster.

1. Introduction

Surface color of aquatic animals, as the most intuitive phenotypic trait, is one of the important factors influencing consumers' preference at the seafood market, thus, having a direct impact on product value (Alfnes et al., 2006; Clydesdale, 1993). Molluscan shellfish have traditionally been a major product of world aquaculture, and their shell color also influences consumer preference. Moreover, molluscan shells that have high phenotypic variation and striking coloration and patterns have attracted many collectors and scientists for hundreds of years (Williams, 2016). Therefore, whether shell color can be integrated into the breeding target of molluscan shellfish has attracted the attention of breeders and scientists (Brake et al., 2004; Brown et al., 2008; Evans et al., 2009). To answer this question, it is critical to understand the genetic basis of different shell colorations.

Fortunately, the availability of different shell phenotypes from wild stocks in most taxa provides the possibility of studying the inheritance patterns by controlled crossing. In some cases, shell color polymorphism is attributable to a major single locus. For example, a one-locus three-allele model for shell trait was proposed for *Pinctada margaritifera*, whereby the black allele is dominant to red coloration, which is dominant to the white shell (Ky et al., 2016). In addition, it has been reported that multiple alleles at a single locus control the shell coloration polymorphism in *Haliotis discus hannai* Ino (Kobayashi et al., 2004; Liu et al., 2009), *Macoma balthica* (Luttikhuizen and Drent., 2008) and *Hyriopsis cumingii* (Wen et al., 2013). However, some color variation was controlled by a second locus, epistatic over loci governing the expression of other colors, such as the orange shell coloration in *Chlamys nobilis* (Zheng et al., 2013) and purple shell coloration in *Argopecten purpuratus* (Winkler et al., 2001). It has been suggested that some color variation might be controlled by more than two interacting loci, such as the orange shell in *Argopecten purpuratus* (Winkler et al., 2001). Crossing studies in both bivalves and gastropods have shown that shell coloration in many species is a heritable trait, but the modes of inheritance of various color morphs differ significantly depending on the species and color morphs.

The Pacific oyster (*Crassostrea gigas*) is one of the most widely farmed aquaculture species worldwide (FAO, 2016). With the increasing demand for live and half-shell oysters, there is a strong incentive to improve oyster shell traits (Mizuta and Wikfors, 2018). Shell color, as an intuitive character of oyster shells, plays an important role in increasing the commercial value of oysters (Nell, 2001). However, little is known about the genetic basis of shell color determination in *C. gigas* compared with the other Mollusca species. Shell pigmentation of naturally *C. gigas* exhibits a continuous variation from near-white, pigment-free shells to near-black, fully pigmented shells, with some mixed with some golden and purple shell colors (Brake et al., 2004). Black pigmentation was identified as a foreground color while golden

* Corresponding author. 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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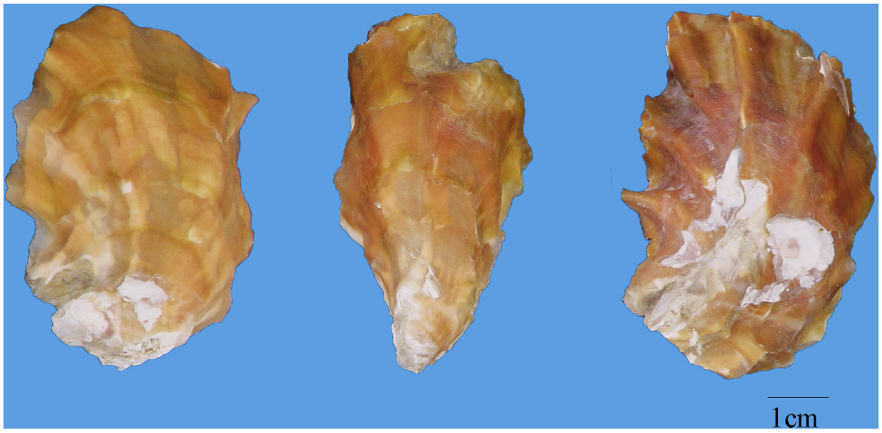

Fig. 1. Orange shells with different saturations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and white were background colors (Ge et al., 2015). Black pigmentation was initially considered as a quantitative trait that is controlled by multiple genes (Brake et al., 2004). However, some evidences suggested that the existence of a major locus responsible for the black shell (Evans et al., 2009; Ge et al., 2014). Interestingly, it appeared that the secretion and distribution of black pigmentation are controlled by two independent loci (Xu et al., 2019). Golden shell color was inherited in a different pattern compared with black pigmentation. The golden coloration in the shell is dominant over white and has an epistatic effect on black pigmentation (Ge et al., 2015). At present, only the inheritance patterns of black and one special shell color (golden) have been analyzed in *C. gigas*. Considering the complexity and variety of shell pigmentation in *C. gigas*, it is critical to study the inheritance patterns of other colors to better understand the genetic basis of shell coloration in *C. gigas*.

In our previous work of *C. gigas* family breeding, a novel shell color mutant with both left and right orange shells was obtained that has not been reported in the natural population before (Fig. 1) (Han and Li, 2018; Han et al., 2019). Unlike the natural population of *C. gigas* with black and golden shell colors that exhibit a continuous variation in pigmentation, the orange-shell mutant possesses a pure orange shell without any other pigmentation.

In order to explore the inheritance pattern and the number of genes controlling orange coloration in *C. gigas*, we carried out controlled crosses between oysters with orange and two extreme shell colors (white and black). The color morphs were analyzed in the first- and second-generations as well as progenies of test crosses. We showed that the genetic basis of orange coloration differed from the black and golden coloration reported previously. Understanding the inheritance pattern of orange coloration will bring new insights into the genetic basis of shell coloration in *C. gigas*.

2. Material and methods

2.1. Parental source

Four individual oysters with solid orange shell (about 0.2%) were accidentally identified in the offspring of individuals with solid purple-black left and right shells that were originally produced by crossing females with solid black left shell and males with solid purple left shell selected from the cultured population of *C. gigas* in Rushan, Shandong Province, China (36.5° N, 121.4° E) (Ge et al., 2005; Han et al., 2019; Xu et al., 2019). The four individuals (two males and two females) with left and right orange shells were used to establish the orange shell *C. gigas* line through three generations of family selection and followed by three generations of mass selection (Han et al., 2019). *C. gigas* with relatively white shell and black shell were collected from wild populations in Rushan as base populations, respectively. Breeding program for pure shell color (white and black) and fast growth traits were implemented through four generations of family selection from 2010 to 2013 and three generations of mass selection from 2014 to 2016 (Xing et al., 2018; Xu et al., 2017). In 2017, one-year-old oysters with pure orange, white and black shell (Fig. 2A) randomly selected from their respective strains were used to produce first-generation (F1) crosses.

2.2. Mating experiment

In 2017, a total of 27 F1 families with nine experimental crosses and three replicates were produced by 3 × 3 factorial mating among oysters with orange, white and black shell. In 2018, 18 second-generation (F2) families were produced by single-pair mating between F1 crosses. Six were lost during the grow-out stage. Among the F2 families, five families were from orange shell ♀ × white shell ♂, two from white shell ♀ × orange shell ♂, and five from black shell ♀ × orange shell ♂. In addition, 18 test-crosses (TC) were produced by single-pair mating between orange-shell *C. gigas* selected from eighth selection generations (O_{G8}) and F1 crosses. Five were lost during the grow-out stage. Among the survived families, five of which were from orange shell ♀ × white shell ♂, three from white shell ♀ × orange shell ♂, three from black shell ♀ × orange shell ♂, and two from orange shell ♀ × black shell ♂ (Fig. 3).

For all mating experiments, artificial spawning and rearing management in larvae, spat and adult stage was conducted following the standard procedure described as (Li et al., 2011).

2.3. Shell coloration record

Samples of all F1 families were randomly collected on day 270 in 2017, and samples from all F2 and TC families were randomly collected on day 210 in 2018. All samples were shucked and their shells were cleaned and air-dried in the dark. Since three shell morphs had striking difference, the offspring from each family were classified by eye observation according to the color of the left valve. The shell was defined as “white” when there was no additional pigmentation covering the shell. Similarly, the shell was defined as “orange” or “black” only when orange or black covering the entire shell. Because oysters grow together in long-line culture method, the shells were easily damaged when separated. The lack of pigment in a small portion of the shell caused by the physical damage of the shell was ignored.

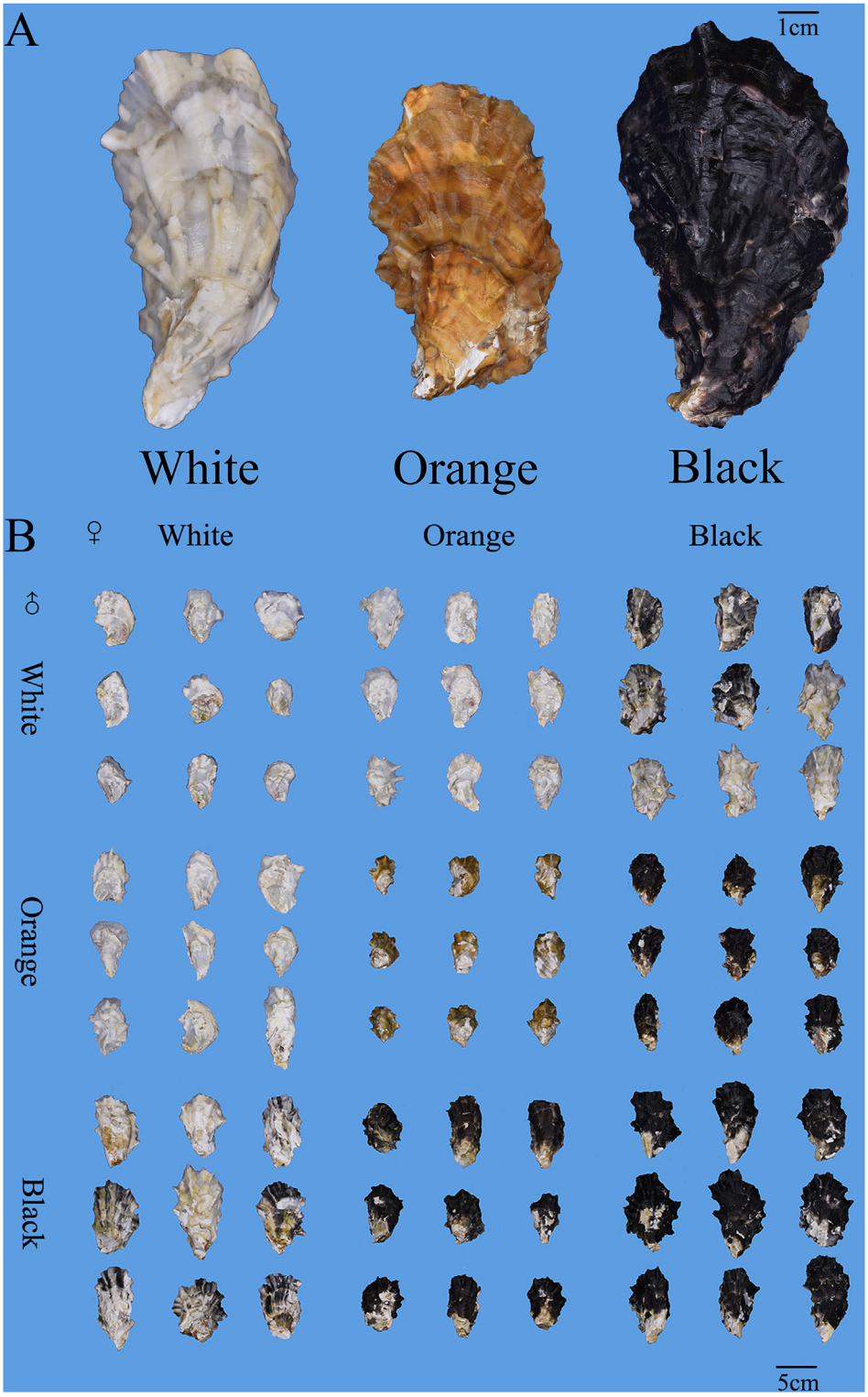

Fig. 2. Representative parents of white, orange and black shell color patterns (A) and representative offspring of first-generation crosses produced by 3 × 3 factorial mating among oysters with three shell color (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.4. Statistical analysis

The proportions of individuals with discrete shell colors in the F2 and TC were analyzed under the assumption of a two-locus, two-allele model. The parental genotypes were inferred from the parental and progeny phenotypes. The fit of the observed segregation ratios to the expected under the proposed pattern of inheritance were tested using the chi-squared test for goodness-off-it. The heterogeneity between families within each cross-type was quantified by I^2, which describes the percentage of total variation across studies that is due to heterogeneity rather than chance (Higgins et al., 2003).

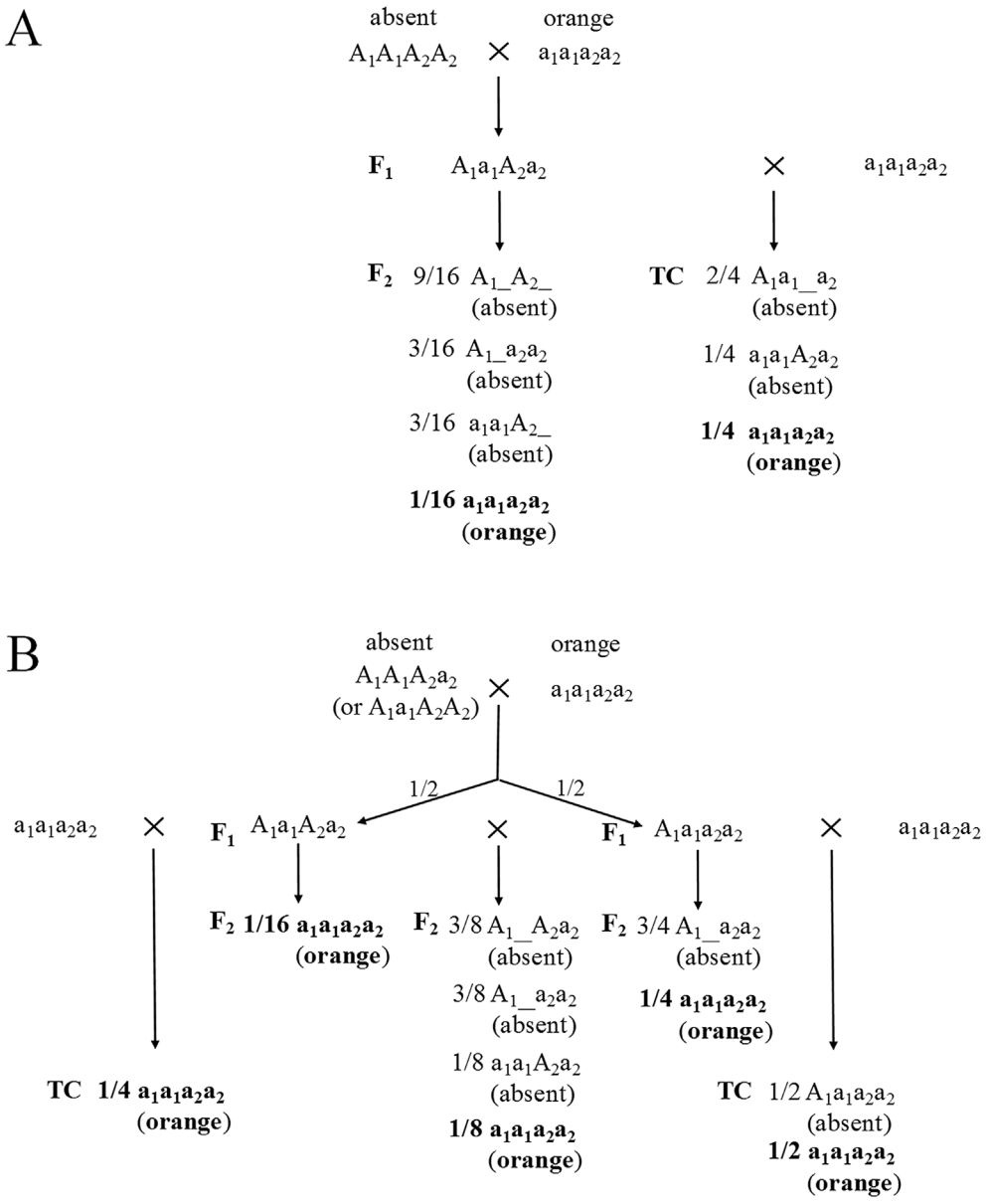

Fig. 3. Proposed inheritance pattern of orange shell color ($a_1a_1a_2a_2$) when $a_1a_1a_2a_2$ was crossed to $A_1A_1A_2A_2$ (A) and $a_1a_1a_2a_2$ was crossed to $A_1a_1A_2A_2$ or $A_1A_1A_2a_2$ (B). F1 = first-generation; F2 = second-generation; TC = test crosses.

3. Results and discussion

The shell color phenotypes in the offspring of 27 F1 families were shown in Table 1 and Fig. 2B. Only orange offspring were observed in three orange families (OO_1, OO_2 and OO_3). Similarly, each of the black (BB_1, BB_2 and BB_3) or white (WW_1, WW_2 and WW_3) families produced only black and white offspring, respectively. In crosses between oysters with orange shell and oysters with white shell (OW_1, WO_1, OW_2, WO_2, OW_3 and WO_3), only white offspring were observed in all crosses regardless the parental orange shell oysters were male or female. Similarly, out-crossing oysters of orange shell with black shell oysters (OB_1, BO_1, OB_2, BO_2, OB_3 and BO_3) produced only black offspring. Moreover, oysters with distinct orange shell were observed in the offspring of each F2 families (Table 2, Table 3). Results from F1 and F2 indicate that the orange coloration is a recessive trait inherited in a Mendelian pattern and it is not sex linked. This is consistent with our observation that non-orange oysters have never been found during the breeding of orange shell line in our study. F1 crossing white shell oysters with black shell oysters produced F1 individuals of black pigmentation on the external surface with varying degrees of mottles and bands (Fig. 2B) as reported by Ge et al. (2015) and Xu et al. (2019). In contrast, dark pigmentation covered the entire shell surface from crosses of orange shell oysters with black shell oysters (Fig. 2B, Table 1). And distinct black offspring were observed in each of the F2 families of cross orange shell × black shell (Table 3). Collectively, these data indicate that the genetic locus controlling the orange shell color has no effect on the black locus. Moreover, it suggested that the inheritance pattern of orange shell color is different from that of the golden color as previously reported by Ge et al. (2015), because the golden is dominant over white and has an epistatic effect on its black foreground pigmentation.

The number of loci controlling orange coloration in *C. gigas* can be inferred from the segregation ratios of individuals in the F2 and test crosses (Tables 2 and 3). The heterozygous F1 oysters from the out crosses of orange shell with white shell were used for in-cross to produce F2 families (Table 2). The data revealed that each of the F2 crosses of orange shell × white shell (crosses 1–7) produced discrete orange and white offspring close to a ratio of 1:15 ($P > 0.05$), suggesting that two independent recessive genes control the appearance of orange shell

Table 1
Shell color of the offspring from first-generation crosses among individuals with orange shell (O), white shell (W) and black shell (B) color of *C. gigas*.

Family	Parents		shell color of offspring		
	Female	Male	Orange	White	Black
OO_1	O-1	O-4	72		
OO_2	O-2	O-5	100		
OO_3	O-3	O-6	54		
WW_1	W-1	W-4		87	
WW_2	W-2	W-5		95	
WW_3	W-3	W-6		66	
BB_1	B-1	B-4			120
BB_2	B-2	B-5			71
BB_3	B-3	B-6			80
OW_1	O-1	W-4		96	
WO_1	W-1	O-4		91	
OW_2	O-2	W-5		112	
WO_2	W-2	O-5		59	
OW_3	O-3	W-6		74	
WO_3	W-3	O-6		69	
BO_1	B-1	O-4			65
OB_1	O-1	B-4			71
BO_2	B-2	O-5			89
OB_2	O-2	B-5			55
BO_3	B-3	O-6			95
OB_3	O-3	B-6			114

Since black pigmentation with varying degrees of mottles and bands appeared on the external surface of first-generation crosses between oysters with white shell and oysters with black shell (WB or BW; see Fig. 1B), the data of these crosses were excluded from the Table 1.

(Fig. 3A). The shell color appear orange when both loci carry recessive homozygous mutants ($a_1a_1a_2a_2$). The orange coloration is absent when either of these two loci has at least one copy of a dominant allele A_1 or A_2. This hypothesis was supported by the result of all test crosses (crosses 13–20) in our study that produced offspring of two distinct groups with a 1:3 of orange to white ratio ($P > 0.05$).

In crosses of orange shell × black shell, all offspring from F2 and test crosses had discrete orange or black shell. However, only two F2 crosses (crosses 8 and 12) had orange and black offspring close to a ratio of 1:15 ($P > 0.05$) and three test crosses (cross 22–24) fitted a 1:3 orange to black ratio ($P > 0.05$), respectively (Table 3). The excepted segregation ratio 1:15 for F2 crosses and 1:3 for test crosses were based on the assumption that the black color founders were homozygote. However, recessive a_1 or a_2 alleles may persist in the black shell line. As stated in the Materials and Methods, the four founder individuals with orange shell color were initially identified in the offspring of parents with black-purple shell in *C. gigas*. Therefore, the deviations ($P < 0.05$) from the expected ratio in the separation ratio of offspring in F2 (cross 9–11) and test crosses (cross 21 and 25) maybe due to one of loci in black shell parent is heterozygote ($A_1A_1A_2a_2$ or $A_1a_1A_2A_2$). If the black shell parent is a heterozygote, the shell color of the F1 will still be black, but its genotype will be $A_1a_1A_2a_2$ or $A_1a_1a_2a_2$ (Fig. 3B). The F2 will have a segregation of 1 orange shell to 15 black shell oysters in the cross of $A_1a_1A_2a_2 \times A_1a_1A_2a_2$, while segregated into a 1:3 ratio for orange shell to black shell in cross of $A_1a_1a_2a_2 \times A_1a_1a_2a_2$. In addition, in the cross of $A_1a_1A_2a_2 \times A_1a_1a_2a_2$, they could produce oysters with a 1:7 ratio of orange shell to black shell. As showed in Table 3, the segregation ratio for orange shell and black shell of cross 9 was approximately 1:3 ($P > 0.05$), and cross 10 produced both orange and black offspring in a ratio close to 1:7 ($P > 0.05$). The segregation ratios in the cross 11 fitted nicely with the ratios of 1:3 ($\chi^2 = 1.210$, $P > 0.05$) and 1:7 ($\chi^2 = 1.788$, $P > 0.05$) for orange shell and black shell, respectively. These results suggest that the black parent is likely a heterozygote ($A_1A_1A_2a_2$ or $A_1a_1A_2A_2$) (Fig. 3B). Meanwhile, the results of test crosses that crosses 21 and 25 fitted to a ratio of 1:1 and crosses 22–24 fitted to ratio of 1:3 for orange oysters and black oysters corroborated this hypothesis. However, the genotypes of F1 hybrids could not be determined from the available data because the observed segregate ratios of crosses 8, 11 and 12 were not significantly different from two expected ratios according to the chi-square test ($P > 0.05$).

In addition, there seems to be another explanation for the observed separation ratio in F2 crosses of orange shell × black shell. All the F2 crosses (crosses 8–12) produced discrete orange and black offspring close to a ratio 3:13 ($P > 0.05$) (Supplementary Table S1), which is characteristic of a recessive suppressor acting on a recessive mutation (Supplementary Fig. S1). A suppressor is an allele that reverses the effect of a mutation of another gene, resulting in the normal (wild-type) phenotype. If this hypothesis is true then the test crosses will have a segregation of 1 orange shell to 1 black shell oysters (Supplementary Fig. S1). However, three out of five test crosses significantly different from 1:1 ($P < 0.05$) (Table 3). Therefore, the results of this study do not support the hypothesis that suppressor existence.

From the segregation ratio of all F2 and test crosses, we can

Table 2
Shell color of offspring from second-generation crosses (F_2) and test crosses produced by orange shell (O) × white shell (W) of *C. gigas*, with indications of expected ratios and chi-square test.

Cross no.	Parents[a]		Observed color segregation			Chi-square test	
	Female	Male	Orange	White	SUM	Expected ratio	χ^2
F_2			$I^2 = 49\%$				
1	OW_1-1	OW_1-5	4	52	56	1:15	**0.076**
2	OW_1-2	OW_1-6	8	120	128	1:15	**0.000**
3	OW_1-3	OW_1-7	5	48	53	1:15	**0.917**
4	OW_3-2	OW_3-8	7	64	71	1:15	**1.579**
5	OW_3-3	OW_3-9	8	100	108	1:15	**0.247**
6	WO_2-2	WO_2-7	1	60	61	1:15	**2.213**
7	WO_2-3	WO_2-8	9	69	78	1:15	**3.723**
Test cross			$I^2 = 47\%$				
13	OW_1-4	O_{G8}-3	19	34	53	1:3	**3.327**
14	O_{G8}-1	OW_1-9	22	87	109	1:3	**1.349**
15	OW_3-4	O_{G8}-4	35	86	121	1:3	**0.994**
16	OW_3-5	O_{G8}-5	11	23	34	1:3	**0.980**
17	OW_3-6	O_{G8}-6	7	36	43	1:3	**1.744**
18	WO_2-4	O_{G8}-7	15	53	68	1:3	**0.314**
19	WO_2-5	O_{G8}-8	45	95	140	1:3	**3.810**
20	O_{G8}-2	WO_2-9	20	38	58	1:3	**2.782**

[a] O_{G8}, individuals of the eighth generation of orange-shell line of Pacific oyster. I^2, the heterogeneity between families within each cross-type. Bold indicates $P > 0.05$.

Table 3
Shell color of offspring from second-generation crosses (F_2) and test crosses produced by orange shell (O) × black shell (B) of *C. gigas*, with indications of expected ratios (Exp. ratio1, Exp. ratio2 and Exp. ratio3) and chi-square test.

Cross no.	Parents[a]		Observed color segregation			Chi-square test					
	Female	Male	Orange	Black	SUM	Exp. ratio1	χ^2	Exp. ratio2	χ^2	Exp. ratio3	χ^2
F_2			I^2 = 39%								
8	BO_1-2	BO_1-8	5	49	54	1:15	**0.835**	1:3	7.136	1:7	**0.519**
9	BO_1-3	BO_1-9	24	90	114	1:15	42.632	1:3	**0.947**	1:7	7.624
10	BO_2-1	BO_2-7	17	90	107	1:15	16.963	1:3	4.738	1:7	**1.123**
11	BO_2-2	BO_2-8	10	44	54	1:15	13.872	1:3	**1.210**	1:7	**1.788**
12	BO_2-3	BO_2-9	4	38	42	1:15	**0.768**	1:3	5.365	1:7	**0.341**
Test cross			I^2 = 75%								
21	BO_1-6	O-G_8-9	19	24	43	1:3	8.442	1:1	**0.581**	-	-
22	BO_2-4	O-G_8-10	5	28	33	1:3	**1.707**	1:1	16.030	-	-
23	BO_2-5	O-G_8-11	24	66	90	1:3	**0.133**	1:1	19.600	-	-
24	BO_2-4	O-G_8-12	15	45	60	1:3	**0.000**	1:1	15.000	-	-
25	BO_2-6	O-G_8-13	33	43	76	1:3	13.754	1:1	**1.316**	-	-

[a] $O_{G8,}$ individuals of the eighth generation of orange-shell line of Pacific oyster. Exp. ratio1, expected ratio for the hypothesis that the black shell colored founder individuals were homozygous (see Fig. 3A); Exp. ratio2 and Exp. ratio3, expected ratio for the hypothesis that the black shell colored founder individuals were heterozygote (see Fig. 3B). I^2, the heterogeneity between families within each cross-type. Bold indicates $P > 0.05$.

conclude that the orange coloration is governed by two independent recessive genes, a1 and a2. Both genes are required to be in the homozygous recessive form to produce the orange shell coloration. Similar inheritance pattern has been reported in many studies on flower (Pahlavani et al., 2004; Yue et al., 2008) and seed coat colorations (Vera et al., 1979). Further research is needed to investigate how these two genes, a1 and a2, play their roles in the formation of orange phenotype in *C. gigas*.

The proposed model presented in Fig. 3 provides a posssible explanation of the observations in this study, but this does not exclude other possible mechanisms, considering the limited number of crosses and moderate to substantial heterogeneity (I^2: 39–75%) within each cross-type in this study. In addition, this study only attempted to explain the inheritance pattern of orange shell relative to black and white shell in *C. gigas*. The inheritance pattern relative to other shell colors (e.g., golden) needs further investigation. Furthermore, the proposed model is based on the progenies from lines selected for several generations. The artificial selection may lead to the loss of polymorphism at some loci besides the two revealed in the present study (Xu et al., 2019). An initially rapid response to selection could result from the fixation of major genes followed by a more gradual and sustained response on polygenes. The genetics of shell pigmentation in the *C. gigas* might be more complex than our assumption. Therefore, further verification experiments need to be designed. Also, comparative studies on other important traits like growth or survival among different color types would be desired in future to apply the inheritance mode of shell color to commercial program of selective breeding of *C. gigas* as seen in other bivalve specie (Wada and Komaru, 1994, 1996).

In conclusion, our studies demonstrated that the orange shell color of Pacific oyster is recessive to black and white. Our data support the idea that the orange shell is controlled by two independent recessive genes and both genes exist in the homozygous recessive form to produce the orange coloration. In addition, the loci controlling orange shell have no effects on the loci controlling black foreground pigment. These results can be used to guide the oyster breeding with desired shell color and the cross breeding among oyster lines with different shell colors.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734616.

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