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# Analysis of protein kinase C (*HcPKC*) gene expression and single-nucleotide polymorphisms related to inner shell color traits in *Hyriopsis cumingii*

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## Abstract

**Background:** Protein kinase C (PKC) is a multifunctional serine and PKC can phosphorylate serine residues in the cytoplasmic domain of tyrosinase, thereby regulating the activity of tyrosinase. Activated PKC is bound to the melanosome membrane, and unactivated PKC is free in the cytoplasm of melanocytes. In this study, we study the role of PKC gene in the melanin synthesis pathway and its effect on the color of the nacre of *H. cumingii*.

**Results:** In this study, a *HcPKC* gene in *H. cumingii* was cloned and its effects on melanin synthesis and nacre color were studied. *HcPKC* was expressed in both purple and white mussels, and the level of mRNA expression was higher in the purple mussels than in white mussels. Strong and specific mRNA signals were detected in the dorsal epithelial cells of the mantle pallial layer, indicating that *HcPKC* may be involved in nacre formation. After SNP association with inner shell color related traits, according to the principle that $0.25 < PIC < 0.5$ is medium polymorphism and $PIC < 0.25$ is low polymorphism, the A + 332G site on the *HcPKC* gene was a site of moderate polymorphism, and the other four sites were low polymorphism sex sites. There was strong linkage disequilibrium among the five loci. A haplotype was constructed and it was found that the frequency of T1 (AGGAA)in the white population was significantly higher than that in the purple population ($P < 0.05$).

**Conclusion:** The study found that *HcPKC* of *H. cumingii* can be used as a candidate gene related to inner shell color, and some of the SNP sites can be used for molecular-assisted breeding in the spinnaker mussel, providing a reference for cultivating high-quality freshwater pearls.

**Keywords:** Hyriopsis cumingii, Nacre color, HcPKC, SNP

## Background

*Hyriopsis cumingii* is a unique freshwater mussel in China, which can cultivate high-quality pearls [1]. Mussels of this species produce pearls of high quality in terms of color, luster, and shape [2]. The freshwater pearls produced by purple *H. cumingii* have a very high economic value [3]. Recently, due to overexploitation, habitat loss, and environmental pollution, wild populations have declined significantly and are facing local extinction [2]. Therefore, the need to harvest high-quality freshwater pearls artificially is imminent. However, there are few

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relevant studies on the mechanisms involved in the formation of shell nacre color, but it is still in its infancy. Studies have shown that in addition to environmental factors, the color of pearls is also affected by the donor and recipient mussels [4]. Some studies suggest that some metal ions can also affect the formation of pearl color [5, 6], and other studies show that pearl color is related to organic pigments, such as porphyrin [7], carotenoids [8], and melanin [9–11], which produce pearls of different colors.

Protein kinase C (PKC) is a lipid- and $Ca^{2+}$-dependent serine/threonine kinase consisting of a single polypeptide chain [12]. Because the structure of each subtype has a certain conservation and specificity, the functions of the subtypes are also diverse [13]. PKC widely exists in animal tissues and cells, is the main mediator of signal transduction pathway, and also plays a role that cannot be underestimated in physiological processes such as cell proliferation and differentiation [14]. In the melanin metabolism pathway, PKC activates the tyrosinase by phosphorylation of its two serine residues [15]. Activated PKC is bound to the melanosome membrane, and unactivated PKC is free in the cytoplasm of melanocytes [16]. The physiological activation of PKC reportedly stimulates melanin production [17], whereas the inhibition of PKC activity or depletion of cellular PKC has been shown to inhibit melanin synthesis [18]. Park et al. [19] paired cultures of primary human melanocytes treated with PKC inhibitors, found that the PKC inhibitor bisindolylmaleimide can reduce skin pigmentation, and demonstrated that the inhibition of PKC-β activity can reduce pigmentation. Jung et al. [20] found that syndecan- 2 overexpression increased the membrane localization of PKCbII, and that activated PKCbII associates with the melanosome through RACK1 to regulate melanogenesis.

In this study, a PKC gene (*HcPKC*) was identified in *H. cumingii*, and its full length was cloned. The expression level of the *HcPKC* gene was detected in different tissues. In situ hybridization was used to detect the distribution of mRNA expression in the mantle. Single-nucleotide polymorphism (SNP) mutation sites were detected in *H. cumingii* using *HcPKC* as a candidate gene and correlation analysis was performed with color traits. The molecular markers related to the color traits of the shell nacre were screened and then *H. cumingii* were selected. This selection and breeding process provides basic data for further research.

## Results

### Full-length and sequence analysis of *HcPKC* gene

The full length of the *HcPKC* (GenBank accession MW241548) gene was obtained by 3′ and 5′ RACE cloning. The *HcPKC* gene sequence is 2134 bp in total, of which the 5′-UTR was 12 bp, the 3′-UTR was 1246 bp, and the ORF was 876 bp long, encoding a total of 291 amino acidsThe molecular weight of the mature protein corresponding to the amino acid sequence was 117.04 kDa, and the isoelectric point was calculated as 4.73. S_TKc and S_TK_X domains typical of serine- and threonine-specific kinase families were found. No signal peptide was found (Fig. 1).

### Quantitative gene expression analysis

The relative expression of the *HcPKC* gene in purple and white mussels was detected by qPCR. As shown in Fig. 2, the expression of *HcPKC* in purple mussels was higher than that in white mussels, with an extremely significant difference in the marginal membrane ($P < 0.01$), and no significant difference in other tissues. In purple mussels, the highest expression was in the marginal membrane, and it was significantly different from other tissues ($P < 0.05$). In the white mussel, the highest expression was in the adductor muscle, but there was no significant difference between the tissues.

### In situ hybridization results

The location of the specific expression of the *HcPKC* gene in the mantle tissue was determined by in situ hybridization. The results are shown in Fig. 3, The positive hybridization signal mainly appeared in the dorsal membrane epithelial cells of the outer fold of the mantle (arrow in Fig. 3 A), and no obvious signal was seen in other parts. No positive signal was detected in the negative control group.

### SNP site screening

The samples were amplified with the designed primers, and a total of five SNP sites were found in the amplified fragments. Starting from the ATG start codon, each SNP site is named by the number of bases from the mutation site to the start codon.

### Polymorphism analysis

The *HcPKC* gene was amplified and sequenced from 70 purple mussels and 70 white mussels to screen for the SNP loci. The polymorphic genetic parameters of the five SNP loci of the *HcPKC* gene obtained after the sequencing results were analyzed by software (Table 1). Their observed heterozygosity was in the range of 0.0143–0.0929, the expected heterozygosity was in the range of 0.0624–0.3254, the polymorphic information content(PIC) was in the range of 0.060–0.272, and the effective number of alleles was in the range of 1.0663–1.4799. A $0.25 < PIC < 0.5$ was considered moderate polymorphism and $PIC < 0.25$ was considered low polymorphism, the A + 332G site on the *HcPKC* gene was a

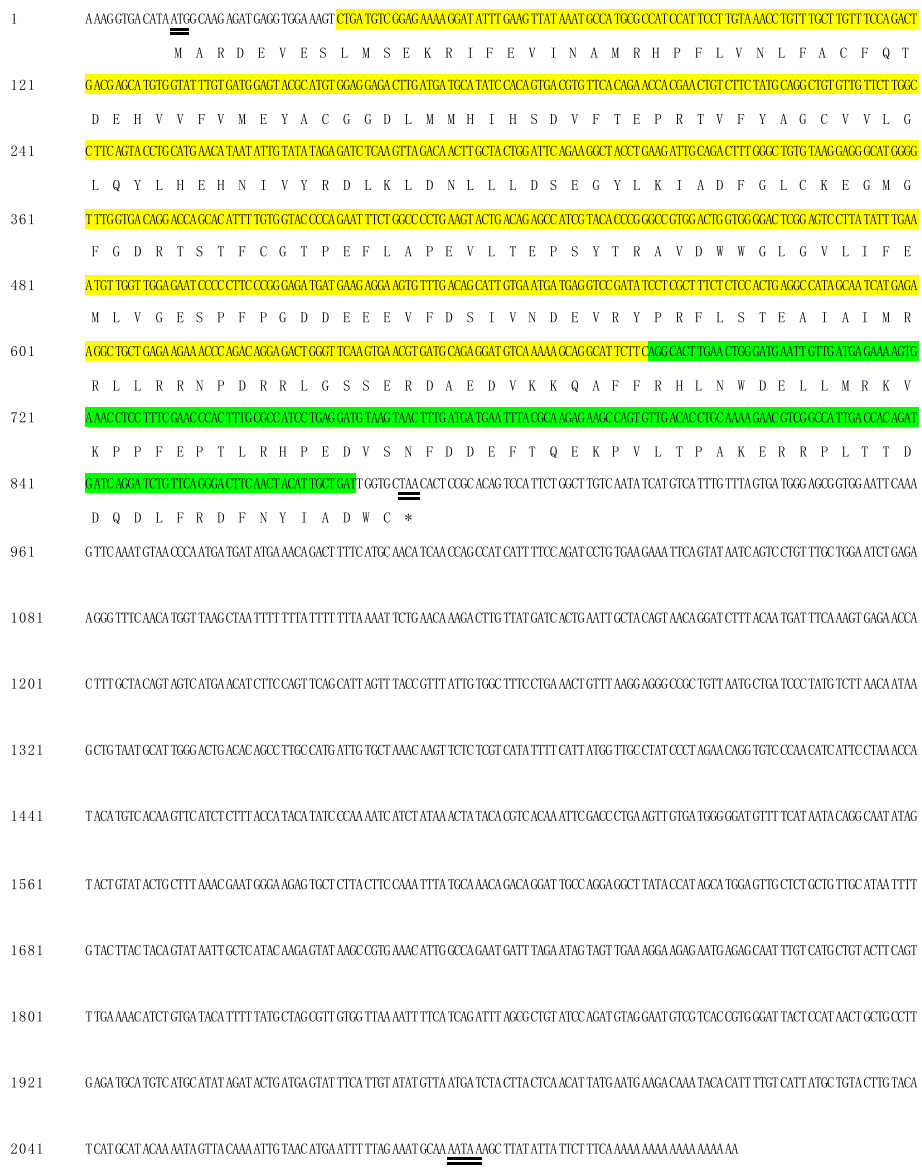


**Fig. 1** cDNA sequence analysis of the HcPKC gene in H. cumingii. The shaded part is the domain. The start codon, stop codon, and the poly-A tail are underlined. The shaded part of yellow represents S_TKc domains typical of serine- and threonine-specific kinase families. The green shaded part represents S_TK_X domains typical of serine- and threonine-specific kinase families

site of moderate polymorphism, and the other four sites were low polymorphism locus.

### Association analysis between the SNP loci of the *HcPKC* gene and inner shell color traits

The genotypes of the SNPs found on the *HcPKC* gene were correlated with the inner shell color traits (*L*, *a*, *b*, and *dE*) of 140 mussels (Table 2). The results showed that among the five SNPs in the *HcPKC* gene, there was no significant difference between the genotypes of the three loci A + 87 T, G + 145 T, and A + 328G, and the parameters of the four inner shell color traits. The genotypes of the G + 217 T locus had significant differences in *b* and *dE* parameters ($P < 0.05$) and the genotypes of the A + 332G loci had significant differences in *L*, *b* and *a*, *dE* parameters ($P < 0.05$).

### Linkage disequilibrium and haplotype analysis of the SNP loci in the *HcPKC* gene

Linkage disequilibrium analysis was performed on the five SNP loci (Table 3), and it was found that there was a strong linkage disequilibrium between all the

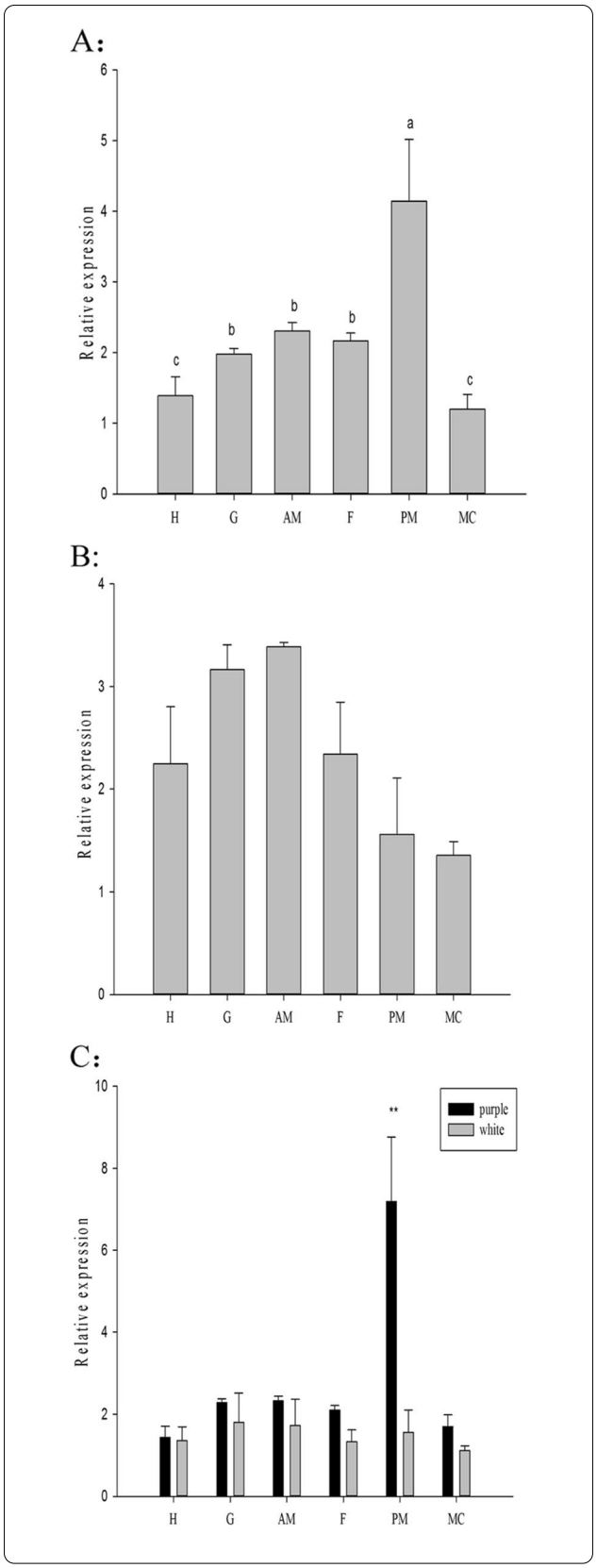


**Fig. 2** Relative expression level of HcPKC. The relative expression level of PKC in various tissues of purple (**A**) and white (**B**) mussels. Comparison of PKC expression in white and purple mussels (**C**). H: hepatopancreas, G: gill, AM: adductor muscle, F: foot. PM: marginal membrane, MC: central membrane. Data from the qPCR experiments are expressed as the means ± SD ($n = 6$). Bars with different letters indicate significant differences ($p < 0.05$). ** represents a highly significant difference at $P < 0.01$

loci ($D' > 0.75$, $r^2 > 0.33$). After haplotype construction (Table 4), it was found that T1 appeared more frequently in the white population than in the purple cultivar.

## Discussion

In this study, a *HcPKC* gene was fully cloned in *H. cumingii* and investigated for the first time. The tissue quantification results showed that the expression level of *HcPKC* in the marginal membrane of purple mussels was significantly higher than that of other tissues ($p < 0.05$). Relevant studies have shown that the outer fold of the mantle is directly involved in the formation of shell nacre [21, 22]. Protein kinase C not only plays a role in the process of melanin synthesis, but also plays a role in other physiological activities, such as nerve and immunity [23], the specific location of *HcPKC* expression in the mantle tissue was determined by in situ hybridization, and a positive hybridization signal mainly appeared in the mantle. The results from the dorsal membrane epithelial cells at the outer fold suggest that *HcPKC* may be involved in the formation of the nacre in *H. cumingii* [24]. Further comparative analysis found a higher expression of *HcPKC* in the tissues of purple mussels than in those of white mussels, and there was a very significant difference in the marginal membrane ($p < 0.01$). Luo et al. [25] found similar results in the phenotypic difference of the *HcCUBDC* gene in white and purple *H. cumingii*, this indicates that the *HcPKC* gene may have a positive effect on the formation of purple nacre.

Studies have shown that the color of shells is heritable [26], and the inner shell color is a breeding target that can improve breeding efficiency [27]. The addition of small pieces of mantle with different inner shell colors will have a significant impact on the color of the pearls produced [28, 29]. Compared with traditional breeding methods, molecular marker-assisted breeding as an emerging breeding method can greatly improve breeding efficiency [30] and has been studied in a variety of aquatic animals [31–33]. In this experiment, primers were designed using the known full-length cDNA sequence of *PKC* in the *H. cumingii*. After primer amplification and sequencing, five SNP sites were found in the exons of the *HcPKC* gene, which was significantly higher than the 1SNP/1000 bp in the previous study [34]. This indicates that there are

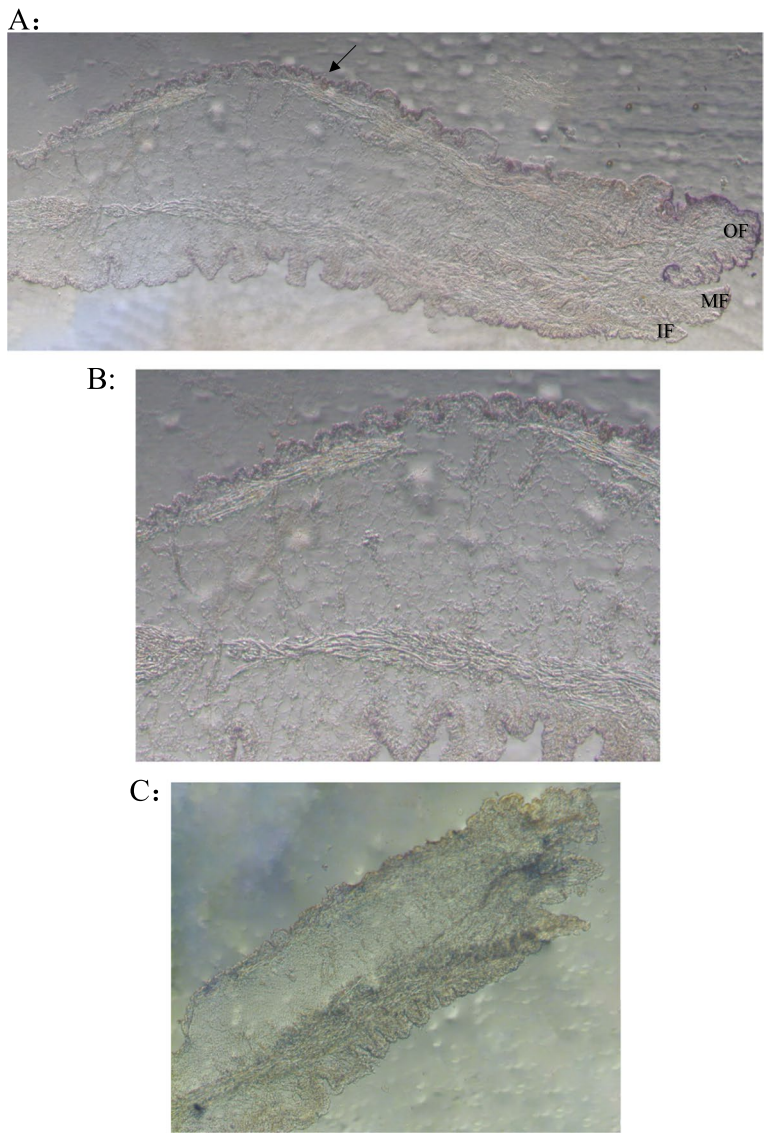


**Fig. 3** In situ hybridization analysis of HcPKC in the mantle (**A**), the arrow indicates the position of the hybridization signal. **B** is a higher magnification of **A**, **C** is background. IF, inner fold; MF, middle fold; OF, outer fold

**Table 1** The polymorphic parameters of five SNP sites in the *HcPKC* gene

| | **Observed heterozygosity** | **Expected heterozygosity** | **Polymorphic information content** | **Effective number of alleles** |
|---|---|---|---|---|
| Suit | $H_0$ | $H_e$ | PIC | $N_e$ |
| A + 87 T | 0.0214 | 0.0624 | 0.060 | 1.0663 |
| G + 145 T | 0.0500 | 0.1513 | 0.139 | 1.1776 |
| G + 217 T | 0.0143 | 0.0953 | 0.090 | 1.1050 |
| A + 328G | 0.0786 | 0.1432 | 0.149 | 1.1942 |
| A + 332G | 0.0929 | 0.3254 | 0.272 | 1.4799 |

**Table 2** Association of the five SNP sites of *HcPKC* polymorphisms with nacre color

| Locus | Genotype | No | M*L* | M*a* | M*b* | M*dE* |
|---|---|---|---|---|---|---|
| A + 87 T | AA | 134 | 54.17 ± 0.70$^{A}$ | 3.38 ± 0.22$^{A}$ | 0.36 ± 0.56$^{A}$ | 47.51 ± 0.72$^{A}$ |
| | AT | 3 | 54.92 ± 5.70$^{A}$ | 3.81 ± 1.64$^{A}$ | -1.73 ± 3.62$^{A}$ | 46.16 ± 6.19$^{A}$ |
| | TT | 3 | 59.14 ± 4.36$^{A}$ | 2.62 ± 1.49$^{A}$ | -4.96 ± 2.19$^{A}$ | 41.73 ± 4.42$^{A}$ |
| G + 145 T | GG | 125 | 54.44 ± 0.74$^{A}$ | 3.36 ± 0.23$^{A}$ | 0.39 ± 0.58$^{A}$ | 47.20 ± 0.76$^{A}$ |
| | GT | 7 | 50.92 ± 2.71$^{A}$ | 4.52 ± 0.85$^{A}$ | -0.25 ± 2.95$^{A}$ | 50.97 ± 2.90$^{A}$ |
| | TT | 8 | 54.98 ± 2.44$^{A}$ | 2.73 ± 0.89$^{A}$ | -2.24 ± 2.16$^{A}$ | 46.69 ± 2.47$^{A}$ |
| G + 217 T | GG | 132 | 54.21 ± 0.70$^{A}$ | 3.42 ± 0.23$^{A}$ | 0.39 ± 0.57$^{AB}$ | 47.46 ± 0.73$^{A}$ |
| | GT | 2 | 41.21 ± 6.74$^{A}$ | 3.13 ± 2.35$^{A}$ | 6.90 ± 3.42$^{B}$ | 60.89 ± 6.28$^{B}$ |
| | TT | 6 | 60.52 ± 3.03$^{A}$ | 2.49 ± 0.90$^{A}$ | -6.20 ± 2.22$^{A}$ | 40.60 ± 3.25$^{A}$ |
| A + 328G | AA | 122 | 54.44 ± 0.74$^{A}$ | 3.31 ± 0.23$^{A}$ | 0.30 ± 0.58$^{A}$ | 47.19 ± 0.76$^{A}$ |
| | AG | 11 | 52.57 ± 2.57$^{A}$ | 4.55 ± 0.76$^{A}$ | 0.72 ± 2.07$^{A}$ | 49.30 ± 2.64$^{A}$ |
| | GG | 7 | 54.44 ± 2.70$^{A}$ | 2.61 ± 1.03$^{A}$ | -2.71 ± 2.38$^{A}$ | 47.17 ± 2.73$^{A}$ |
| A + 332G | AA | 105 | 55.14 ± 0.78$^{B}$ | 3.07 ± 0.24$^{B}$ | 0.10 ± 0.63$^{AB}$ | 46.44 ± 0.81$^{A}$ |
| | AG | 13 | 56.27 ± 2.34$^{B}$ | 3.57 ± 0.71$^{AB}$ | -2.79 ± 1.68$^{A}$ | 45.19 ± 2.42$^{A}$ |
| | GG | 22 | 49.07 ± 1.71$^{A}$ | 4.72 ± 0.65$^{A}$ | 2.47 ± 1.30$^{B}$ | 53.04 ± 1.77$^{B}$ |

*Notes*: Different superscript letters in a column of the same two loci indicate significant difference at $P < 0.05$

**Table 3** Linkage disequilibrium analysis of the five SNP sites of the *HcPKC* gene

| | A + 87 T | G + 145 T | G + 217 T | A + 328G | A + 332G |
|---|---|---|---|---|---|
| A + 87 T | - | 1.000 | 1.000 | 1.000 | 1.000 |
| G + 145 T | 0.371 | - | 1.000 | 0.952 | 1.000 |
| G + 217 T | 0.631 | 0.588 | - | 1.000 | 1.000 |
| A + 328G | 0.339 | 0.827 | 0.537 | - | 1.000 |
| A + 332G | 0.130 | 0.350 | 0.206 | 0.384 | - |

*Notes*: The figure above the diagonal represents D′, the figure below the diagonal represent $r^2$

spinnaker mussels and the *HcPKC* gene showed that the genotypes of the G + 217 T locus had significant differences in *b* and *dE* parameters ($P < 0.05$), A + 332G. The genotypes of the loci were significantly different in *L*, *b* and *a*, *dE* parameters ($P < 0.05$). It is speculated that this gene may play a certain role in the formation of nacre color in the *H. cumingii* [36, 37]. Due to the limitation of the number of samples, this experiment can explain the problem to a certain extent, and subsequent experiments need to further expand the sample size to verify the results of this study.

To further investigate whether the polymorphism of the *HcPKC* gene is associated with nacre color traits, we analyzed linkage disequilibrium [38] and haplotype analysis [39]. The results showed that among the haplotypes constructed by the *HcPKC* gene, therefore, the dominant type can be selected according to demand to speed up breeding efficiency and provide a reference for the rapid selection of the target shell color [40].

**Table 4** Haplotype analysis of the five SNP sites of the *HcPKC* gene

| Haplotype | Sequence | Purple strain(frequency) | White strain(frequency) | χ2(*P* value) |
|---|---|---|---|---|
| T1 | A G G A A | 99.00(0.707) | 113.00(0.807) | 5.379(0.020) |
| T2 | A G G A G | 22.00(0.157) | 13.00(0.093) | 2.681(0.101) |
| T3 | A T G G G | 7.00(0.050) | 2.00(0.014) | 2.879(0.089) |

relatively abundant single nucleotide polymorphisms in the *HcPKC* gene. According to the polymorphism analysis, it was found that in the *HcPKC* gene, the A + 332G site is a moderate polymorphism site, and the other four sites are low polymorphism sites, but no high polymorphism was found in this gene. This is because SNP markers are DNA sequence polymorphisms caused by single nucleotide variation, and it is difficult to show higher polymorphisms such as in Simple Sequence Repeat (SSR) markers [35]. Preliminary analysis of the SNP correlation between the purple and white inner shell color of the

**Fig. 4** Purple (left) and white (right) H.cumingii mussels used in the experiment

**Table 5** Primers used in the study

| primers | (5′-3′)sequence of primers | purpose |
|---|---|---|
| *HcPKC*-F | GCTTGTTTCCAGACTGACGA | Partial fragment amplification of *HcPKC* |
| *HcPKC*–R | CGTCAGTCTGGAAACAAGCAA | Partial fragment amplification of *HcPKC* |
| *HcPKC*-3′ | CCATCCATTCCTTGTAAACCTG | 3′RACE |
| *HcPKC*-5′ | GTCAGTCTGGAAACAAGCAAAC | 5′RACE |
| HcPKC-RT-F | AGTGAACGTGATGCAGAGGA | qPCR |
| HcPKC-RT-R | GGTGTCAACACTGGCTTCTC | qPCR |
| HcPKC-Y-F | AGTGAACGTGATGCAGAGGA | In situ hybridization |
| HcPKC-Y-R | TAATACGACTCACTATAGGGGGTGTCAACACTGGCTTCTC | In situ hybridization |
| EF1α-F | GGAACTTCCCAGGCAGACTGTGC | qPCR internal control |
| EF1α-R | TCAAAACGGGCCGCAGAGAAT | qPCR internal control |

## Conclusions

In this study, a *HcPKC* gene was fully cloned in *H. cumingii* and investigated for the first time. Validation of the effect of *HcPKC* gene on shell nacre by fluorescence quantification, in situ hybridization experiments, and discovery of single-nucleotide polymorphisms (SNPs) associated with inner shell color-related traits that *HcPKC* of *H. cumingii* can be used as a candidate gene related to inner shell color, and some of the SNP sites can be used for molecular-assisted breeding in the spinnaker mussel, providing a reference for cultivating high-quality freshwater pearls.

## Methods

### Ethical approval statement

*H. cumingii* were treated according to animal care and use guidelines for scientific purposes established by the Institutional Animal Care and Use Committee of Shanghai Ocean University, Shanghai, China.

### Experimental materials

Two-year-old healthy *H. cumingii* mussels (average shell length of 10 cm) with purple and white inner-shell colors were obtained from Weimin Aquaculture Base, Jinhua City, Zhejiang Province, China (Fig. 4). Before the experiment, the mussels were placed in a laboratory water tank for oxygenation for about a week, and then fresh mantle samples were stored at − 80 °C for later use.

### Experimental method

#### *Total RNA extraction and cloning of the full-length HcPKC*

The TRIzol method was used to extract total RNA from healthy mantle tissue samples. The SMARTer RACE 5′/3′ kit was used to synthesize RACE-Ready cDNA as a gene cloning template. Specific primers (Table 5) were designed based on the *HcPKC* (*HcPKC*-F and *HcPKC*-R) expressed sequence tags (ESTs) of *H. cumingii* which were obtained from the *H. cumingii* mantle transcriptome library [41]. The PKC gene fragment was obtained from a mantle transcriptome library of *H. cumingii* (Table 5), and the specific primers were designed by Primer 5.0 to perform PCR amplification and verify the sequence. According to the SMARTer RACE 5′/3′ kit instructions, 5′-RACE and 3′-RACE specific primers were designed, RACE cloning was performed, and the DNA was sequenced by Sangon (Shanghai, China) to obtain the full-length PKC gene.

#### *Gene sequence analysis*

ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict the ORF (open reading frame) of the *HcPKC* gene sequence and the encoded amino acid sequence [42]. Smart Blast was used to predict amino acid sequence homology analysis [43]. The amino acid inclusion domains were analyzed by Simple Modular Architecture Research Tool SMART (http://smart.embl-heidelberg.de/). The Protparam online tool (https://www.expasy.org/) was used to obtain information on physical parameters such as amino acid sequence composition, molecular weight, isoelectric point, etc. [44]. ClustalX software was used for multiple sequence alignment analysis [45] and MEGA 5.2 (Arizona State University, USA) was used to construct a phylogenetic tree [46].

#### *Tissue-specific expression analysis of the HcPKC gene*

Hepatopancreas, gill, adductor muscle, foot, marginal membrane, central membrane samples were taken from six healthy *H. cumingii* individuals and were used for RNA extraction. The RNA was then reverse-transcribed to cDNA by using SYBR®Premix Ex Taq II (TliRNaseH Plus, TaKaRa). Bio-Rad-CFX-96 (Bio-Rad, USA) was used for fluorescence quantitative PCR. The PCR reaction mixture was as follows: SYBR®Premix Ex Taq II (TliRNaseH Plus), 10 μL; upstream and downstream primers, 0.8 μL; $ddH_2O$, 6.8 μL and cDNA template 1.6 μL. Each reaction was performed in three replicates. The reaction parameters were: pre-denaturation at 95 °C for 30 s; followed by 40 cycles of 95 °C for 5 s; 56 °C for 35 s; and 72 °C for 30 s. Referring to the previous research results of our laboratory, EF1α was used as an internal reference gene [47] (Table 5).

#### *In situ hybridization*

Specific primers were designed and the T7 promoter sequence TAATACGACTCACTATAGGG (Table 5) was added at the 5′ end. The target fragment was obtained after PCR amplification and product purification, and in vitro transcription was performed using a Complete Gold in vitro transcription kit. The fresh mantle tissue of the mussel was placed in 4% paraformaldehyde to fix and dehydrate for 4 h (in a 4 °C refrigerator), then placed in 25% sucrose solution at 4 °C overnight. The tissue was cut into ~ 10 μm sections. They were marked and stored on glass slides at − 80 °C for later use. Follow-up in situ hybridization experiments were performed later.

#### *Extraction of genomic DNA*

For SNP experiments, 70 white mussels and 70 purple mussels were selected randomly. The genomic DNA of the experimental samples was extracted using a TIANamp Marine Animals DNA Kit and coagulated with 1% agarose. The quality of DNA was detected by gel electrophoresis and a NanoDrop 2000C spectrophotometer, and the samples were placed in a − 20 °C refrigerator for later use.

**Table 6** The primers of SNP in the *HcPKC* gene of *H. cumingii*

| primers | (5′-3′)sequence of primers |
|---|---|
| F1 | CTTTATTGACAATGGCAGAGCA |
| R1 | AGTTCTGCTAAACCCCTCCAT |
| F2 | TAACCATGATGATTTGTCTTCCTCT |
| R2 | TTCCAGCAAACAGGACTGATTAT |

#### *Data measurement*

Using a Lovibond-RT200 surface colorimeter to measure the inner shell color of purple and white experimental mussels, and according to the uniform color space determined by the International Commission on Illumination (CIE), $L^*$ represents the brightness. $L^*>0$ indicated that the color was bright, $L^*<0$, darker color; $a^*>0$, redder color, $a^*<0$, greener color; $b^*>0$, yellowish color, and $b^*<0$, bluer color [48]. The anterior, middle, and posterior margins of the right shell of 140 mussels were measured, and the difference in the color parameter was calculated as follows: $dE=(L^2+a^2+b^2)^{½}$, $L=Lx\text{-}L0$, $a=ax\text{-}a0$, $b=bx\text{-}b0$. *L*x, *a*x, and *b*x are the color parameter values of different shells. *L*0, *a*0, and *b*0 are the color parameters of standard white inner shell mussels and M*L*, M*a*, M*b*, and M*dE* represent the average value of *L*, *a*, *b*, and *dE*.

#### *Screening of SNP loci in the HcPKC gene of H. cumingii*

The *HcPKC* gene was compared with the *PKC* gene in the genome of the *H. cumingii* to determine the exon and intron regions. Primers specific to exonic regions were designed (Table 6). The DNA samples of 10 white mussels and 10 purple mussels were selected randomly for sequence amplification, and the amplified products were sent to MAP BIOTECH (Shanghai) for sequencing. Sequence 5.4.6 was used to obtain the SNP site from the compared sequencing results.

#### *Data analysis*

Genetic parameters such as observed heterozygosity, expected heterozygosity, and polymorphism content were analyzed using Popgene software [49]. The chi-square test was performed using SPSS software to analyze the correlation between the genotypes of different SNPs in the *HcPKC* gene fragment and the inner shell color of the mussels [50]. Analysis of linkage disequilibrium

and haplotype construction with SHEsis online software (http://analysis.bio-x.cn/) [51, 52].

### *Statistical analysis*

Data are shown as the mean ± SD and was analysed using SPSS 17.0 software. Differences were recognized as significant when $p < 0.05$ and highly significant when $p < 0.01$.

#### Abbreviations

PKC: Protein kinase C; SNP: Single-nucleotide polymorphisms; H. cumingii: Hyriopsis cumingii; HcPKC: A PKC gene in Hyriopsis cumingii.

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#### Authors' contributions

MYZ, ZYB designed the experiments. MYZ, JPZ, XJC and BYG carried out the experiments. XJC, JLL and ZYB conducted the statistical analysis and discussion. MYZ and ZYB organized and wrote the manuscript. All authors read and approved the final manuscript.

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#### Availability of data and materials

All data generated during this study are included in this published article.

## Declarations

#### Ethics approval and consent to participate

*H. cumingii* were treated according to animal care and use guidelines for scientific purposes established by the Institutional Animal Care and Use Committee of Shanghai Ocean University, Shanghai, China.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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#### References

1. Li J, Li Y: Aquaculture in China--freshwater pearl culture. World Aquaculture 2009, 41(60).
2. Bai Z, Luo M, Zhu W, Lin J, Wang G, Li J. Multiple paternity in the freshwater pearl mussel Hyriopsis cumingii (Lea, 1852). J Molluscan Stud. 2011;78(1):142–6.
3. Hu H, Sun C, Bai Z, Li J. Genotype by environment interactions for inner shell color and growth traits in the purple freshwater pearl mussel, Hyriopsis cumingii, reared with different water depths and mud substrates. Aquaculture. 2021;531: 735942.
4. Jiale L, Yue L. The main influencing factors on the quality of cultured pearls. J Fish China. 2011;35(11):1753–60.
5. Xueying L, Haizeng W, Shengli S, Jibiao Z. Analysis on Fourier Transform Infrared and Graphite Furnace Atomic Absorption Spectrometry of Pearls with Different Colours. J Gem Gemmol. 2007;01:15–8.
6. Genfang Z, Ronghui Y, Aiping F. Research Progress about the Color of Pearl and Shell Nacre. Chin J Zool. 2014;49(01):137–44.
7. Yuntao Z: The contribution of porphyrin and metalloporphyrin to the color of Pearl and its mechanism. China University of Geosciences 2006.
8. Jinpan Z, Zhiyi B, Mengying Z, Ling Y, Fenghui L, He W. Functional analysis and SNP screening of lysophosphaticly lcholine acyltransferase 1 HcLPCAT1 gene and its association analysis with shell color traits in Hyriopsis cumingii. J Fish Sci China. 2021;28(11):1373–84.
9. Zhang M, Chen X, Zhang J, Li J, Bai Z. Cloning of a HcCreb gene and analysis of its effects on nacre color and melanin synthesis in Hyriopsis cumingii. PLoS ONE. 2021;16(5): e0251452.
10. Chen X, Liu X, Bai Z, Zhao L, Li J. HcTyr and HcTyp-1 of Hyriopsis cumingii, novel tyrosinase and tyrosinase-related protein genes involved in nacre color formation. Comp Biochem Physiol B: Biochem Mol Biol. 2017;204:1–8.
11. Shen J, Huang D, Sun C, Li J, Bai Z. Cloning of a microphthalmia-associated transcription factor gene and its functional analysis in nacre formation and melanin synthesis in Hyriopsis cumingii. Aquaculture and Fisheries. 2018;3(6):217–24.
12. Azzi A, Boscoboinik D, Hensey C. The protein kinase C family. Eur J Biochem. 1992;208(3):547–57.
13. Olive MF, Messing RO. Protein kinase C isozymes and addiction. Mol Neurobiol. 2004;29(2):139–53.
14. Qiang D, Dingxin L: Research progress of protein kinase C. 1013488/jsmhx20180314 2018, 38(03).
15. Lee DW, Kim HJ, Choi CH, Shin JH, Kim EK. Development of a Protein Chip to Measure PKC beta Activity. Applied Biochemistry And Biotechnology. 2011;163(6):803–12.
16. Zhang J. Advances in the research of melanogenesis-related proteins. Henan Medical Research. 2009;18(3):257–61.
17. Allan AE, Archambault M, Messana E, Gilchrest BA. Topically Applied Diacylglycerols Increase Pigmentation in Guinea Pig Skin. J Investig Dermatol. 1995;105(5):687–92.
18. Ando H, Oka M, Ichihashi M, Mishima Y. Protein kinase C activators inhibit melanogenesis in B16 melanoma cells. J Dermatol Sci. 1990;1(3):228.
19. Park H-Y, Lee J, Kapasi S, Peterson S, Gilchrest BA, González S, Middelkamp-Hup MA. Topical Application of a Protein Kinase C Inhibitor Reduces Skin and Hair Pigmentation. J Investig Dermatol. 2004;122(1):159–66.
20. Jung H, Chung H, Chang SE, Choi S, Oh ES: Syndecan-2 regulates melanin synthesis via protein kinase C βII-mediated tyrosinase activation. Pigment Cell Melanoma Res 2014, 27(3).
21. Liu X, Dong S, Jin C, Bai Z, Wang G: Silkmapin of Hyriopsis cumingii, a novel silk-like shell matrix protein involved in nacre formation. Gene 2014, 555.
22. Zhang C, Xie L, Huang J, Chen L, Zhang R. A novel putative tyrosinase involved in periostracum formation from the pearl oyster (Pinctada fucata). Biochem Biophys Res Commun. 2006;342(2):632–9.
23. Oka M, Kikkawa U. Protein kinase C in melanoma. Cancer And Metastasis Reviews. 2005;24(2):287–300.
24. Chen X, Liu X, Bai Z, Zhao L, Li J: HcTyr and HcTyp-1 of Hyriopsis cumingii , novel tyrosinase and tyrosinase-related protein genes involved in nacre color formation. Comparative Biochemistry and Physiology, Part B 2017, 204.
25. Hongrui L, Zhiyi B, Xiaojun L, Qingqing L, Shaojian D, Shimei Z, Jiale L. Full-length c DNA cloning and expression analysis of Hc CUBDC gene from Hyriopsis cumingii. J Fish China. 2015;39(09):1313–23.
26. Jerry D, Kvingedal R, Lind C, Evans B, Taylor J, Safari A. Donor-oyster derived heritability estimates and the effect of genotype × environment interaction on the production of pearl quality traits in the silver-lip pearl oyster. Pinctada maxima Aquaculture. 2012;338–341:66–71.
27. Zhaoqi W, Xuekai H, Zhiyi B, Jiale L. Estimates of genetic parameters for inner shell color and growth straits during one year old stage in the

purple strain of Hyriopsis cumingii using microsatellite based parentage assignment. J Fish China. 2014;38(05):644–50.
28. Jebelli A, Khalaj-Kondori M, Bonyadi M, Feizi MAH, Rahmati-Yamchi M. Beta-Boswellic Acid and Ethanolic Extract of Olibanum Regulating the Expression Levels of CREB-1 and CREB-2 Genes. Iran J Pharm Res. 2019;18(2):877–86.
29. Qingqing L, Zhiyi B, Xiaojun L, Xuekai H, Hongrui L, Shaojian D, Jiale L. Correlation analysis of non-nucleated pearl quality parameters with growth traits and inner shell color of Hyriopsis cumingii. J Fish China. 2015;39(11):1631–9.
30. Cuiyun L, Youyi K, Xianhu Z, Chao L, Xiaowen S. Advances of molecular marker-assisted breeding for aquatic species. J Fish China. 2019;43(01):36–53.
31. Xuekai H, Xiajun C, Zhiyi B, Xiaojun L, Jiale L. Detection of shell nacre colour-related SNP and gene mapping of HcTyr gene in Hyriopsis cumingii. J Fish China. 2017;41(07):1044–53.
32. Xiajun C, Xuekai H, Zhiyi B, Jiale L. Detection of nacre colour-related SNPs and genetic mapping of HcTyp-1 gene in Hyriopsis cumingii. J Fish China. 2019;43(02):467–73.
33. Chao L: Screening and SNP analysis on carotenoid metabolism related genes in Pinctada fucata martensii. Guangdong Ocean University; 2018.
34. Vignal A, Milan D, SanCristobal M, Eggen A. A review on SNP and other types of molecular markers and their use in animal genetics. Genet Sel Evol. 2002;34(3):275–305.
35. Hubert S, Bussey JT, Higgins B, Curtis BA, Bowman S. Development of single nucleotide polymorphism markers for Atlantic cod (Gadus morhua) using expressed sequences. Aquaculture. 2009;296(1–2):7–14.
36. Mengying Z, Xiajun C, Jinpan Z, Jiale L, Zhiyi B. Cloning of a HcCreb gene and analysis of its effects on nacre color and melanin synthesis in Hyriopsis cumingii. PloS One. 2021;16(5):e0251452.
37. Li X, Bai Z, Luo H, Liu Y, Wang G, Li J. Cloning, differential tissue expression of a novel hcApo gene, and its correlation with total carotenoid content in purple and white inner-shell color pearl mussel Hyriopsis cumingii. Gene. 2014;538(2):258–65.
38. Daly MJ, Rioux JD, Schaffner SF, Hudson TJ, Lander ES. High-resolution haplotype structure in the human genome. Nat Genet. 2001;29(2):229–32.
39. Clark AG. The role of haplotypes in candidate gene studies. Genet Epidemiol. 2004;27(4):321–33.
40. Fuping L, Junjie B. Single nucleotide polymorphisms and its application in genetic breeding of aquatic animals. J Fish Sci China. 2008;04:704–12.
41. Bai ZY, Zheng HF, Lin JY, Wang GL, Li J. Comparative Analysis of the Transcriptome in Tissues Secreting Purple and White Nacre in the Pearl Mussel Hyriopsis cumingii. PLOS ONE. 2013;8(1):e53617.
42. Rong OU: A New PubMed Search Tool——NCBI Search Toolbar and Its Applications. Researches in Medical Education 2006.
43. Ivica L, Tobias D, Peer B. SMART: recent updates, new developments and status in 2015. Nuclc Acids Research. 2015;D1:257–60.
44. Gasteiger E, Hoogland C, Gattiker A, Duvaud SE, Wilkins MR, Appel RD, Bairoch A: Protein Identification and Analysis Tools on the ExPASy Server. 1999.
45. J D T: The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic acids research 1997, 24(25).
46. Kumar S, Nei M, Dudley J, Tamura K. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform. 2008;9(4):299–306.
47. Zhiyi, Bai, Jingyun, Lin, Keyi, Ma, Guiling, Wang, Donghong, Niu: Identification of housekeeping genes suitable for gene expression analysis in the pearl mussel, Hyriopsis cumingii, during biomineralization. Molecular Genetics & Genomics 2014.
48. Hunt R: The Specification of Colour Appearance. II. Effects of Changes in Viewing Conditions. Color Research & Application 2007, 2:109–120.
49. Francis CY, Rong CY, Boyle T: POPGENE, Microsoft Window-based freeware for population genetic analysis. University of Alberta 1999:1–31.
50. Shanzhao Y: SPSS statistical software application basis: SPSS statistical software application basis; 2001.
51. YonYon Sh, n H: SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Research 2006.
52. Li ZQ, Zhang Z, He ZD, Tang W, Li T, Zeng Z, He L, Shi YY: A partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers: update of the SHEsis (http://analysis.bio-x.cn). Cell Research 2009, 19(4):519–523.

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