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Characterization and Function Analysis of β, β-carotene-9′, 10′-oxygenase 2 (*BCDO2*) Gene in Carotenoid Metabolism of the Red Shell Hard Clam (*Meretrix meretrix*)

Lulu Fu[1,2], *Heming Shi*[2], *Wenfang Dai*[2,3], *Hanhan Yao*[2]*, *Yongbo Bao*[2], *Zhihua Lin*[2,3] *and Yinghui Dong*[2]*

[1] College of Fisheries, Henan Normal University, Xinxiang, China, [2] Zhejiang Key Laboratory of Aquatic Germplasm Resources, College of Biological & Environmental Sciences, Zhejiang Wanli University, Ningbo, China, [3] Ninghai Institute of Mariculture Breeding and Seed Industry, Zhejiang Wanli University, Ningbo, China

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***Correspondence:**
Hanhan Yao
yaohanhan1020@126.com
Yinghui Dong
dongyinghui118@126.com

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The relationship between carotenoid and shellfish shell color has gained increasing attention. β, β-carotene-9′,10′-oxygenase 2 (BCDO2) is a key enzyme in animal carotenoid metabolism, and its accumulation affects the change in body color, as demonstrated in mammals, birds, and fish. However, it is unclear whether BCDO2 is involved in the formation of the red shell color of clam. To explore the molecular structure and biological function of *BCDO2* gene in the process of carotenoids accumulation, in this study, the *BCDO2* from hard clam *Meretrix meretrix* (designated as *Mm-BCDO2*) was cloned and characterized, and the single-nucleotide polymorphisms (SNPs) associated with shell color were detected. The results of qRT-PCR indicated that *Mm-BCDO2* gene was expressed in all six tested tissues, and the expression of mantle was significantly higher than other tissues ($P < 0.05$). The association analysis identified 20 SNPs in the exons of *Mm-BCDO2*, among which three loci (i.e., c.984A > C, c.1148C > T, and c.1187A > T) were remarkably related ($P < 0.05$) to the shell color of clam. The western blot analysis revealed that the expression level of Mm-BCDO2 in the mantle of red shell clams was stronger than that of white shell clams ($P < 0.05$). Further, the immunofluorescence analysis indicated that the single-layer columnar cells at the edge of the mantle were the major sites for the Mm-BCDO2 secretion. This study explored the potential impacts of *BCDO2* gene on the shell color of *M. meretrix*, which provided a theoretical basis for a better understanding of the important role of *BCDO2* in carotenoid metabolism.

Keywords: *Meretrix meretrix*, *BCDO2*, carotenoids, shell color, SNP

INTRODUCTION

Carotenoids are a class of fat-soluble pigments, which can be classified into two groups: the xanthophylls (oxygenated-contained group) and the carotenes (hydrocarbons without oxygenated group) (Wu et al., 2016). It has been reported that carotenoids play an important role in various aspects of animal life. For example, it acts as precursors of vitamin A and antioxidants to involve

in host physiological response (Svensson and Wong, 2011; Milani et al., 2017). The zeaxanthin, lutein, and astaxanthin have a positive effect on antioxidant and light filtering by preventing vision loss and eye diseases (Li et al., 2017), hence protecting the eyesight to a certain extent (Felix et al., 2011). Moreover, carotenoids and their derivatives are involved in the coloration of feathers, skin, and shells, which play a signal role in mate selection, social competition, and species recognition (Blount and Mcgraw, 2008).

Notably, animals that are unable to *de novo* synthesize carotenoids can get them from the diet (Zheng et al., 2010). The metabolic process of carotenoids in animals includes a series of metabolic reactions. Enzyme degradation is the key step for the utilization of carotenoids. So far, two types of carotene oxygenase, β-carotene 15, 15′-monooxygenase (BCMO1) and β-carotene 9′, 10′-dioxygenase (BCDO2), have been identified in vertebrates (Wu et al., 2016). BCMO1 is a key enzyme that converts β-carotene into vitamin A. Most β-carotene is symmetrically cleaved to form metabolites under the action of BCMO1 (Georg et al., 2010). BCDO2 displays broad substrate specificity and can cleave β-carotene, generating beta-10′-apocarotenal and beta-ionone; contrarily, beta-10′-apocarotenal can be cleaved by BCMO1 to produce retinaldehyde and downstream derivatives including retinol and retinoic acid (Seña et al., 2016; Jin et al., 2020). In addition, BCMO1 catalyzes the asymmetric cleavage of xanthophylls to generate rosafluene and ionone (Seña et al., 2016). Studies have found that the expression level of *BCDO2* affects the body color of animals (Eriksson et al., 2008). Similarly, researches on the body surface color of wall lizard have showed that the content of lutein and zeatin is remarkably higher in yellow skin lizard compared with that in white skin lizard, which corresponds to the significantly lower expression level of *BCDO2* in yellow skin lizard (Andrade et al., 2019). Genetic studies on the effect of *BCDO2* mutations on "yellow fat" phenotype in cattle, sheep, and chicken have showed that *BCDO2* is implicated in the carotenoid homeostasis of milk color, fat color, and skin color (Eriksson et al., 2008; Berry et al., 2009; Tian et al., 2009; Vge and Boman, 2010). Genetic disruption (low-temperature expression and detergent choice) of *BCDO2* can significantly affect binding and turnover rates of the recombinant enzymes with various xanthophyll substrates and then increase the concentration of dietary pigment in plasma and blood of the mice (Babino et al., 2015).

Currently, researches on the role of carotenoids on the coloring of tissues and shells have been reported in *Meretrix meretrix* (Lin and Dong, 2015; Cui et al., 2018), *Crassostrea gigas* (Ge et al., 2016; Wang et al., 2016, 2018), *Pinctada fucata* (Zhang et al., 2019), *Hyriopsis cumingii* (Li et al., 2014b), *Paphia textile* (Deng et al., 2018), *Chlamys nobilis* (Zheng et al., 2010), and *Exopalaemon carinicauda* (Jin et al., 2020). The hard clam (*M. meretrix*) mainly distributed in the coastal area of Asia is one of the most important commercially cultured bivalves in China (Lin, 2015). Notably, clam shell displays two kinds of typical and differentiated colors, including white and red (Lin, 2015). The Raman spectra of the shell layer of *M. meretrix* showed that the characteristic peaks of the carotenoids of the red shell clams are stronger than those of the white shell clams (Zhan, 2015). And the total carotenoid content in the marginal membrane of the red clam mantle is about 3.4 times that of the white clam, which is a very significant difference (Qi, 2014). Especially, the ability of individual clams to ingest, absorb, and metabolize carotenoids is different, which may be one of the main reasons for the difference in shell color (Meilisza et al., 2017). However, it is unclear whether this difference attributes to the expression of the *BCDO2* gene in *M. meretrix*. To address this gap, the expression level of the *BCDO2* gene was compared between red and white shell color *M. meretrix*. Further, we investigated the distribution and SNPs of the *BCDO2* gene in the two groups. Our findings will provide valuable information for further studies on the mechanism of clam shell color formation.

MATERIALS AND METHODS

Ethics Statement

The adult hard clams (*M. meretrix*) at the age of 2 years were collected from the genetic breeding research center of Zhejiang Wanli University, China. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Wanli University, China.

Sample Collection and Preparation

Six tissues including gill, siphon, digestive gland, adductor muscle, mantle, and foot were dissected, frozen immediately in liquid nitrogen, and then stored at −80°C.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the foot, mantle, digestive gland, siphon, gill, and adductor muscle by using the Trizol method. The first-strand cDNA was synthesized using the prime script RT reagent kit with gDNA Eraser (TAKARA, Japan) according to the instructions of the manufacturer. In brief, the amplification reactions were performed in a 20 μL volume containing 7 μL tissue RNA (1,500 μg/μL), 1 μL gDNA eraser, 2 μL 5× gDNA eraser buffer, 1 μL prime script RT enzyme mix 1, 4 μL 5× prime script buffer 2, 1 μL RT prime mix, and 4 μL RNase free dH_2O. The first-strand cDNA was synthesized by incubating the mixture at 42°C for 2 min, 37°C for 15 min, and finally, heating at 85°C for 5 s.

Cloning of Full-Length cDNA

The expressed sequence tag (EST) homologous to *Mm-BCDO2* was detected through the cDNA library of *M. meretrix* (GenBank accession no. SRX023927). A pair of gene-specific primers, *Mm-BCDO2*-F and *Mm-BCDO2*-R (**Table 1**), was designed on the basis of EST sequence of *Mm-BCDO2* gene. The PCR amplification was synthesized following the instructions of SMARTer^{TM} RACE cDNA amplification kit (Clontech, San Francisco, USA). The RACE PCR was performed in a 25 μL volume, which contained DEPC water, 10× advantage 2 PCR buffer, 10 mM of dNTPs, 10 μM of gene-specific primer, 10× universal primer A mix (UPM), 1 μL of diluted RACE cDNA, and 50× advantage 2 polymerase mix. The amplification was carried out as follows: 5 cycles of 94°C for 30 s, 72°C for 3 min; 5 cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 3 min; and 25 cycles of 94°C for 30 s, 68°C for 30 s, 72°C 3 min. PCR products were examined on 1.0% agarose gels and purified by gel extraction kit (Tiangen,

TABLE 1 | Primers and sequences used in this experiment.

Primer	Primer sequence 5′-3′	Applications
Mm-BCDO2-F	AAGGAATGGGCAGCGGATGACTGTTAC	3′ RACE
Mm-BCDO2-R	GCGTCCACCACCAACAAGAAAGCA	5′ RACE
Mm-BCDO2-F1	GACCTTGCCCACACCAG	cDNA identification
Mm-BCDO2-R1	CATCATTTTGTTCAGCCAGA	cDNA identification
Mm-BCDO2-SNP-F	ACAGAAATGGACCGGGC	SNP detection
Mm-BCDO2-SNP-R	TCCTTCATAGATCTTGCGTCCAC	SNP detection
Mm-BCDO2-qRT-F	CATACGAGGATAACGGTCACG	qRT-PCR
Mm-BCDO2-qRT-R	TTTCCGCTTTGGCTGCTG	qRT-PCR
18s-F	CTTTCAAATGTCTGCCCTATCAACT	qRT-PCR
18s-R	TCCCGTATTGTTATTTTTCGTCACT	qRT-PCR

Beijing, China). The purified PCR products were cloned into pEasy-T1 (Trans, Beijing, China), and T1 vector was transformed into *E. coli* DH5a, and the positive plasmids were selected and sequenced. A pair of gene-specific primers, *Mm-BCDO2*-F1 and *Mm-BCDO2*-R1 (**Table 1**), was designed based on the full-length cDNA sequence and used to confirm the accuracy of cloning and sequencing of *Mm-BCDO2*.

Sequence and Phylogenetic Analysis

The cDNA sequence was assembled using the BLASTX search program of the National Center for Biotechnology Information (NCBI). The open reading frame (ORF) of *Mm-BCDO2* cDNA was identified using the ORF Finder program from NCBI. The theoretical isoelectric point (p*I*) and molecular weight (Mw) of Mm-BCDO2 protein were calculated using Compute p*I*/Mw Tool. Conserved domain of the deduced Mm-BCDO2 was predicted by simple modular architecture research tool (SMART). Tertiary structure of the amino acid sequence of deduced Mm-BCDO2 was analyzed using the SWISSMODEL tool. Multiple alignments of BCDO2 proteins between *M. meretrix* and other species were performed using ClustalW2 program. A phylogenetic tree was constructed by the neighbor-joining (NJ) method with MEGA X software.

Quantitative Expression Analysis

The mRNA expression levels of *Mm-BCDO2* at different adult tissues ($n = 4$, four sets of samples per tissue) were analyzed by real-time quantitative reverse transcription PCR (qRT-PCR). A pair of gene-specific primers, *Mm-BCDO2* qRT-F/R (**Table 1**), was used to quantitatively detect the expression level of *Mm-BCDO2* with 18S rRNA as an internal reference. The qRT-PCR was performed in 12 μL of iTaq universal SYBR green super mix (Bio-Rad, Beijing, China), 7.2 μL deionized water, 0.8 μL of the first-strand cDNA, and 1 μL of each primer. The amplification was carried out at the following condition: incubation of 20 s at 94°C; 40 cycles of 3 s at 94°C, 15 s at 60°C, and 10 s at 72°C; and a final extension of 7 min at 72°C. All amplifications were performed in triplicate as biological replicates, and negative controls were run in the absence of cDNA templates. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of *Mm-BCDO2*. The expression levels of *Mm-BCDO2* among different tissues and between red and white shell clam groups were compared using the one-way ANOVA and Student's *t*-tests, respectively.

SNPs of *Mm-BCDO2* and Their Association With Clam Shell Color

A pair of primers, *Mm-BCDO2*-SNP-F/R (**Table 1**), was designed based on the cDNA sequence of *Mm-BCDO2* gene; 180 adult hard clams at 2 years of age were used for the SNP detection, collected from one population in Ningbo, Zhejiang Province, China. Total RNA was extracted from the mantle of clams, and then, the cDNA was synthetized using the methods described above. SNPs were detected by directly sequencing the PCR products using Sanger technology and the ABI3730 platform (Applied Biosystems, Foster City, CA, USA). The SNP genotypes were analyzed by using the Mutation Surveyor software version 4.0.8. The software PopGen32 was used to identify deviations from Hardy–Weinberg equilibrium and polymorphism information content (*PIC*) of significantly different SNP sites. The associated loci linkage disequilibrium (*LD*) was analyzed by the SHEsis online (http://analysis.bio-x.cn/myAnalysis.php). The difference in the genotype frequency of SNP locus between red and white shell clam populations was examined using the chi-square (χ^2) text. The correlation between the SNPs of *Mm-BCDO2* and clam shell color traits was analyzed by using the SPSS software version 20.0. The one-way ANOVA was adopted to compare the difference among different genotypes.

Immunofluorescence Analysis

The marginal tissues of the mantle and digestive gland were chosen from red and white shell clams, respectively. These samples were fixed with 4% paraformaldehyde, then dehydrated by gradient ethanol and xylene/*n*-butanol transparent, and paraffin-embedded sections (thickness 4 μm) were prepared. After baking, the tissue slices were deparaffinized with xylene, rehydrated with gradient ethanol, washed with PBS, and retrieved with EDTA antigen. Sections were then blocked for 1 h in blocking solution (5% albumin from bovine serum) at room temperature, followed by primary antibody (antibody rabbit anti-Mm-BCDO2, produced privately by HuaBio, Zhejiang, 1:200) incubation (overnight, 4°C). Primary antibody was detected by secondary antibodies Alexa Fluor 488 donkey anti-rabbit immunoglobulin G (Invitrogen, Carlsbad, California, U.S.) and diluted at 1:150. Nuclei were stained with DAPI (Beyotime, Shanghai, China). The cells were observed and photographed under a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan).

Western Blot Detection

The mantle and digestive gland tissues were dissected from red and white shell clams, respectively. The samples were added with

an appropriate amount of RIPA lysate and then homogenized. The suspension was collected to measure protein concentration by BCA method, configured SDS-PAGE gel with appropriate concentration and loaded the same amount. The protein glue with the target protein was transferred to PVDF membrane and then blocked for 1 h in blocking solution (5% skimmed milk powder solution) at room temperature, followed by primary antibody (antibody rabbit anti-Mm-BCDO2, produced privately by HuaBio, Zhejiang, 1:1,000) incubation (overnight, 4°C). Primary antibody was bound by secondary antibodies [anti-rabbit labeled with biotin HRP (1:8,000)] for 1 h. ECL luminescent substrate mixture was dropped on the PVDF membrane. The proteins were observed and photographed under gel imagers (Bio-Rad, Beijing, China).

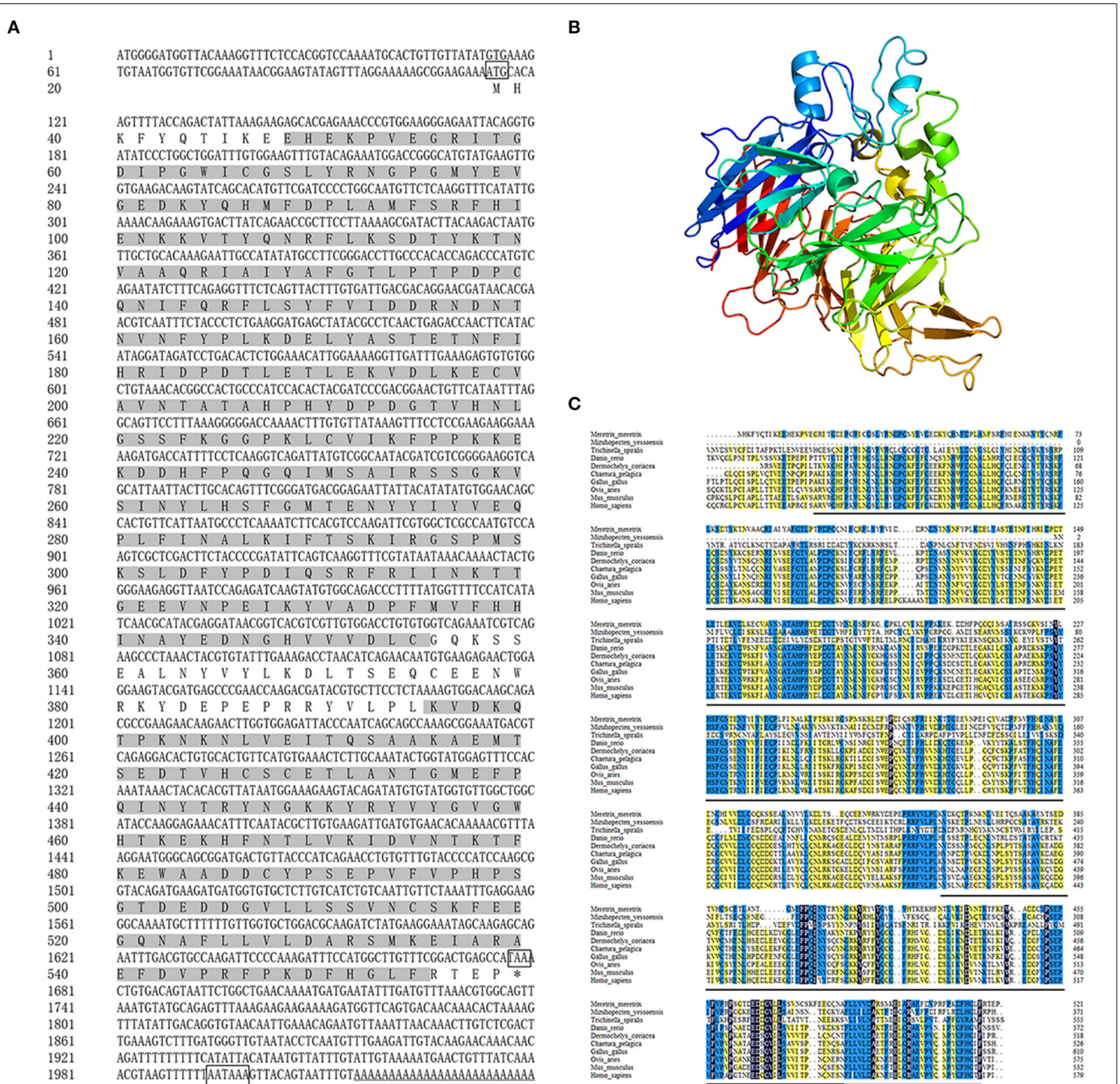

FIGURE 1 | The information of the *Mm-BCDO2* gene. **(A)** The full length of cDNA and deduced amino acid sequence of *Mm-BCDO2*. The frames represented the start codon, the stop codon, and polyadenylation signal. The gray background fill is the functional domain of RPE65. **(B)** The structure of Mm-BCDO2 protein. The arrow structure elements are β-strand, the helical structure elements are α-helix, and the others are random coils and turns. **(C)** Multiple alignments of amino acid sequences of BCDO2 from *M. meretrix* and other model species. Among these, the completely (=100%), strongly (≥75%), and weakly (≥50%) conserved residues are shaded in black, blue, and yellow, respectively. The black line represents the domain RPE65.

RESULTS

Molecular Characterization of *Mm-BCDO2*

The full-length cDNA of *Mm-BCDO2* gene was 2,040 bp, including a 5' untranslated region (UTR) of 113 bp, an open reading frame (ORF) of 1,566 bp that encoded 521 amino acids, and a 3' UTR of 361 bp existing a tail signal (AATAAA) after terminator codon (**Figure 1A**). Based on the amino acid sequence encoded by the *BCDO2* gene, the protein molecular weight was predicted to be 60.06 kDa, corresponding isoelectric point (p*I*) = 5.83, including a RPE65 domain (11–308, 356–517 aa). Mm-BCDO2 was a hydrophilic protein with a large proportion of polar amino acids. Protein structure prediction showed that Mm-BCDO2 protein composed of 9 α helices, 38 β-sheets, and a large number of random coils and had the typical characteristics of RPE65 protein family with a cleft and a right-handed twisted anti-parallel β-barrel (**Figure 1B**). The multiple comparisons between the BCDO2 of mollusks and model animals showed that Mm-BCDO2 had a relatively low similarity with other species (15−44%). Among them, Mm-BCDO2 had the highest homology with *Tauraco erythrolophus* (43.36%), the lowest homology with *Trichinella spiralis* (15.15%), and the homology with shellfish *Mizuhopecten yessoensis* (27.34%) (**Figure 1C**).

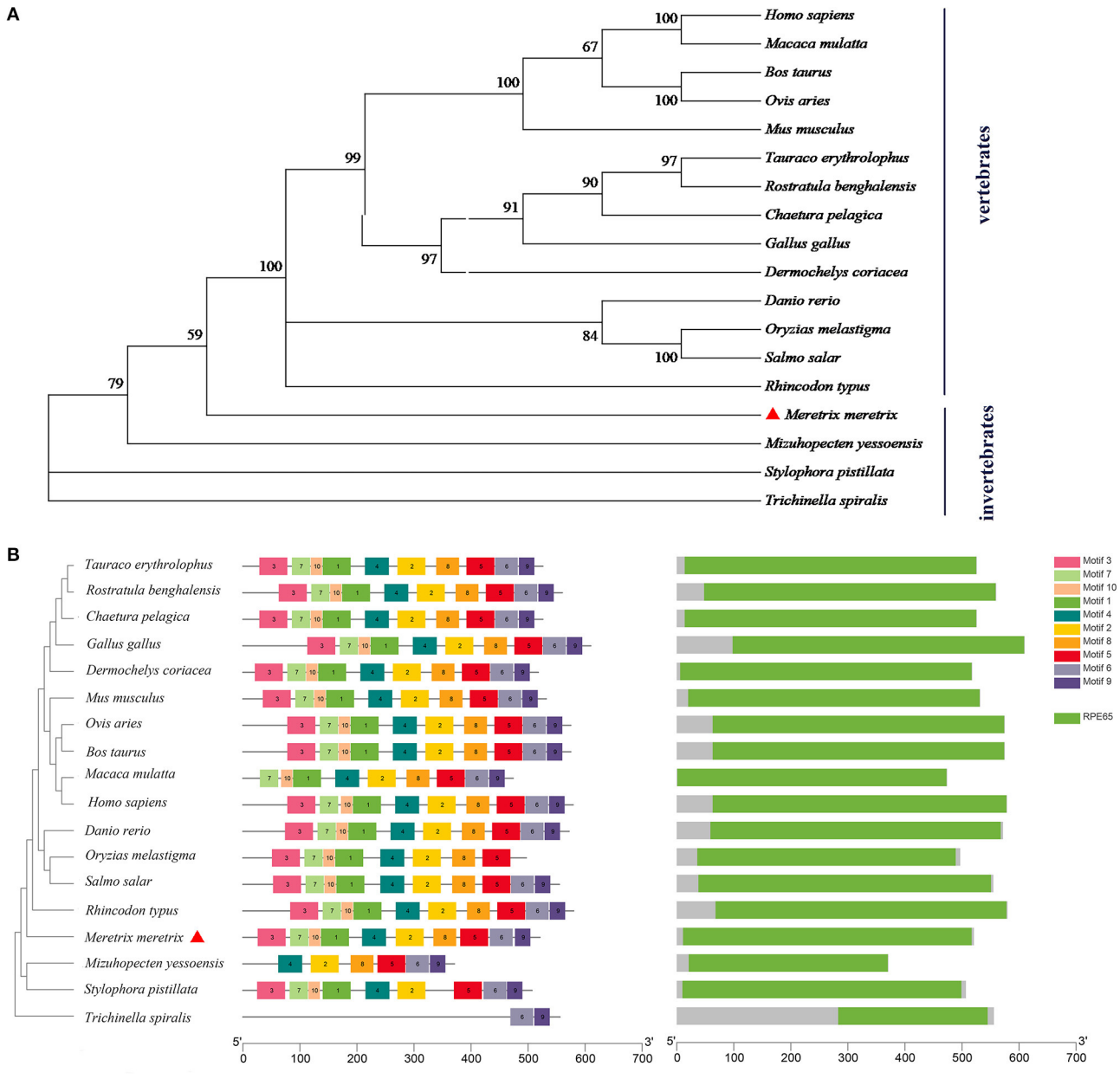

FIGURE 2 | Analysis of evolutionary relationship among species. **(A)** Phylogenetic tree analysis of BCDO2 protein between *M. meretrix* and other species. **(B)** The structure of BCDO2 of *M. meretrix*, other bivalves and vertebrates.

The phylogenetic tree showed that the BCDO2 proteins from vertebrates were clustered together as one branch, while that from *M. meretrix* and other invertebrates were gathered into one branch (**Figure 2A**). Eighteen BCDO2 protein sequences were analyzed and identified 10 conserved motifs, ranging in length from 6 to 50 amino acid residues. Most of the BCDO2 proteins contained motif 1–10 with the exception of *M. yessoensis*, *Stylophora pistillata,* and *T. spiralis*. The domain prediction analysis showed that there was a PRE65 functional domain in all tested vertebrates and invertebrates except *T. spiralis* (**Figure 2B**), indicating that the functions between them might be extremely similar.

High mRNA expression levels of *Mm-BCDO2* were observed in the mantle and foot, followed by the digestive gland, siphon, adductor muscle, and gill (**Figure 3**). Notably, the expression level of *Mm-BCDO2* was significantly higher ($P < 0.05$) in the mantle of red shell clams than that of white shell clams (**Figure 3**).

Associations Between the SNP of *Mm-BCDO2* and Shell Color of Clam

A total of 20 polymorphic loci were screened in the coding sequence of *Mm-BCDO2* (**Supplementary Table 1**), of which three SNPs (c.984A > C, c.1148C > T, and c.1187A > T) were significantly associated with clam shell color formation (**Table 2**). The c.984A>C and c.1187A>T sites were non-synonymous mutations, which led to the mutation of amino acids from Lys to Gln and from Lys to Asn, respectively. The c.1148C>T site was a synonymous mutation. Statistical analysis of the mutation types of SNP sites found that there existed conversion and transversion types in the SNP of *Mm-BCDO2* exons (**Table 3**).

Both c.1148 C>T and c.1187 A>T were low polymorphism sites, while c.984 A>C was a moderate polymorphism site in the red shell clams. On the contrary, all the three SNPs were moderately polymorphic sites in the white shell clams (**Table 4**). The range of observed heterozygosity (H_o) and expected heterozygosity (H_e) in the red shell clams was 0.193–0.237 and 0.232–0.468, respectively, whereas the range of H_o and H_e in the white shell clams was 0.146–0.265 and 0.335–0.497, respectively. Further, the linkage disequilibrium results showed that the three sites were strongly linked ($D' > 0.75$; **Table 5**).

Three-locus haplotypes consisting of three SNPs were constructed to estimate the haplotype frequencies using SHEsis software. The analytical results showed that ACA was a common haplotype. CCA, CCT, and CTT haplotype frequencies for these three polymorphisms showed significant differences between the red shell and white shell ($P < 0.05$; **Table 6**).

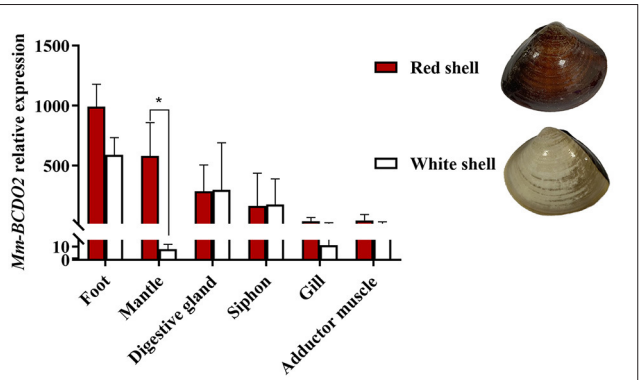

FIGURE 3 | Relative expression levels of *Mm-BCDO2* in different tissues from *M. meretrix*. Significant differences between red shell and white shell are indicated with an asterisk for $P < 0.05$.

Distribution of Mm-BCDO2 Protein in the Mantle and Digestive Gland

The immunofluorescence was used to assess the subcellular distribution of Mm-BCDO2 in the mantle and digestive gland

TABLE 3 | Variation type of the SNPs of *Mm-BCDO2* gene.

Variation type	Genotype	Sites	Total number
Transition	GA	500, 1,047, 1,406, 1,436, 1,454, 1,496	6
	CT	1,148, 1,211, 1,403, 1,439, 1,589	5
Transversion	TA	663, 707, 1,118, 1,187, 1,535	5
	AC	899, 984, 1,376, 1,376	4

TABLE 2 | Analysis of association between the SNPs of *Mm-BCDO2* and clam shell color.

Locus	Genotype	Number (Genotype frequency)		Allele	Allele frequency		χ^2 (*P*-value)
		Red shell	White shell		Red shell	White shell	
c.984A>C	AA	69 (51.11)	72 (47.68)	A	62.96	54.97	6.855 (0.032)*
	AC	32 (23.7)	22 (14.57)	C	37.04	45.03	
	CC	34 (25.19)	57 (37.75)				
c.1148C>T	CC	104 (77.04)	107 (70.86)	C	86.67	79.14	7.228 (0.027)*
	CT	26 (19.26)	25 (16.56)	T	13.33	20.86	
	TT	5 (3.7)	19 (12.58)				
c.1187A>T	AA	100 (74.07)	85 (56.29)	A	85.19	69.54	16.026 (0.0003)**
	AT	30 (22.22)	40 (26.49)	T	14.81	30.46	
	TT	5 (3.7)	26 (17.22)				

Significant differences between red shell and white shell are indicated with an asterisk for P < 0.05, and with two asterisks for P < 0.01.

TABLE 4 | Polymorphic parameters of the SNP loci of *Mm-BCDO2* gene.

Strain	Locus	Ho	He	Ne	PIC
Red shell color	c.984 A>C	0.237	0.468	1.874	0.358
	c.1148 C>T	0.193	0.232	1.301	0.204
	c.1187 A>T	0.222	0.253	1.338	0.220
White shell color	c.984 A>C	0.146	0.497	1.981	0.373
	c.1148 C>T	0.172	0.335	1.502	0.278
	c.1187 A>T	0.265	0.425	1.735	0.334

TABLE 5 | Linkage disequilibrium analysis among three SNP of *Mm-BCDO2* gene.

Locus	c.984A>C	c.1148C>T	c.1187A>T
c.984 A>C		1.000 (1.000)	1.000 (1.000)
c.1148 C>T	0.262 (0.328)		1.000 (1.000)
c.1187 A>T	0.296 (0.543)	0.885 (0.543)	

The value above the diagonal represents D′, and the value below the diagonal represents R^2. The number outside the parentheses and inside the parentheses is the linkage disequilibrium analysis of red and white shell clams, respectively.

TABLE 6 | Haplotype analysis of three SNPs of *Mm-BCDO2* gene.

Haplotype sequence	All subjects	Red shell (frequency)	White shell (frequency)	χ^2 (*P*-value)
ACA	336	166.00 (0.550)	170.00 (0.630)	3.760 (0.052)
CCA	103	43.00 (0.142)	60.00 (0.222)	6.156 (0.013*)
CCT	33	29.00 (0.096)	4.00 (0.015)	17.298 (0.000**)
CTT	100	64.00 (0.212)	36.00 (0.133)	6.102 (0.013*)
CTA	0	0.00 (0.000)	0.00 (0.000)	

Significant differences between red shell and white shell are indicated with an asterisk for $P < 0.05$, and with two asterisks for $P < 0.01$.

of red and white shell clams. The result revealed that the Mm-BCDO2 was an extranuclear enzyme (**Figure 4**). According to the green fluorescence signal, the expression level of Mm-BCDO2 protein in single-layer columnar cells at the edge of the mantle of red clam was higher than that of white clam, but there was no difference in the muscle fibers scattered in the middle of the mantle (**Figure 4A**). Mm-BCDO2 was expressed on the glandular epithelium of digestive gland, while there was no difference in the expression level of Mm-BCDO2 between red and white shell clams (**Figure 4B**).

Expression of Mm-BCDO2 Protein in the Mantle and Digestive Gland

Western blot analysis of the Mm-BCDO2 protein in the mantle and digestive gland showed that the target band appeared at the predicted molecular weight. There was no significant difference in the expression level of BCDO2 in the digestive gland between red and white shell clams (**Figure 5A**). Conversely, Mm-BCDO2 was highly expressed in the mantle of the red shell clams compared with that of the white shell clams ($P < 0.05$; **Figure 5B**).

DISCUSSION

Studies on mammals, fish, and birds have revealed that *BCDO2* can control carotenoid homeostasis, thereby affecting changes in body surface color or tissue color (Amengual et al., 2013; Costabile et al., 2016). In this study, the full-length cDNA sequence of *Mm-BCDO2* was successfully cloned. Further, *Mm-BCDO2* displayed low identity in sequence size with other species such as mammal and shellfish, but all of them contained the RPE65 domain. It is still controversial whether the function of *BCOD2* gene is conserved in evolution. For example, RPE65 was molecularly identified as the first member of the vertebrate carotenoid cleavage dioxygenase family member (Hamel et al., 1993) and initially characterized as retinoid-binding protein rather than an enzyme catalyst (Gollapalli et al., 2003; Mata et al., 2004). Some studies question whether there exist the BCDO2 enzyme that produces vitamin A (Hansen and Maret, 1988). It has been reported that *BCDO2* gene encodes an enzymatically active protein, but it showed no carotenoid cleavage activity in human (Li et al., 2014a; Seña et al., 2016). Indeed, studies have proved that BCDO2 is a catalytically competent enzyme and displays broad substrate specificity by transferring human *BCDO2* splice variants into ARPE-19 cells and *E. coli* (Thomas et al., 2020). Furthermore, primate BCDO2 displayed a conserved structural fold and enzymatic function (Babino et al., 2015). Consistently, the *BCDO2* gene in vertebrate was conserved in evolution, while that in invertebrate was quite different here. Therefore, whether the function of *BCDO2* gene is conserved in evolution needs to be further explored.

Increasing studies have shown that the polymorphism of *BCDO2* gene is related to color traits (Wu et al., 2020). For instance, the nonsense mutations of *BCDO2* gene in the cattle lead to increased carotenoids in milk (Berry et al., 2009), while the yellow fat traits of sheep and rabbit are correlated with mutations in the coding region of *BCDO2* gene (Vge and Boman, 2010; Strychalski et al., 2015, 2019; Niu et al., 2016). In addition, studies have found that some polymorphic loci of *BCDO2* gene are completely linked to the skin color traits of chickens (Eriksson et al., 2008; Xu et al., 2017). These findings reveal that *BCDO2* mutations cause the alterations in its function on mediating carotenoids cleavage. Non-synonymous single-nucleotide polymorphisms (SNPs) result in changes in the encoded amino acids, which in turn may affect the function of corresponding proteins (Katsonis et al., 2015; Cao et al., 2016). This is considered to be an important cause that leads to changes in animal phenotypes. In this study, the positions c.984 A>C and c.1187 A>T were non-synonymous mutations, and the position Lys984Gln caused the amino acid coded to be mutated from positively charged to uncharged, which was located near the conservative site. Similarly, the site Lys1187Asn led to the amino acid mutation from positively charged to negatively charged, which was located in the starting region of the functional domain. Three haplotype frequencies for these three polymorphisms showed significant differences between red shell clams and white shell clams ($P < 0.05$). We think the different genotypes of the three SNPs may associate with mRNA stability or affect *Mm-BCDO2* gene expression through

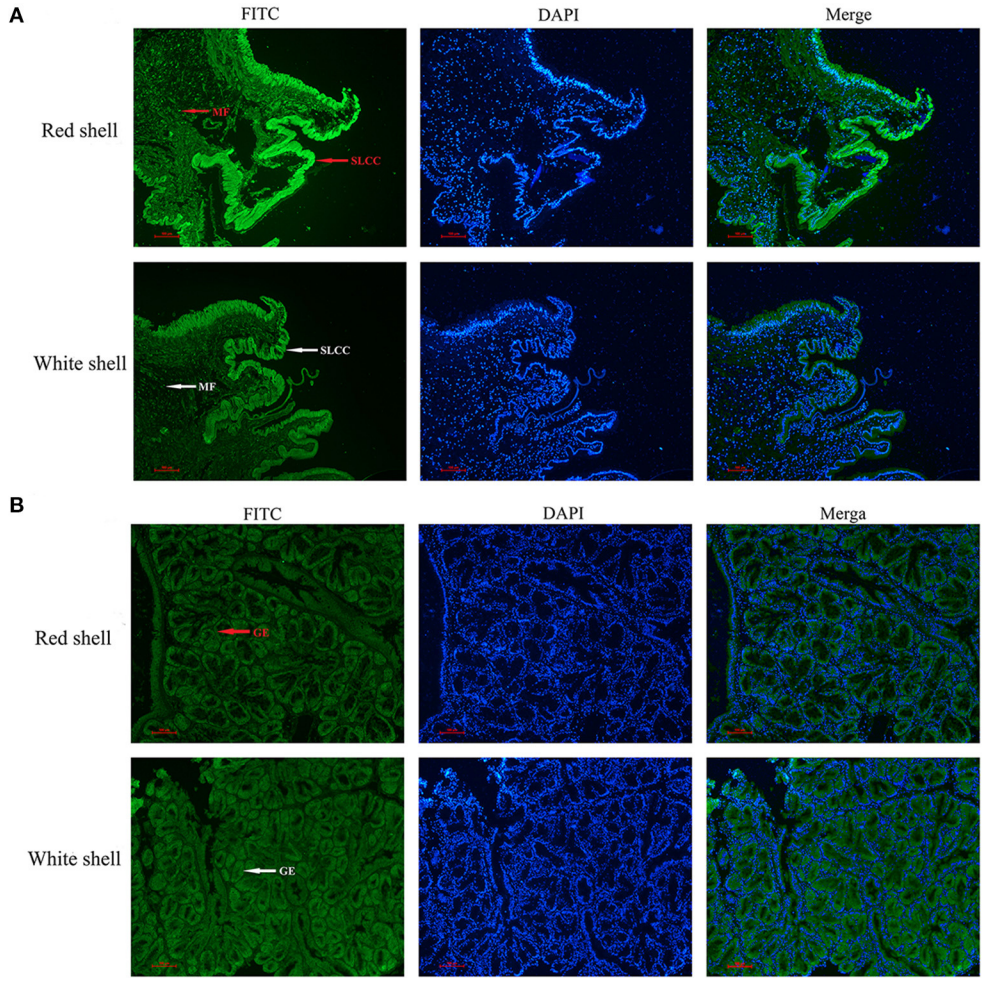

FIGURE 4 | Distribution of BCDO2 in the mantle and digestive gland from red and white shell *M. meretrix*, respectively. **(A)** Localization of BCDO2 in single-layer columnar cells (SLCC) and muscle fibers (MF) of the mantle. **(B)** Localization of BCDO2 in glandular epithelium (GE) of digestive gland. Green fluorescence signal, FITC; blue fluorescence signal, DAPI.

interaction with other transcription factors that would disturb carotenoids metabolism. It has been shown that changes in the charge of amino acids can lead to changes in redox intensity (Jiao et al., 2016). Consistent with the notion, a non-synonymous mutation in the *BCDO2* gene was found to disrupt the carotenoid cleavage activity of the enzyme in a study of the coloration mechanism on birds, thereby causing a significant increase in the concentration of intact carotenoids in tissues (Anna et al., 2020). These findings suggest that *BCDO2* gene plays an important role in the accumulation of carotenoids in animal tissues. Therefore, we speculate that changes of amino acid at multiple sites may affect the spatial collision between the side chain of corresponding site of the clam BCDO2 protein and the surrounding side chain, thereby changing the protein structure or causing protein folding defects, which in turn altered enzyme activities and resulted in different metabolic capabilities between red and white shell clams. Intriguingly, there was a strong linkage between c.984 A>C and c.1187 A>T, which could be inherited stably and could be an important target of selection in the evolution of color traits.

Changes in gene expression are the basis for differential phenotypes between the same species (Fallahshahroudi et al., 2018). In this study, the *Mm-BCDO2* gene was expressed in all six tested tissues, with significantly higher expression in the mantle of red shell clams than white shell clams, and there was no difference in the digestive gland. These findings were further verified by western blot and immunofluorescence analysis. Consistently, the expression level of *BCDO2* gene in the red feathers and beaks of *Quelea quelea* was significantly higher than that in the pale yellow feathers and beaks of the population (Walsh et al., 2012). The expression level of *BCDO2* decreased in the skin, muscle, and adipose tissue of the white leghorn compared with that of the *Red Junglefowl*, but no change was observed in the liver and other internal organs. It has been proposed that the expression level of *BCDO2* gene in the livers did not decrease because BCDO2 was not sensitive to the

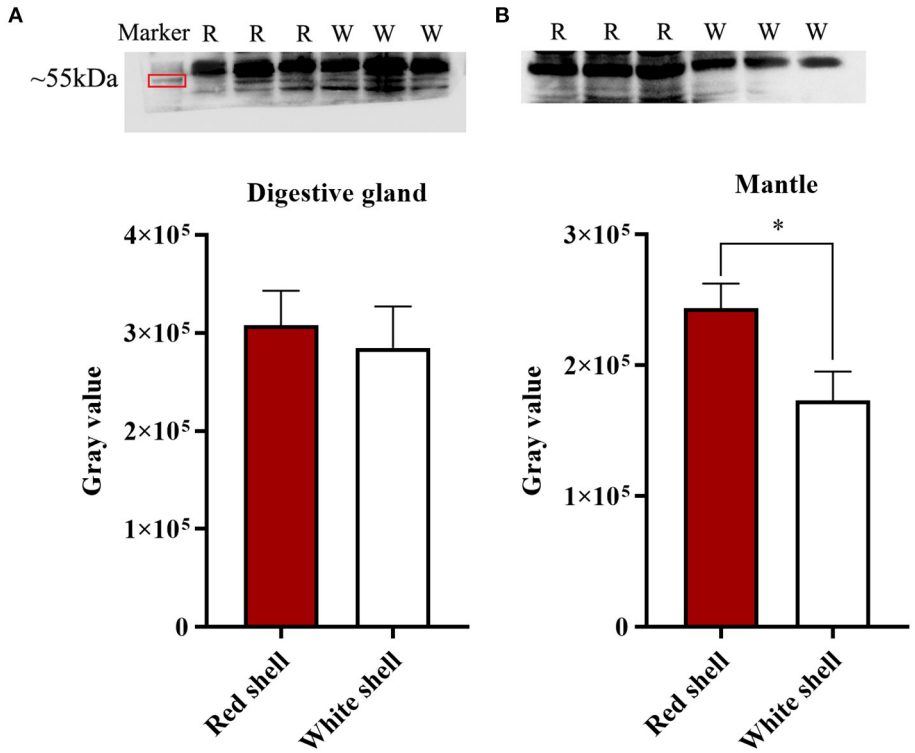

FIGURE 5 | Western blotting analysis of Mm-BCDO2 protein in the digestive gland **(A)** and mantle **(B)** of red and white shell clams. Significant differences between red shell and white shell are indicated with an asterisk for $P < 0.05$.

toxicity of excess carotenoids (Fallahshahroudi et al., 2019). On the contrary, the transcript levels of *BCDO2-l* gene was elevated in the white Chinook salmon compared with that in the red Chinook salmon (Lehnert et al., 2019). The protection of cells against apoptosis induced by carotenoid is strictly dependent on BCDO2 enzymatic function (Lobo et al., 2012). Our previous study on the clam shell color found that the genes and proteins that are related to carotenoid transport were significantly higher in the red shell clams than that in the white shell clams (Zhan, 2015). Therefore, we speculate that there are two explanations for this phenomenon. On the one hand, it is to prevent excessive carotenoid accumulation in the body from causing cellular oxidative stress, which will have side effects on the body, so more carotene oxygenases are needed to decompose carotenoids. Previous studies in mice have shown that high concentration of carotenoids can force the expression of the *BCDO2* gene, so the high-level expression of carotene oxygenase in the body can balance the carotenoid concentration without causing oxidative stress (Babino et al., 2015). On the other hand, it may be that the body needs to metabolize carotenoids to show different colors (red, yellow, etc.). "Red" pigments are instead often metabolically derived from the yellow precursors, typically by oxygenation (also known as allylic oxidation), adding double-bonded oxygen atoms ("keto-groups") to the end rings, thereby shifting the absorptance λ max toward longer wavelengths (Britton, 1995). For example, researchers have found that some birds produce red feathers by metabolizing carotenoids into derived keto carotenoids, while other birds produce red feathers by depositing high concentrations of yellow dietary carotenoids (Andersson et al., 2006). And the method of depositing carotenoids in clams needs further research.

In summary, we characterized the cDNA of *Mm-BCDO2* and analyzed the relationship between its sequence characteristics and phylogenetic trait. The expression level of *Mm-BCDO2* gene was significantly higher in the mantle of the red shell clams than that in the white shell clams. Moreover, the association analysis identified three shell color-related SNPs in the exons of *Mm-BCDO2*. Among them, the non-synonymous mutations of c.984 A>C and c.1187 A>T sites may be correlated with the accumulation of carotenoids. Overall, these findings contribute to understanding the role of *Mm-BCDO2* gene in the formation of shell color via regulating carotenoid levels and provide molecular marker for shell color breeding in *M. meretrix*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Wanli University, China.

AUTHOR CONTRIBUTIONS

YD, HY, and ZL designed the study. LF performed the experiments under the support of YB. LF, HS, WD, and YD analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.746026/full#supplementary-material

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