Hindawi
Aquaculture Research
Volume 2023, Article ID 7412887, 9 pages
https://doi.org/10.1155/2023/7412887

Research Article

Cloning, Characterization, and Expression Prolife Analysis of Tyrosinase Genes: Insights into Black Shell Formation in *Cyclina sinensis*

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Received 11 November 2022; Revised 28 January 2023; Accepted 3 February 2023; Published 16 February 2023

Academic Editor: Hisham Abdelrahman

Shell colors that exhibit a positive relationship with excellent traits can be employed as marker colors for breeding new varieties of bivalves. The clam *Cyclina sinensis* is an economically important marine bivalve that has three main colors: black, white, and purple. In the present study, we cloned and analyzed the full-length cDNA of the tyrosinase gene (TYR), which is the key gene for melanin synthesis, to explore the formation mechanism of black shells. The full-length cDNA of TYR was cloned by RACE-PCR, and the results showed that the full-length cDNA of TYR was 2993 bp, including a 2262 bp open reading frame encoding 753 amino acids. The results of functional domain prediction showed that deduced TYR protein contains a typical functional domain of tyrosinase, the common central domain. Phylogenetic analysis was conducted using neighbor-joining and maximum likelihood methods, and the results indicated that the evolutionary positions of *C. sinensis* and *Meretrix meretrix* were closest. The qRT–PCR results showed that TYR mRNA was highly expressed in the outer edge of the mantle, which suggested that TYR was involved in the synthesis of melanin in the mantle of *C. sinensis* and might play an important regulatory role in the formation of melanin in black clam shells.

1. Introduction

Cyclina sinensis is an economically important shellfish aquaculture species in the coastal areas of China and is distributed in North Korea and Japan [1, 2]. Its meat is tender and delicious and rich in nutrition and contains a variety of amino acids and trace elements required by the human body, and this species has the characteristics of fast growth, excellent quality, strong adaptability, and so on. In recent years, artificial clam breeding has become more difficult due to breeding diseases coupled with the degradation of germplasm resources, resulting in reduced quality and yield [3, 4]. Therefore, it is of great significance to improve the breeding of *C. sinensis* [5]. At present, the characteristics of nutrition, disease resistance, growth, shell color, and other traits can be used to determine specific molecular markers to screen out superior species of *C. sinensis* [6]. Among those characteristics, shell color has been widely studied in regards to its molecular inheritance [7]. The polymorphism of shell color is affected by genes, the environment, food, and other factors. It is also linked to survival, growth, development, and disease resistance [8, 9]. Freshly excavated shells of *C. sinensis* are purplish black, and they gradually oxidize to white or brown as the time of

exposure to air increases. Studies have shown that a darker shell color of *C. sinensis* indicates a high degree of freshness and reproductive ability [10]. Therefore, shell color can be used as a marker of excellent traits to improve variety breeding [11].

Previously, three types of pigments were found in the shells of mollusks, namely, melanin, tetrapyrroles, and carotenoids [12, 13]. Among them, melanin is a polymer with a complex structure and high molecular weight in living organisms [14]. It is the pigment with the widest distribution range among many biological pigments. Melanin can be divided into eumelanin, pheomelanin, and allomelanin according to the source, generation pathway, and structural characteristics of the pigment [15]. Melanin has a variety of biological functions, such as antiaging and antioxidant functions, scavenging of free radicals, and reducing the oxidative damage of the adductor muscle [16]. In organisms, polyphenols or indole compounds undergo a series of oxidation and polymerization reactions to produce melanin [17], resulting in final coloration of black or tan, and this process mainly requires the participation of tyrosine and tyrosinase [15]. Most species in nature have melanin in their bodies or on their surfaces, including fungi [18], sponges, and various bacteria [19, 20]. It is also widely found in various animals, such as mammals [21–23], poultry and birds [24], and reptiles and amphibians [25, 26]. Melanin has also been observed in mollusks, including *Crassostrea gigas*, *Sepia officinalis*, and *Patinopecten yessoensis* [27–29].

In melanocytes, the three major pigmentation enzymes required for melanin synthesis are TYR (tyrosinase), TYRP1 (tyrosinase-related protein 1), and TRP2/DCT (dopachrome tautomerase) [30]. Among them, tyrosinase is widely found in microorganisms, animals, plants, and the human body. It is a glycosylated, multifunctional, copper metalloenzyme that is one of the most key enzymes in the process of melanin formation [31]. Its expression controlling gene is a tyrosinase gene. Tyrosinase in plants is called polyphenol oxidase, in insects, it is called phenol oxidase, and in eukaryotes and microorganisms, it is known as tyrosinase [32, 33]. A large number of studies have confirmed that tyrosinase gene mutations can cause abnormal body color in a variety of animals (mice, rabbits, *Oryzias latipes*, etc.) [34–37], usually manifested as albinism [38] or abnormal visual function of the eyes [39–41]. In addition, studies of the TYR gene in bivalves has also been widely reported, including studies in *C. gigas* [42, 43], *P. yessoensis* [29], *Meretrix meretrix* [44], *Pinctada fucata* [45], and *Ruditapes philippinarum* [46]. However, the TYR gene has not been reported in *C. sinensis*.

In this study, the full-length cDNA sequence of the TYR gene was cloned in *C. sinensis*, and analyses of its sequence characteristics, including functional domain prediction of the encoded protein and multiple amino acid sequence alignment, were performed. In addition, phylogenetic analysis and gene expression pattern analysis were also carried out. This study provides a theoretical basis for exploring the molecular mechanism of *C. sinensis* black shell formation.

2. Materials and Methods

2.1. Animal and Sampling. Two-year-old clams were obtained from Lianyungang, Jiangsu Province, China. They were cultured for three weeks in an indoor-circulating seawater system. Six individuals of *C. sinensis* (body weight, 8.06 ± 1.33 g; shell length, 2.84 ± 0.18 cm) were randomly selected for subsequent anatomical experiments. The mantle and gill tissue samples were removed and immediately stored in liquid nitrogen for subsequent total RNA extraction and ready-strand cDNA synthesis.

For the extraction of total RNA from different tissues, six clams were randomly selected and dissected to collect six tissues in their bodies, including the mantle (inner and outer edges), siphon, adductor muscle, visceral mass, foot, and gills (Figure 1). The samples were immediately stored in a centrifuge tube containing RNA preservation solution at −80°C, and 200 mg of each sample was used for RNA extraction.

2.2. Total RNA Extraction, cDNA Synthesis, and RACE-PCR Amplification. Total RNA was extracted using the TRIzol kit (Invitrogen, USA) according to the kit instructions [47]. The concentration and quality of total RNA evaluated by the 28S/18S ratio were determined using an Infinite F200 microplate reader (TECAN, Switzerland). The cDNA of different tissues was synthesized using 800 ng total RNA of each sample according to the instructions of the reverse transcription kit. Total RNA extracted from 6 tissues of *C. sinensis* was mixed and used as a template for 3′- and 5′- RACE ready cDNA synthesis using the SMARTer™ RACE cDNA amplification kit (Takara, Beijing, China; CAT: 634860). The specific synthesis steps were performed according to the kit instructions. The ORF sequence and the genomic sequence of the TYR gene were obtained from the whole genome sequence of *C. sinensis* [8] (NCBI GenBank accession number: JAAONU000000000). To obtain 5′ and 3′ cDNA ends of TYR, the optimal two pairs of specific primers (outer and inner primers, Table 1) were designed and selected using Oligo v7.5 software (Molecular Biology Insights, Inc., Colorado, USA) based on the CDS sequence of TYR from *C. sinensis* genome sequencing. The universal primers (UPM-long and UPM-short) and the outer specific primers were applied to the outer PCR amplifications, and the universal primer (NUP) and the inner specific primers were applied to the next inner PCR amplifications. The procedure of touchdown PCR was performed with two steps. The first step was as follows: 15 cycles of 95°C for 30 s, the annealing temperature starting from 72°C for 30 s, then reducing 2°C per 5 cycles, and 72°C for 2 min for extension. The second step was as follows: 25 cycles of 95°C for 30 s, 64°C for 30 s, 72°C for 2 min, and then 72°C for 7 min for PCR terminating [10]. The purification steps of 3′- and 5′- cDNA PCR amplification products and the methods of ligation and transformation of recovered products were described by Wei et al. [47].

2.3. Sequence Analysis. To obtain the full-length cDNA of TYR, sequence assembly and splicing of 5′- and 3′ -cDNA were performed using SeqMan software (DNASTAR v7.0,

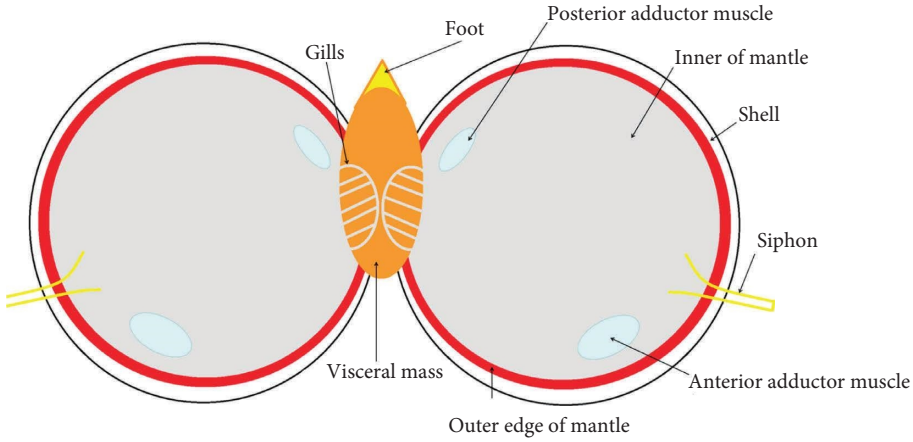

Figure 1: Sketch map of *C. sinensis* internal organization.

Table 1: Primers and their sequences for TYR used in this study.

Primer	Sequence (5′-3′)	Purpose
TYR-5GSP-R	AGCTGGTCCGAAGTGCGCTCGACGAAT	5′ RACE outer primer
TYR-5NGSP-R	GGTAAGTCGCCTGCTGTTGAGAT	5′ RACE inner primer
TYR-3GSP-F	CGAATCCACGCTTTAGGCCGGAGCACAG	3′ RACE outer primer
TYR-3NGSP-F	TTTCGGGCGGTATCAGGGTATCTT	3′ RACE inner primer
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE
UPM-short	CTAATACGACTCACTATAGGGC	RACE
NUP	AAGCAGTGGTAACAACGCAGAGT	RACE
TYR-qRT-F	GAGCAGAATAGCGAGACACG	qRT-PCR
TYR-qRT-R	TCTCTCTTTGCTTCACCCAAC	qRT-PCR
β-actin-F	ACGAGGACGTAGCTGCTTTGGT	Internal reference
β-actin-R	GAGTTAGGTTTCCGATTGGCAC	Internal reference

Wisconsin, USA; https://www.dnastar.com/). The ORF region and the amino acid sequence were predicted by using EditSeq software (DNASTAR v7.0). The online software Pfam domain (https://pfam.xfam.org/search#tabview=tab1) was used to predict the amino acid sequence of the functional structure. The ORF sequence of the gene and the genome sequence of the gene were analyzed with the Splign online platform (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi?textpage=online&level=form) to predict the number of TYR introns and exons in the genome. Multiple amino acid sequence alignment of TYR was performed using the Clustal W method in MEGA v6.0 software (https://www.megasoftware.net/).

2.4. Phylogenetic Analysis. The phylogenetic tree was constructed by using MEGA v6.0 software by neighbor-joining (NJ) and maximum likelihood (ML) methods with default parameters [48]. The amino acid sequences of other species were downloaded from the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov/gene/?term=) (Table 2). Then, the amino acid sequences were aligned using the MUSCLE method in MEGA v6.0 software. The bootstrap value was set to 1000, and the final tree result was edited using FigTree v1.4.2 software.

Table 2: List of species and amino acid sequence accessions used in this study.

NO	Species name	NCBI GenBank accession number
1	*Cyclina sinensis*	OP616710
2	*Meretrix meretrix*	ALG64484.1
3	*Crassostrea virginica*	XP_022334965.1
4	*Mytilus galloprovincialis*	VDI13701.1
5	*Hyriopsis cumingii*	APC92581.1
6	*Mus musculus*	BAA00079.1
7	*Homo sapiens*	AAB60319.1
8	*Bos taurus*	AAL02331.2
9	*Canis lupus familiaris*	AAQ17535.1
10	*Oryzias latipes*	BAA06155.1
11	*Danio rerio*	AAN17339.1
12	*Amphilophus citrinellus*	ANE23829.1
13	*Xiphophorus maculatus*	XP_005803437.1
14	*Ovis aries*	NP_001123495
15	*Chelmon rostratus*	XP_041796342.1

2.5. Real-Time PCR. To determine the expression pattern of TYR in different tissues of *C. sinensis*, quantitative real-time PCR (qRT-PCR) was conducted using a Takara SYBR® green I Premix Ex Taq™ kit (Takara, Dalian, China) in a StepOnePlus real-time PCR detection system. The qRT-PCR volume was 20 μl, containing 10 μl SYBR Premix Ex

Taq™ (Takara), 2 μl cDNA template, 0.4 μl ROX I fluorescent dye, 0.5 μl TYR-qRT-F (forward primer), and 0.5 μl TYR-qRT-R (reverse primer) (Table 1). The qRT-PCR amplification procedure was performed according to the method by Wei et al. [47]. β-actin was selected as the reference gene, and the relative mRNA expression level of TYR was calculated by the $2^{-\Delta\Delta Ct}$ method [49]. One-way ANOVA in SPSS v18.0 software (IBM, New York, NY, USA) was used for statistical analysis.

3. Results

3.1. Cloning of the Full-Length cDNA Sequence of TYR. The full-length cDNA of TYR was 2993 bp, containing 112 bp 5′-UTR (noncoding region), 619 bp 3′-UTR, and a 2262 bp coding region (ORF), which encoded a protein containing 753 amino acid residues (TYR). At the end of the sequence, two typical mRNA sequences were found: a 28 bp polyA tail at 3′-UTR and a polyadenylation signal sequence (AATAAA) at 162 bp upstream of the polyA tail (Figure 2). In addition, the molecular weight of the deduced TYR protein was 85.4 kDa, and the isoelectric point (PI) was 8.878.

3.2. Analysis of TYR Sequence Characteristics

3.2.1. Genome Structure. To study the genomic structure of TYR, the ORF sequence was mapped to its genomic DNA sequence. After comparative analysis, it was found that the genomic sequence of TYR consisted of 6 exons of 15, 142, 269, 113, 1619, and 104 bp in length and was segmented by 5 introns of 511, 1013, 458, 234, and 239 bp in length (Figure 3).

3.2.2. Multiple Amino Acid Sequence Alignment. For multiple sequence alignment analysis, we used amino acid sequences of TYR proteins from eight species, including four mollusks (*C. sinensis*, *M. meretrix*, *Hyriopsis cumingii*, and *Mytilus galloprovincialis*), a bony fish (*Danio rerio*), and two mammals (*Mus musculus and Homo sapiens*). The results predicted a typical functional domain of TYR, namely, the common central domain of tyrosinase. This typical TYR domain is conserved in eight species (Figure 4).

The identical or similar amino acids were shaded and colored by using ESPript software. The conserved domain is underlined by the red solid line, and the amino acid sequences used in this study are listed in Table 2.

3.2.3. Phylogenetic Analysis. Phylogenetic analysis was performed using the NJ and ML methods based on the TYR amino acid sequences of 15 species. The bootstrap value option from the software was selected to test the credibility of building a branch of the phylogenetic tree. Amino acid sequences of 14 species were downloaded from the GenBank database, including five species of teleost fish (*D. rerio*, *O. latipes*, *Amphilophus citrinellus*, *Xiphophorus maculatus*, and *Chelmon rostratus*), 5 species of mammals (*Canis lupus familiaris*, *H. sapiens*, *M. musculus*, *Bos taurus*, and *Ovis aries*), *and* 4 species of mollusks (*M. meretrix*, *M. galloprovincialis*, *H. cumingii*, and *Crassostrea virginica*) (Table 2). The results showed that vertebrates were clustered into one group and that mollusks were clustered into another group. In addition, *C. sinensis* is close to *M. meretrix* in the mollusk group (Figure 5) because they form a monophyletic clade with high confidence (100), which is supported by both the NJ and ML analyses.

NJ and ML bootstrap values are presented at the nodes of the tree. The amino acid sequences from 15 species are listed in Table 2.

3.2.4. Tissue Expression Distribution. The mRNA expression levels of TYR in various tissues from *C. sinensis* were analyzed by qRT-PCR. As shown in Figure 6, the mRNA of TYR was widely expressed in all tissues, with the highest and most significant expression in the outer edge of the mantle of *C. sinensis* ($p < 0.05$), followed by the visceral mass and gill. Lower mRNA expression levels were detected in other tissues, including the inner mantle, adductor muscle, foot, and siphon.

4. Discussion

During the development of shellfish, pigment cells in the outer epidermis of the mantle constantly secrete pigment granules and pigment particles reach the surface of the mantle through microvilli to form pigment bands [50]. Some researchers have also found that pigment-secreting cells at the mantle edge of mollusks contribute to the formation of pigmented columnar shells and are closely related to the production of melanin [51–53]. Melanin exists in the nacreous layer and cuticle of the *C. sinensis* shell. Under different environmental conditions (inside and outside the sediment), the change in shell color is reversible, which may be due to the change in redox activity of melanin in the shell [8]. In the process of shell formation, how melanin is secreted from the mantle into the shell remains unclear. In this study, the full-length cDNA sequence of TYR from *C. sinensis* was cloned by RACE-PCR. The full-length sequence of TYR is 2993 bp, which contains 2262 bp ORF encoding 753 amino acids. Sequence analysis showed that the genomic structure of the TYR gene consisted of six exons and five introns. The encoded polypeptide chain contains a typical functional domain of tyrosinase, the common central domain of tyrosinase, which is conserved in mollusks and vertebrates. In addition, the evolutionary status of the TYR gene of *C. sinensis* is close to that of *M. meretrix*, which is consistent with the results of traditional taxonomy (*C. sinensis* and *M. meretrix* are both species of Veneridae) [6]. Therefore, it can be preliminarily inferred that the shell pigment of *C. sinensis*, which is homologous to *M. meretrix*, may also be secreted by the mantle epidermal cytochrome. This inference is consistent with the report of Saenko and Schilthuizen [12].

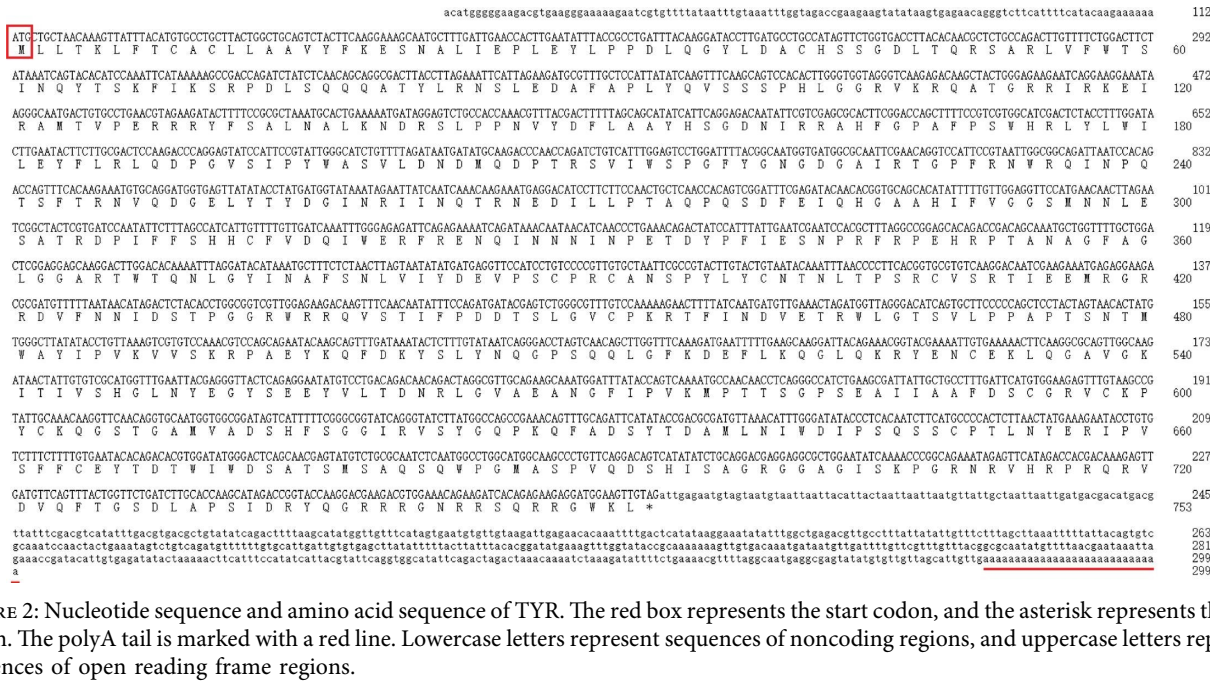

FIGURE 2: Nucleotide sequence and amino acid sequence of TYR. The red box represents the start codon, and the asterisk represents the stop codon. The polyA tail is marked with a red line. Lowercase letters represent sequences of noncoding regions, and uppercase letters represent sequences of open reading frame regions.

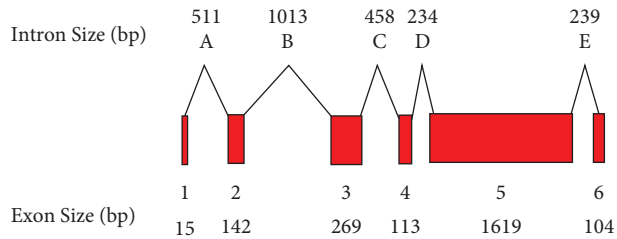

FIGURE 3: Genomic structure of the TYR gene in *C. sinensis*. The solid boxes (1–6) represent the 6 exons, and the capital letters (A–E) represent the 5 introns.

The TYR gene encodes a melanosome transmembrane protein that oxidizes tyrosine or DOPA substrates to melanin [54]. Most cases of albinism are caused by mutations in the tyrosinase gene [55–57]. At present, the study of TYR in bivalves has also been widely reported. The TYR gene was involved in the formation of juvenile and adult golden shells in *C. gigas* [42, 43], and its expression product, tyrosinase, had the highest content in the pigment band [58]. The tyrosinase family has also been reported to be involved in melanin formation in *Patinopecten yessoensis* [29]. There were significant differences in the expression level of TYR and the total melanin content at the edge of the mantle in *M. meretrix* with three different shell colors, indicating that TYR was involved in the formation of shell color [44]. The TYR gene was shown to be involved in the wound healing process of *P. fucata* by specific expression in mantle tissue, and this expression was speculated to be involved in the formation of the cuticle and prismatic layer of *P. fucata* [45]. In addition, the expression of different TYR family genes in *R. philippinarum* with different shell colors was studied by Jiang et al. [46]; and the results showed that TYR2, TYR6, and TYR10 were highly expressed in orange clams; TYR2, TYR3, TYR4, and TYR9 were highly expressed in white zebra clams; and TYR11 was highly expressed in zebra clams. It was speculated that the different expression patterns of differentTYR family genes resulted in the different shell colors of *R. philippinarum*.

To date, studies of the involvement of TYR in shell color formation has also been widely reported, including studies in *C. gigas* [42, 43], *P. yessoensis* [29], *M. meretrix* [44], *P. fucata* [45], and *R. philippinarum* [46]. In this study, qRT-PCR results showed that the mRNA of the TYR gene was widely expressed in all tissues of *C. sinensis*, with the highest and most significant expression in the outer mantle. This is consistent with the tissue expression pattern of TYR in *R. philippinarum*. The highest expression of TYR2 in seven tissues of *R. philippinarum* with different shell colors was found in the mantle [46]. Therefore, we speculate that TYR may be involved in the synthesis of melanin in the mantle of *C. sinensis*, and the melanin of the shell may be generated by the secretion of melanin in the mantle during shell formation [10]. In addition to the formation of shell color, TYR

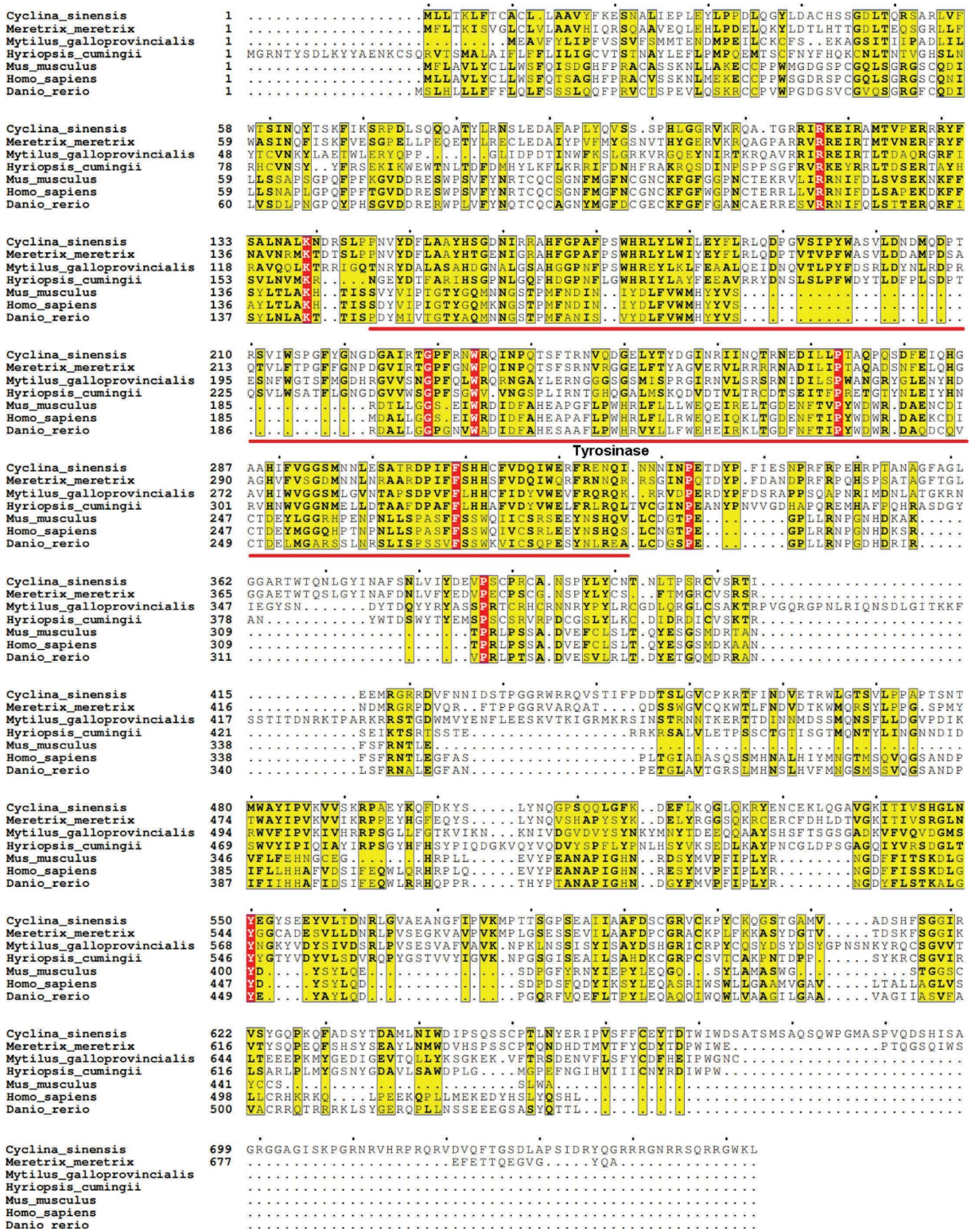

FIGURE 4: Multiple amino acid sequence alignment of TYR protein sequences.

was also found to be involved in the immune response of shellfish. The activity of TYR in the hemolymph and blood cells of bivalves was significantly increased after infection with parasites, indicating that TYR was involved in the natural immune response of shellfish [59]. In the present study, the TYR gene was also highly expressed in gill tissue. In addition to respiratory filtration, the gill is also a major immune organ that can regulate autoimmunity. Therefore,

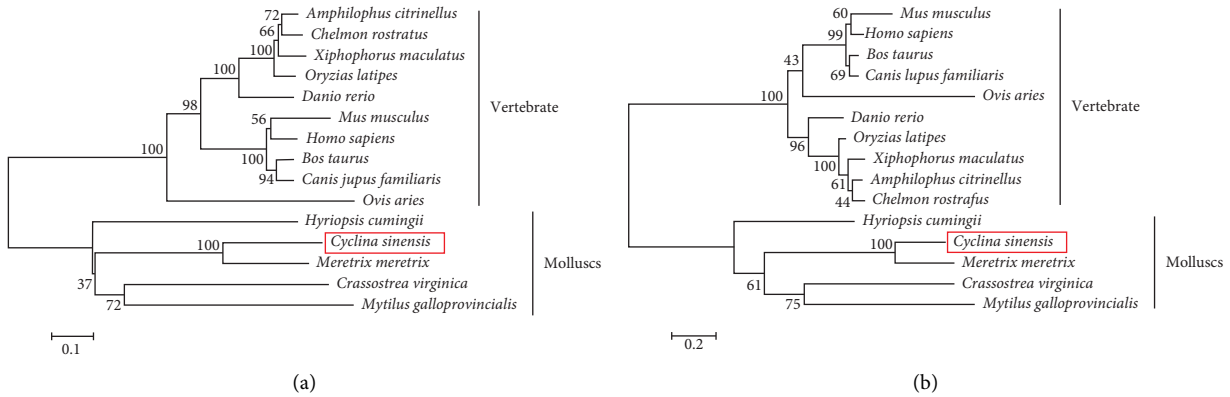

Figure 5: Phylogenetic analysis of the TYR gene from *C. sinensis* and 14 other species using NJ (a) and ML (b) methods.

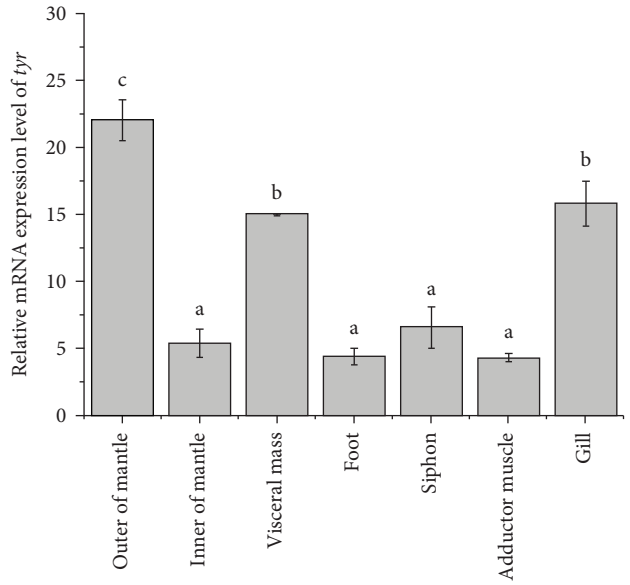

Figure 6: The expression levels of the TYR gene in different tissues of *C. sinensis*. Note: values are presented as the mean ± standard error, and the different lowercase letters above histogram columns represent significant difference between different tissues ($p < 0.05$).

the TYR gene may also be involved in the immune regulation of *C. sinensis*, and this inference has been verified in other species [60, 61].

5. Conclusions

In the present study, the full-length cDNA sequence of TYR was cloned, characterized, and analyzed. It was found that the mRNA of TYR was highly expressed in the outer mantle of *C. sinensis*, indicating that TYR might be involved in the synthesis of melanin in the outer mantle of *C. sinensis* and might be related to the formation of black shells; however, these results require further experimental verification.

Data Availability

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

Ethical Approval

The collection and sampling of the experimental animals utilized in this study were approved by the Animal Care and Use Committee of Jiangsu Ocean University. All handling of animals was performed in accordance with the guidelines for the Care and Use of Laboratory Animals at Jiangsu Ocean University.

Disclosure

Yuyan Sun and Xin Shan are the co-first authors. The funding bodies played a key role in the design of the study, collection, analysis, interpretation of the data, and writing of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

MW and ZD conceived and designed the project. YS, XS, DL, and XL analyzed the data. PQ, LT, SX, XL, JQ, ZH and BG performed experiments. YS and XS wrote the manuscript. All the authors read, reviewed, and approved the manuscript. Yuyan Sun and Xin Shan contributed equally to this work.

Acknowledgments

This work was supported by the Natural Science Foundation of Jiangsu Province (BK20191008), the Six Talent Summit Program of Jiangsu Province (NY-113), a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, and the Postgraduate Research and Practice Innovation Program of Jiangsu Province (KYCX2022-63 and KYCX2021-067).

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