BODY COLOR REGULATION OF *LEPTOBOTIA TAENIOPS* THROUGH TYROSINASE GENE EXPRESSION

JIANG, H.[1,2,3*] – LIU, S.[1,2,3] – XIAO, T. Y.[1,2,3*] – XIE, M.[4]

[1]*College of Animal Science and Technology, Hunan Agricultural University Changsha 410128, China*

[2]*Hunan Engineering Technology Research Center of Featured Aquatic Resources Utilization Hunan Agricultural University, Changsha 410128, China*

[3]*Collaborative Innovation Center for Efficient and Health Production of Fisheries in Hunan Province, Changde 415000, China*

[4]*Hunan Fisheries Science Institute, Changsha 410153, China*

[*]*Corresponding authors*
e-mail: 1176139630@qq.com (H. Jiang), tyxiao1128@163.com (T. Xiao)

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Abstract. Body color plays important roles in various behaviors of fish. *Leptobotia taeniops* exhibits two body color phenotypes at the same habitat. However, the mechanism is unclear. Tyrosinase plays a significant role in regulating synthesis of melanin. Therefore, we inferred that expression differences of tyrosinase gene was the primary reason for the different body colors of *L. taeniops*. To verify our inference, we firstly cloned and sequenced the whole-length cDNA sequence of *L. taeniops* tyrosinase gene through the rapid-amplification of cDNA ends cloning technology, and compared the expressions of the tyrosinase gene at different tissues between light and dark phenotypes using real-time quantitative reverse transcription PCR. The results showed the cDNA sequence was very similar to those from Cyprinidae fish. The expressions of tyrosinase gene in eyeball, dorsal fin, skin, muscle, liver and gill of the light phenotype were significantly lower than these in the dark phenotype. These results implied the expression of tyrosinase gene played an important role in regulating the body color of *L. taeniops*. The results provided important reference information to further elaborate the molecular mechanism of *L. taeniops* to adapt to their surrounding environment through changing color, protect wild *L. taeniops* resource, and cultivate new ornamental fish varieties.
Keywords: *Leptobotia taeniops, tyrosinase, melanin, tissue differential expression, rapid-amplification of cDNA ends*

Introduction

Body color plays important roles in various behaviors of fish, such as competition, courtship, avoiding predators and warning (Moretz and Morris, 2003; Hubbard et al., 2010; Culumber, 2013). Changes of body color is considered an adaptive response to environment changes. The way that the mechanism of body color changing works is a fundamental question in conservation ecology.

Leptobotia taeniops (Cyprinformes: Cobitidae) is an indigenous species in China, which mainly distributes in the Yangtze River and its tributary (Chen, 1980). There are two body color phenotypes of *L. taeniops* at the same freshwater habitat. The light phenotype of *L. taeniops* is near golden color. There are small black stripes and splashes on their body. The dark phenotype of *L. taeniops* is yellowish-brown. There are massive black stripes and splashes on their body. No obvious genetic differentiation is detected

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between the light and the dark phenotypes (Meng, 2011). So far, the mechanism that regulated the body color phenotypes is still unclear.

Body color of fish is controlled by types, proportion and distribution of pigment cells in epidermis. The pigment cells mainly include melanocytes, xanthophore, erythrophore, iridophore and leucophore (Schartl et al., 2016). Melanocytes contain melanin and are widely distributed in skin, hair, retina and bone of vertebrates. Melanin plays important roles in preventing damage to DNA and proteins from ultraviolet rays (Slominski et al., 2015), enhancing antioxidant capacity (Tu et al., 2009), maintaining intracellular calcium homeostasis (Parekh, 2016), and regulating immunization of organisms through NF-κB signaling pathway (Zhou et al., 2013). It is synthetized through tyrosine-tyrosinase reaction system and tyrosinase (TYR, EC 1.14.18.1) catalyzes tyrosine to the L-dihydroxyphenylalanine through hydroxylation and then converts to the dopaquinone. Therefore, tyrosinase is an iconic enzyme to melanin synthesis (Sánchez-Ferrer et al., 1995; Chen et al., 2015).

Therefore, we inferred that synthetic amount of melanin controlled the body colors of *L. taeniops* that living in the same freshwater habitat. To verify the inference, we firstly cloned and sequenced the whole-length cDNA sequence of tyrosinase gene through the rapid-amplification of cDNA ends (RACE) cloning technology. Then we compared the expression differences of the tyrosinase gene at different tissues between light and dark sub-populations of *L. taeniops* using real-time quantitative reverse transcription PCR (qRT-PCR). The results provided important reference information to further elaborate the molecular mechanism of *L. taeniops* to adapt to their surrounding environment through body color, protect wild *L. taeniops* resource, as well as cultivate new ornamental fish variety.

Materials and Methods

Sample collection

Total of 40 *L. taeniops* specimens (20 light phenotypic specimens and 20 dark phenotypic specimens) were collected from the Yueyang section of the Yangtze River during July to September, 2017. The specimens were transported to the Aquaculture Laboratory of Hunan Agricultural University, and temporary cultured 7 days in a culture system with circulating water at 25 ± 1°C before anaesthetized. The specimens were starved during the temporary cultured period. To eliminate the effect of healthy status on gene expression, each 6 specimens of light and dark phenotypes were chosen to further analyze according to their healthy status and exercise ability. The specimens were anaesthetized using MS-222, taken pictures, and measured their body lengths and body weights before dissected. After dissected, their sex was identified through sex gland. Their eyeball, skin at dorsal fin base, muscle, dorsal fin, gill, brain, liver and fish blubber were collected into freezing tubes and stored at -80°C for further analysis.

RNA extraction and sequencing cDNA of tyrosinase gene

The RNA used to synthetize cDNA of *L. taeniops* tyrosinase gene were extracted from 50-80 mg of skin using E.Z.N.A. Total RNA I kit (OMEGA, China) according to the manufacturer's introduction. The concentration and purity of RNA were measured using a nucleic acid and protein detector (Eppendorf, Germany). The degradation was assessed by 1.5% agarose gel electrophoresis with 120 V. The first strand of cDNA was

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synthetized using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, USA) according to the manufacturer's introduction.

The intermediate primers, TYR-F and TYR-R, were designed according to the sequences of Cyprinidae tyrosinase genes in GenBank database using primer 6.0 software. Then the intermediate sequence was amplified using the primers and the first strand of cDNA as a template. The PCR product was purified using a Gel Extraction kit (Omega, China) and bound to pTOPO-T vectors (Aidlab, China) and cloned as a previous report (Ni et al., 2017). Five positive clones were screened and bidirectional sequenced to obtain the intermediate sequence of *L. taeniops* tyrosinase gene using ABI 3730 system at Wuhan Aokedingsheng Bio-Science, Ltd., China.

According to the sequences of Cyprinidae tyrosinase genes and the intermediate sequence of *L. taeniops* tyrosinase gene, the 5′ and 3′ bilateral primers TYR5′-outer and TYR3′-outer and 5′ and 3′ medial primers TYR5′-inner and TYR3′-inner for RACE amplifying were designed using Oligo 7.0 software (*Table 1*). The 5' RACE cDNA and 3' RACE cDNA were synthetized using the primers and approximately 2 μg RNA as the template according to the user manual of the SMARTer RACE 5'/3' kit (Clontech, USA). Subsequently, the 3′ RACE and 5′ RACE of *L. taeniops* tyrosinase gene were amplified using the 3' RACE cDNA and 5' RACE cDNA as the template. The PCR product was purified and sequenced as described above.

Table 1. *The primer sequences used in the present study. RACE, rapid-amplification of cDNA ends; TYR, tyrosinase*

Primers name*	Primer sequence (5′–3′)	Utilization
TYR-F	GTCCTCGGTGTTCTCCTCCT	Amplifying the intermediate sequence of TYR gene
TYR-R	CCTCCTCTTCACTGCTGTTCA	
TYR3′–outer	TTCTGCATCACGCCTTTATTGACA	Amplifying 3′ RACE
TYR3′–inner	GAAACGGAGATTATTTCCTGTCCAC	
TYR5′–outer	GGGAGGCTGGTGTCTCCGTAGCCACT	Amplifying 5′ RACE
TYR5′–inner	TCCAGAGCGTTGCGGAAGCTCATGTTGG	
Universal primer (UPM)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE universal primers
Short universal primer	CTAATACGAC TCACTATAGGGC	
qRT-TYR-F	ATGCCTATTTGCTGGACCCC	Primers for qRT-PCR
qRT-TYR-R	TATGCCGACATCTTCCTGCG	
β-Actin-F	CTGGACTTGGCTGGTCGTG	Primers for qRT-PCR
β-Actin-R	CTCGAAGTCAAGGGCAACAT	

* F: forward primer; R: reverse primer

The sequences were merged to whole-length cDNA sequence of *L. taeniops* tyrosinase using SeqMan 5.01 module of DNAStar software. The amino acid sequence of *L. taeniops* tyrosinase was predicted based on the cDNA sequence using DNAMAN 7.0 software. The similar amino acid sequences of *L. taeniops* tyrosinase were retrieved from GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/blast). The amino acid sequences of tyrosinase from koi carp (*Cyprinus carpio*, ANN11899.1), zebrafish

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(*Danio rerio*, NP_571088.3), goldfish (*Carassius auratus*, ABI93943.1), Atlantic salmon (*Salmo salar*, ABD73809.1), finless eel (*Monopterus albus*, XP_020475631.1), midas cichlid (*Amphilophus citrinellus*, ANE23829.1), snakehead (*Channa argus*, AMQ24769.1), rainbow trout (*Oncorhynchus mykiss*, NP_001117694.1), medaka (*Oryzias latipes*, BAA31348.1), fugu rubripes (*Takifugu rubripes*, AAK13523.1), swordtail fish (*Xiphophorus maculatus*, XP_023207866.1), red crucian carp (*Carassius auratus auratus*, BAH03534.1), channel catfish (*Ictalurus punctatus*, AAF20161.1), sea pineapple (Ha*locynthia roretzi*, BAC76424.1), lancelet (*Branchiostoma japonicum*, AFS33098.1), black-spotted pond frog (*Pelophylax nigromaculatus*, BAA02077.1), soft-shell turtle (*Pelodiscus sinensis japonicas*, BAB79632.1), egret (*Egretta garzetta*, KFP18390.1), chicken (*Gallus gallus*, AAB36375.1), horse (*Equus caballus*, XP_001492610.4), sheep (*Ovis aries*, NP_001123499.1), mouse (*Mus nusculus*, NP_035791.1), and human being (*Homo sapiens*, NP_000363.1) were chose as reference sequences to construct phylogenetic tree. The sequences as well as that from *L. taeniaps* were aligned using Clustal X2.1 and neighbor-joining phylogenetic tree with 1000 times of bootstrap was constructed using MEGA 6.0. Expasy (http://www.expasy.org/) and SMART (http://smart.embl-heidelberg.de) were used to analyze the amino acid sequences of *L. taeniops* tyrosinase and predict functional domains of the protein.

The whole-length cDNA sequence has been submitted to the GenBank database with accession number MG870259.

Expression analysis of tyrosinase gene in different tissues of L. taeniops

Total RNA in eyeball, skin, muscle, dorsal fin, eyeball, gill, brain, liver and fish blubber of light and dark *L. taeniops* phenotypes were extracted using an E.Z.N.A. Total RNA I kit (OMEGA, China). The first strand of cDNA was synthetized using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, USA) with Oligo$(dT)_{18}$ primer. Primers qRT-TYR-F and qRT-TYR-R were designed according to the coding sequence (CDS) of the tyrosinase gene for real-time qRT-PCR (*Table 1*). Primers β-Actin-F and β-Actin-R (*Table 1*) were designed according to the sequence of *β*-Actin gene for endogenous reference to detect the amplification efficiencies (*E*%) and the correlation coefficients (R^2).

Real-time qRT-PCR was conducted on a CFX96 TouchTM real-time PCR detection system (Bio-Rad, USA) as a previous report (Ni et al., 2018) with minor modification. Briefly, each 25 μl reaction mixture consisted of 1 × SYBR Green Ⅱ PCR master mix (TaKaRa, China) containing 200 nM each primer and 20 ng of cDNA. The reactions were performed by incubation for 10 min at 95°C, following by 10 s at 95°C, 10 s at 60°C and 15 s at 72°C for 35 cycles. The program of melt curve was increased 0.5°C each 5 s from 65°C to 95°C. The relative expressions of tyrosinase gene in different tissues were calculated by the comparative Ct ($2^{-\triangle\triangle Ct}$) method (Li et al., 2012; Spivak et al., 2012).

Statistical analysis

One-way analysis of variance (ANOVA) for comparing the expressions of tyrosinase gene among different issues and Duncan's multiple range test for post-hoc test was conducted using R version 3.5.1. Independent *t*-test was conducted using R version 3.5.1 for comparing the body weights, the body lengths, and the expressions of

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tyrosinase gene in specific issue between the light and the dark phenotypes. Binomial test for comparing the sex ratio between the light and the dark phenotypes was also conducted using R version 3.5.1. Results for each parameter are presented as means ± standard deviation (SD) for each group. P-values < 0.05 were considered statistically significant.

Results

Physical characteristics of L. taeniops

The background color of *L. taeniops* was yellowish-brown. A lot of sparse black spots were on back of the light phenotype. Only very small gray spots were on their body sides (*Fig. 1A*). However, there were massive black stripes and splashes on the back and body sides of the dark phenotype (*Fig. 1B*). Body lengths of the light phenotype were 10.30 ± 0.60 cm, and these of the dark phenotype were 10.27 ± 0.95 cm (*Fig. 1*). Body weights of the light phenotype were 14.85 ± 2.30 g, and these of the dark phenotype were 15.33 ± 4.32 g. Neither the body length nor the body weight was detected significant difference between the light and dark phenotypes in the body length (independent *t*-test, $t = -0.073$, $p = 0.94$) and the body weight (independent *t*-test, $t = 0.24$, $p = 0.82$). Sex ratio between the light and dark phenotypes was also no significant difference (♀ : ♂ was 1 : 5 for the light phenotype; ♀ : ♂ was 2 : 4 for the dark phenotype; Binomial test, $p = 0.263$).

Figure 1. *Light (A) and dark (B) phenotypes of Leptobotia taeniops*

cDNA and amino acid sequences of tyrosinase of L. taeniops

The whole-length cDNA sequence of *L. taeniops* tyrosinase was 2643 bp, which was comprised by a 1617 bp of open reading frame, a 22 bp of untranslated region (UTR) at upstream of 5' end, and a 1004 bp of UTR at downstream of 3' end. The cDNA sequence contained the typically polyadenylation signal (AATAAA) and Poly(A)-tail of vertebrates (*Appendix 1*). The complete CDS of *L. taeniops* tyrosinase gene was approximately 90% of similarity to those of Cyprinidae fishes, such as *Cryprinus carpiod*, *C. auratis* and *D. rerio.* Putative amino acid sequence was 538 amino-acid residues and was constituted by 4 conserved domains, i.e. signal peptide, epithelial growth factor-like domain (EGF-like domain), tyrosinase domain, and transmembrane region. Tyrosinase domain included two copper binding sites (CuA and CuB; *Appendix 2*). The structure of the CuA was (H-x(4,5)-F-[LIVMFTP]-x-[FW]-H-R-x(2)-[LVMT]-x(3)-E), which corresponding amino acid sequence was HESAAFLPWHRVYLLFWE. The structure of the CuB was (D-P-x-F-[LIVMFYW]-x(2-)-x(3)-D), which corresponding amino acid sequence was DPIFLLHHAFID. The

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structures of the CuA and CuB were highly conserved among vertebrate species. The structures of CuA and CuB of *L. taeniops* were completely identical with Cyprinidae *Cryprinus carpiod*, *C. auratis* and *D. rerio* (*Appendix 3*). The molecular weight of *L. taeniops* tyrosinase was 60.81 kD. Theoretical isoelectric point of *L. taeniops* tyrosinase was 6.13. The predictive chemical structure formula was $C_{2718}H_{4105}N_{749}O_{788}S_{29}$. The instability coefficient was 56.16, which indicated that the protein was unstable. The prediction atlas of hydrophobicity showed that the tyrosinase of *L. taeniops* contained more hydrophilic regions than hydrophobic regions. Therefore, the tyrosinase was hydrophilic protein (*Appendix 4*). There were 13 cysteines in the tyrosinase of *L. taeniops*. Nine of 13 cysteines were at N-terminal, and other 4 cysteines were in the middle between CuA and CuB (*Appendix 1*).

Neighbor-Joining tree showed that all amino acid sequences of fish tyrosinase were clustered together, and those of amphibians, reptiles, birds and mammals were clustered to another cluster. *H. roretzi* of Urochordata and *B. japonicum* of Cephalochorda was clustered to two separate branches. *L. taeniops* was clustered together to Cyprinidae fishes (*Fig. 2*).

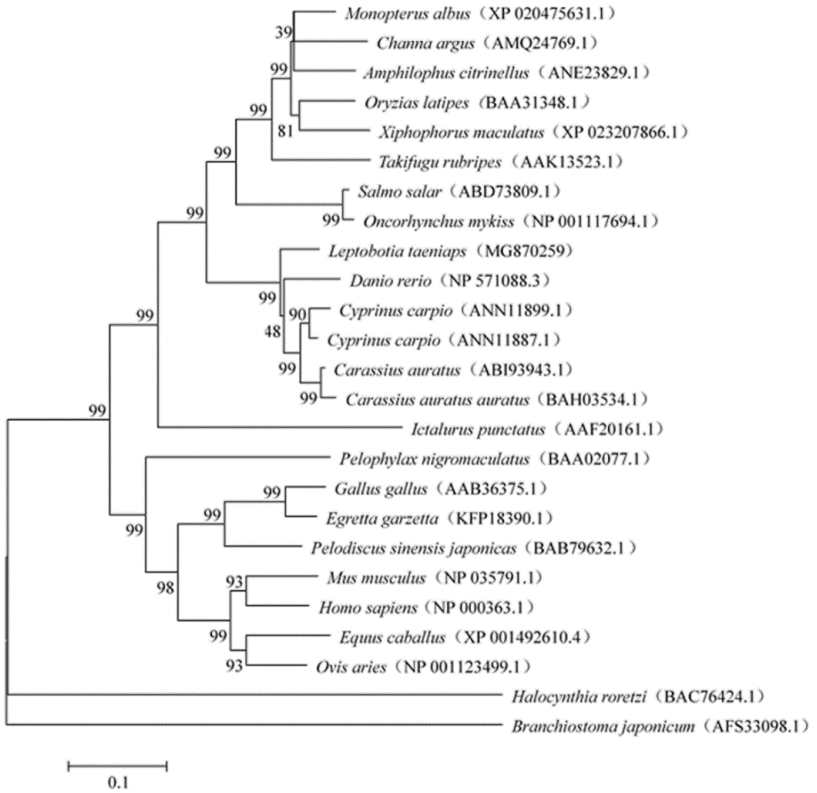

Figure 2. *Phylogenetic relationships of Leptobotia taeniaps and other representative vertebrates constructed based on amino acid sequences of tyrosinase*

Expression of tyrosinase gene in different tissues and organs of L. taeniaps

The amplification efficiencies (E%) and the correlation coefficients (R^2) showed that the primer pairs qRT-TYR-F/R (E% = 104.8%, R^2=0.991) and β-Actin-F/R (E% = 104.7%, R^2 = 0.998) were in sufficient to the requirements of real-time qRT-PCR

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(90% < *E*% < 105%, and R^2 > 0.98). The expressional trend of tyrosinase gene in skin, muscle, dorsal fin, eyeball, gill, brain, liver, and fish blubber was concordant between the light and dark phenotypes of *L. taeniops*. It was highest expressed in eyeball, and significantly different to other tissues (One-way ANOVA, $p < 0.01$), followed by dorsal fin, skin, muscle and brain. The expressions of tyrosinase gene in gill, liver and fish blubber of *L. taeniops* were very low, and no significant difference was detected among the three issues (One-way ANOVA, $p > 0.05$; *Fig. 3*). The expressions of tyrosinase gene in eyeball (independent *t*-test, $t = 34.88$, $p < 0.01$), dorsal fin (independent *t*-test, $t = 19.85$, $p < 0.01$), skin (independent *t*-test, $t = 25.60$, $p < 0.01$), muscle (independent *t*-test, $t = 15.53$, $p < 0.01$), liver (independent *t*-test, $t = 5.77$, $p < 0.01$) and gill (independent *t*-test, $t = 7.35$, $p < 0.01$) of the light phenotype were significantly lower than these in the dark phenotype. No significant difference of the expressions in brain (independent *t*-test, $t = 1.07$, $p = 0.31$) and fish blubber (independent *t*-test, $t = 0$, $p = 1.00$) was found between the light and the dark phenotypes of *L. taeniops* (*Fig. 3*).

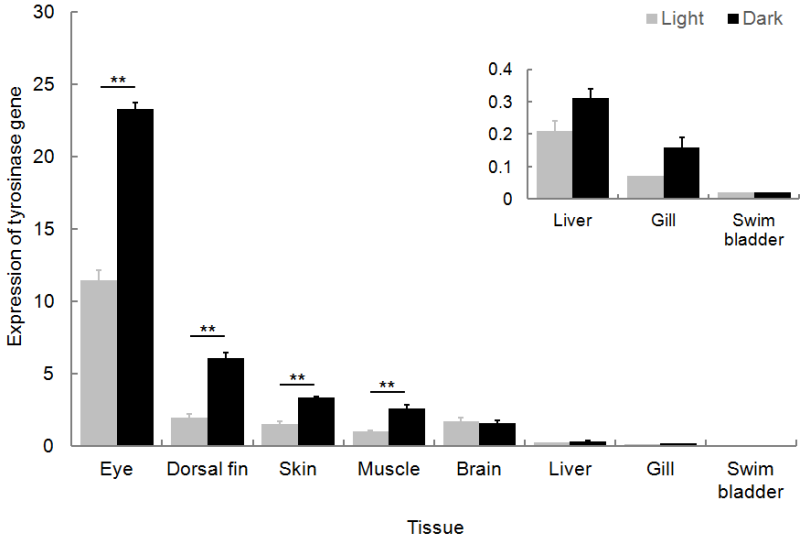

Figure 3. *Expressions of tyrosinase gene in eight tissues of Leptobotia taeniaps. The asterisks indicating differences between light and dark phenotypes. Independent t-test was used to compare the expressions of tyrosinase gene between the light and dark phenotypes of L. taeniaps.* **,* $p < 0.05$*;* ***,* $p < 0.01$

Discussion

Tyrosinase is a key enzyme to synthesize melanin. The expression of tyrosinase gene controls the speed and production of melanin. The higher expression of tyrosinase gene promotes the intracellular synthetization of melanin (Gutiérrez-Gil et al., 2007). The expressions of tyrosinase gene in the peoples with dark skin are higher than those in the peoples with pale skin (Huang et al., 2008). The expressions of tyrosinase gene in skin are differences among different varieties of *Cryprinus carpiod*, and the expression is the highest in dark *C. carpiod* (Wang et al., 2012). The expressions of tyrosinase gene in scale, skin and tail fin of dark *A. citrinellus* are significantly higher than those of bright yellow ones (Jiang, 2016). Our results showed that the expressions of tyrosinase gene in eyeball (independent *t*-test, $t = 34.88$, $p < 0.01$), dorsal fin (independent *t*-test, $t = 19.85$, $p < 0.01$), skin (independent *t*-test, $t = 25.60$, $p < 0.01$), muscle (independent *t*-test,

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$t = 15.53$, $p < 0.01$), liver (independent *t*-test, $t = 5.77$, $p < 0.01$) and gill (independent *t*-test, $t = 7.35$, $p < 0.01$) of the light phenotype were significantly lower than these in the dark phenotype. The result implied that the expressional differences of tyrosinase gene was the major reason that caused the differentiation of *L. taeniops* body color.

Expressional patterns of tyrosinase gene are different among fishes. The gene was expressed in eyeballs and skins of *D. rerio* (Chiu, 2003) and *O. latipes* (Zou et al., 2006), while it is not expressed in their livers. Our results showed that the expression of tyrosinase gene in eyeball of *L. taeniops* was the highest, followed by dorsal fin, skin, muscle and brain. The expressions of tyrosinase gene in gill, liver and fish blubber of *L. taeniops* were the lowest, coincident with the results in *C. auratus* (Chiu, 2003) and *C. carpiod* (Wang et al., 2012).

Melanin participates in many physiological processes of organisms. And tyrosinase is the essential enzyme to synthetize melanin. Therefore, the tyrosinase gene is expressed in various issues of *L. taeniops*. There are lots of melanocytes in retinal pigment epithelium (RPE). As an antioxidant, the melanin protects RPE and nerve cells from oxidative damage (Zhang et al., 2011). Therefore, tyrosinase gene was highly expressed in eyeball. Nerve cells produce melanin during their development, and brain is the tissue in which nerve cells are concentrated. Therefore, tyrosinase gene was also expressed in brain (Sulzer et al., 2000). In addition, the expressions of tyrosinase gene in heart, fish blubber and liver implied that the tyrosinase probably did not only participate in the synthetizing melanin, but also regulating other physiological activities (Wang et al., 2012).

Although melanin plays important roles in preventing damage to DNA and proteins from ultraviolet rays (Slominski et al., 2015), enhancing antioxidant capacity (Tu et al., 2009), maintaining intracellular calcium homeostasis (Parekh, 2016), and regulating immunization of organisms through NF-κB signaling pathway (Zhou et al., 2013), the reason caused the differentiation of *L. taeniops* body color are still needed to further study. Sex and development phase are two major factors that influence body color of fish (Bjerkeng et al., 2000; Suqimoto, 2002; Li et al., 2014). However, no significant difference in sex and development phase (it was indicated by the body length and the body weight) were detected between the light and the dark phenotypes in our study. Therefore, externally special stimulation was probably the reason that caused the expressional differences of tyrosinase gene between the light and dark phenotypes of *L. taeniops* and the differentiation of their body colors. It should be further studied which environmental factors regulate the expression of *L. taeniops* tyrosinase gene.

Our results showed the structure of *L. taeniops* tyrosinase was accordance with other vertebrates (Jagirdar et al., 2014; Ming et al., 2016; Yu et al., 2017). A signal peptide comprised 20 amino-acid residues (MRPSSISPLLFFIQYLGLSL) was in tyrosinase gene of *L. taeniops*, which was the requisite structure to lead the newly synthesized protein to enter the endoplasmic reticulum to complete its modification (Wang et al., 2005). It can influence expression of the protein (Güler-Gane et al., 2016), and control location of the protein (Wei et al., 2006). Compared with amphibians, reptiles, birds and mammals, the tyrosinase gene sequence of *L. taeniops* was highly similar to the tyrosinase gene sequences from other fishes, especially Cyprinidae fishes. There were 13 cysteines in the tyrosinase of *L. taeniops*. Nine of 13 cysteines were at N-terminal, and other 4 cysteines were in the middle between CuA and CuB, in accordance with *C. carpiod* (Wang et al., 2012). This result verified that the amino acid sequence of tyrosinase was highly conservative in fishes, as showed by the phylogenetic tree

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(*Figure 2*). The cysteine in protein promotes the active sites of tyrosinase to bind copper ion during its process (Bijelic et al., 2015; Lai et al., 2018). The EGF-like domain (CQCAGNYMGFDC) was rich in cysteines, which coded protein activating EGF signal pathway and was connected with the multienzyme complex that induces expression of the tyrosinase-related proteins (Jackson et al., 1990, 1992; Dunning et al., 2015). Tyrosinase was type III copper oxidase. Its tyrosinase domain included two domains of histidine residues (HESAAFLPWHRVYLLFWE and DPIFLLHHAFID) that bind to copper ion (Pretzler and Rompel, 2018). Binding by metal ion commonly changes the stability and function of protein. Copper ion endows the redox properties to tyrosinase (Solano, 2018), and plays important role in synthetizing melanin.

In conclustion, we firstly cloned and sequenced the whole-length cDNA sequence of *L. taeniops* tyrosinase gene, and compared the expressions of tyrosinase gene in different issues between the light and dark phenotypes of *L. taeniops*. Tyrosinase gene was mainly expressed in eyeball, skin, dorsal fin, muscle and brain of both the light and dark phenotypes of *L. taeniops*. The expressions of tyrosinase gene in eyeball, dorsal fin, skin and muscle of the light phenotype were significantly lower than these in the dark phenotype ($p < 0.01$). These results implied that the expression of tyrosinase gene played important role in regulating the synthesis of melanin and the body color of *L. taeniops*. Our results provided important reference information to further elaborate the molecular mechanism of *L. taeniops* to adapt to their surrounding environment through body color, protect wild *L. taeniops* resource, as well as cultivate new ornamental fish variety. However, how external and intrinsic factors influence the expression of tyrosinase gene and which genes participate in regulation of the expression of tyrosinase gene should be further studied in the future.

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APPENDICES

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1       ACATGGGGGTGTCTGATGTAAC**ATG**AGGCCTTCGTCCATCTCTCCGCTTCTCTTCTTCATTCAGTACCTGGGTCTCTC
1                                 M  R  P  S  S  I  S  P  L  L  F  F  I  Q  Y  L  G  L  S
79      CCTCCAACAGTTTCCTCGACCCTGCACCACTCCGGAGGTTCTCCAAAGCAAGCAGTGCTGCCCGGTCTGGCCGGGGGA
20       L  Q  Q  F  P  R  P  C. T  T  P  E  V  L  Q  S  K  Q  C. C. P  V  W  P  G  D
157     CGGCTCGGTGTGCGGGGTCCTTTCGGGTCGAGGGTTCTGCCGGGACGTCACGGTCTCGGACCTCCCCAACGGGCCGCA
46       G  S  V  C. G  V  L  S  G  R  G  F  C. R  D  V  T  V  S  D  L  P  N  G  P  Q
235     GTACCCTCACTCCGGCGTGGATGATCGAGAGCGGTGGCCTCTGGTGTTCTACAACCGAACCTGCCAATGCGCCGGTAA
72       Y  P  H  S  G  V  D  D  R  E  R  W  P  L  V  F  Y  N  R  T  C. Q  C. A  G  N
313     CTACATGGGGTTCGACTGCGGTGAGTGTAAGTTCGGCTACTTCGGGCCCAACTGCGGGGAGAGGCGAGAATCCGTGCG
98       Y  M  G  F  D  C. G  E  C. K  F  G  Y  F  G  P  N  C. G  E  R  R  E  S  V  R
391     CAGGGACATCTTCCAGCTGTCGGTGGCGGAGAGACAGCGGTTTGTCTCGTACCTGAATCTCGCCAAGACCACCACCAG
124      R  D  I  F  Q  L  S  V  A  E  R  Q  R  F  V  S  Y  L  N  L  A  K  T  T  T  S
469     CCCCGGTTACATGATCGTGACGGGCACGTACGCGCAGATGAACGACGGCGCGACCCCCATGTTCGCCAACGTCAGCGT
150      P  G  Y  M  I  V  T  G  T  Y  A  Q  M  N  D  G  A  T  P  M  F  A  N  V  S  V
547     GTATGACCTGTTCGTGTGGATGCACTACTACGTGTCCCGTGACGCGCTGCTGGGGGGCCCCGGGAACGTGTGGGCCGA
176      Y  D  L  F  V  W  M  H  Y  Y  V  S  R  D  A  L  L  G  G  P  G  N  V  W  A  D
625     CATCGACTTTGCGCACGAGTCGGCGGCCTTTCTGCCCTGGCACCGCGTGTATCTGTTGTTCTGGGAGCATGAGATCCG
202      I  D  F  A  H  E  S  A  A  F  L  P  W  H  R  V  Y  L  L  F  W  E  H  E  I  R
703     GAAGCTGACCGGTGACTTTAACTTCACCATCCCGTACTGGGACTGGCGCGACGCGGCGGACTGTCAGGTGTGCACGGA
228      K  L  T  G  D  F  N  F  T  I  P  Y  W  D  W  R  D  A  A  D  C. Q  V  C. T  D
781     TGAGCTGATGGGGGCGCGCAGCCCACTGAACCCAAACCTCATCAGCCCGTCCTCGGTGTTCTCCTCCTGGAAGGTGGT
254      E  L  M  G  A  R  S  P  L  N  P  N  L  I  S  P  S  S  V  F  S  S  W  K  V  V
859     GTGTTCCCAACCCGAAGACTACAACAATCGGGAGGCTCTGTGTGACGGGTCTCCTGAGGGCCCGTTACTCCGTAACCC
280      C. S  Q  P  E  D  Y  N  N  R  E  A  L  C. D  G  S  P  E  G  P  L  L  R  N  P
937     TGGTAACCACGACCGGACCCGTGTCAGGCGGCTGCCCACCTCTGCAGACGTGGAATCTGTCCTGAGCCTCACAGAGTA
306      G  N  H  D  R  T  R  V  R  R  L  P  T  S  A  D  V  E  S  V  L  S  L  T  E  Y
1015    CGAGACGGGGTCCATGGACCGGCGGGCCAACATGAGCTTCCGCAACGCTCTGGAAGGTTTTGCGAGTCCAGAAACGGG
332      E  T  G  S  M  D  R  R  A  N  M  S  F  R  N  A  L  E  G  F  A  S  P  E  T  G
1093    ATTGGCAATAACGGGTCAGAGTCTGATGCACAACTCCTTACACGTCTTCATGAATGGATCCATGTCCTCAGTACAGGG
358      L  A  I  T  G  Q  S  L  M  H  N  S  L  H  V  F  M  N  G  S  M  S  S  V  Q  G
1171    CTCGGCCAACGACCCCATTTTCCTTCTGCATCACGCCTTTATTGACAGCATCTTTGAGCAGTGGCTACGGAGACACCA
384      S  A  N  D  P  I  F  L  L  H  H  A  F  I  D  S  I  F  E  Q  W  L  R  R  H  Q
1249    GCCTCCCCGCACACATTACCCGACAGCCAACGCTCCGATCGGACACAACGACGGCTATTTCATGGTTCCCTTCATCCC
410      P  P  R  T  H  Y  P  T  A  N  A  P  I  G  H  N  D  G  Y  F  M  V  P  F  I  P
1327    TCTGTACAGAAACGGAGATTATTTCCTGTCCACCAAAGCTCTGGGATATGAATATGCCTATTTGCTGGACCCCAGCCA
436      L  Y  R  N  G  D  Y  F  L  S  T  K  A  L  G  Y  E  Y  A  Y  L  L  D  P  S  Q
1405    GCGGTTCGTGCAGGAGTTTTTGACGCCGTATCTAGAGCAAGCTCAGCAGATCTGGCGTTGGCTGCTGGCCGCGGGAAT
462      R  F  V  Q  E  F  L  T  P  Y  L  E  Q  A  Q  Q  I  W  R  W  L  L  A  A  G  I
1483    CTTGGGTGCGCTCGTGGCGGGAATCGTCGCAGGGATAATCGCCGTGGCGTGTCGCAGAAGGCAAAAGCGCAGGAAGAT
488      L  G  A  L  V  A  G  I  V  A  G  I  I  A  V  A  C  R  R  R  Q  K  R  R  K  M
1561    GTCGGCATACGGGGAGAGACAGCCGCTCCTGAACAGCAGTGAAGAGGAGGGCTCAACTTCATATCAGACAACGCTG**TG**
514      S  A  Y  G  E  R  Q  P  L  L  N  S  S  E  E  E  G  S  T  S  Y  Q  T  T  L  *
1639    **A**ATCAAAACACACACACACACACACACTACAGGCACGTGTCAGCGAGGACAGATACACTGTACCAACGGGAAAAGACA
1717    CAACAGCCAGTTAACATGTGACAAATGAAGCAAGGCCTCACTCCTGAGCTCAGGTCACGTGATGAGGCCTCGCACCCC
1795    TGTTCCAGTCATGAGACCAGGGCTGTTCGGAACAGAATACCACCATATTAATCTCACCCTATGCAGTATAGAGGGGTT
1873    TTCACCACTTATTATTATATATCTTTTTTATAAAAACTTTGGATTATAATTGCAAAATAACAAAATAGTTGTTTACGA
1951    TGATAATGGGCTGAATTAAATGCAGGTCATCAATTACAGATTGAAACATGAAACACGCATCGGGACGGTTTCCCGGTC
2029    AGGGCTTAAGTTTAGTCCCAGACTGAAATGCATGTTTGAGGTCTTAAATGAAAAGAGCTTCACTGACGTATCTTAACA
2107    TACATCAGTGCCATTGATCTGTCTTGAGATGCACATCAGTCATGTTTTTGGTGAGGTATGTTTGTATAAAACTACATT
2185    TACTGATTGAATTAAGACTTCGTCCTTGTTTAACCTGAGGCCTGTCTGGGAAACCGCCCCATTGTGTTTTGTTTCACT
2263    TACTACTTAAAATAGTCCCCAGTTTTTATTACAAAGTACCCAGTGGTTAGTATTCCGTTCCCAACAGGGTGTCGCCCT
2341    GAATTCAGCCAACCAGTGATAATAATGGTGCTTATGTCGCCAATGTGGCACATGCGTACTGTATGTTAGGGCATAAAA
2419    CATATCCAAATGAAATATTAAACGGATGTTTTTAAGAGGAATGAAGTGTGAAAACGAGATGATATGAAATTTTTGCCA
2497    GTGTTTATAAAATAACATTCCAGTCCATGTTGACATTTACATGGCCAACAATGTTAATGCTTTATCTTTGTTCATAAC
2575    CCAATAGGAATAATAATACAATACGTTCAAA**AATAA**AACAAACCCTCAACAAAAAAAAAAAAAAAAAAA
```

Appendix 1. *Nucleotide sequence and deduced amino acid sequence of the tyrosinase cDNA. The start codon (ATG), the stop codon (TGA) and poly(A) signals(AATAAA) were marked with black bold font, The putative amino acid sequence of Signal Peptides domain (1-21 AA), EGF - like structure domain (AA), 68-115, Tyrosinase domain (173-407 AA) and Transmenbrance region (481-503 AA) were underlined, CuA domain (206-223 AA) and CuB domain (387-398 AA) were shaded; Black dots represent Cys*

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Appendix 2. *The structural features of the Leptobotia taeniaps tyrosinase predicted by SMART*

Appendix 3. *Differences of tyrosinase among different species*

Species	Amount of amino acid residues	Molecular weight	Theoretical pI
Homo sapiens	529	60.39 kD	5.71
Mus musculus	533	60.60 kD	5.67
Equus caballus	534	61.00 kD	6.07
Danio rerio	535	60.73 kD	6.07
Oryzias latipes	540	61.25 kD	5.99
Cyprinus carpio	535	60.63 kD	6.07
Carassius auratus	535	60.83 kD	5.71
Leptobotia taeniaps	538	60.81 kD	6.13

Appendix 4. *The hydrophobicity prediction atlas of Leptobotia taeniaps tyrosinase. The horizontal coordinate is the amino acid position, and the ordinate is the scale value of the hydrophobicity (Hphob. / Kyte & Doolittle scale > 0 values indicates hydrophobicity, <0 values indicates hydrophilicity)*

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