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Involvement of the *mitfa* gene in the development of pigment cell in Japanese ornamental (Koi) carp (*Cyprinus carpio* L.)

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ABSTRACT. A colored phenotype is an important feature of ornamental fish. In mammals, microphthalmia-associated transcription factor (MITF) was found to regulate the development of melanocytes. In this study, the *mitfa* cDNA was first cloned from the Japanese ornamental (Koi) carp (*Cyprinus carpio* L.), an important ornamental freshwater fish. The full-length cDNA of the *mitfa* gene contains 1634 bp, coding for 412 amino acids in Koi. The identity degree of *mitfa* amino acid sequences between the Koi carp and zebrafish is 92.9%. We tested the expression of the *mitfa* gene in several varieties of Koi using reverse transcription-polymerase chain reaction and found that the *mitfa* gene is highly expressed in the skin tissues of the *Taisho sanke* and the *Procypris merus*. Interestingly, the *mitfa* gene was also expressed in the *Kohaku* and *Yamabaki ogon*, although melanocytes were not observed in the skin. Koi carp embryos were transparent and colorless, while

after hatching, different types of pigment cells successively emerged in a fixed order. In *Taisho sanke*, melanocytes first appeared in the trunk at approximately 12 days of age. Subsequently, there was a large area of melanocytes by 30 days of age. The expression level of the *mitfa* mRNA was low in early embryos and newly hatched larvae, and increased to high levels in 30-day-old fry. The results show that the *mitfa* gene is involved in regulating fish body color in the development of both melanocytes and pigment cells.

Key words: Japanese ornamental (Koi) carp; Melanocyte; *mitf* gene; Pigment cell

INTRODUCTION

Color patterns are prominent features of many animals and play important roles in camouflage, shoaling, mate choice, the perception of threatening behavior, or the protection against UV irradiation (Protas and Patel, 2008; Howe et al., 2013; Lin et al., 2013). Pigment phenotypes in fish are more complicated than in amniota. There are 4 types of pigment cells in fish, including melanocytes, erythrocytes, xanthophores, and iridocytes (Kelsh, 2004). Various factors are thought to be involved in regulating these cells, such as Fms, Pax3, Sox10, and Wnt (Parichy and Turner, 2003; Lang and Epstein, 2003; Minchin and Hughes, 2008; Koludrovic and Davidson, 2013). The microphthalmia-associated transcription factor (MITF) is a member of the basic helix-loop-helix leucine zipper (bHLH-Zip) protein family (Hodgkinson et al., 1993). The *mitf* gene is expressed in most cell types, and it plays major roles in neural crest-derived and neuroepithelium-derived pigment cells. In mammals, the *mitf* gene direction regulates the differentiation of melanin cell by controlling the expression of tyrosine gene families (Odenthal et al., 1996; Levy et al., 2006). Mutations in the *mitf* gene cause pigmentation diseases, such as deafness, dystopia canthorum, or melanomas (Tassabehji et al., 1994; Smith et al., 2000; Levy et al., 2010; Yokoyama, et al., 2011).

In humans, the *mitf* gene is located on the chromosome $3P^{14.1}$-$3P^{12.3}$ (Garraway et al., 2005). Because of the different promoters used, diverse transcriptional regulation occurs for the *mitf* gene, resulting in the production of multiple MITF isoforms from alternative transcription or splicing (Tachibana et al., 1996; Amae et al., 1998; Fuse et al., 1999). The *mitf* gene in human produces 18 transcripts, 14 of which can be translated into proteins; there are 9 named subtypes and additional unnamed subtypes. MitfA, MitfD, and MitfH are necessary for retinal pigment epithelium development, while MitfM plays a critical role in the development of melanocytes in humans (Oboki et al., 2002; Li et al., 2013; Koludrovic and Davidson, 2013). The *mitfa* and *mitfb* genes have also been reported, which are located on chromosomes 6 and 23 in zebrafish. Additionally, the *mitfa* gene is closely related to the development of melanocytes, while the *mitfb* gene is involved in the retinal pigment epithelium development in zebrafish (Curran et al., 2010; Li et al., 2014).

The Koi (*Cyprinus carpio*) carp is a variety of common carp and an important ornamental fish in Asia. In this study, we cloned the *mitfa* cDNA from Koi carp. We analyzed the expression of the *mitfa* gene in varieties of Koi carp with different body colors and at different stages of body color development. Our results are useful for determining the molecular mechanism of pigment development in the ornamental fish body.

MATERIAL AND METHODS

Materials

Koi carp (6 months old) were obtained from the Hunan YuYuan biological Technology Co., Ltd. in Changsha, Hunan, China. The fertilized eggs of the Koi carp were raised and hatched in the laboratory.

The tissues of skin, scales, or fins of the Koi carp were quickly dissected and then soaked in phosphate buffer solution for directly observation under a microscope. The skin or embryos of Koi carp were collected and frozen in liquid nitrogen for total RNA extraction.

Molecular cloning of full-length *mitfa* gene

The full-length *mitfa* cDNA was cloned from Koi carp according to the *mitfa* cDNA sequence of zebrafish (GenBank accession No. NM130923). Total RNA was extracted from the skin of Koi carp (*Procypris merus*). Reverse transcription-polymerase chain reaction (RT-PCR) was conducted using 1 μL 10 μM mitfa1$^{+/-}$ primers (Table 1). PCR was conducted in a total volume of 20 μL and run for 35 cycles. The rapid amplification of cDNA 3'-ends (RACE) was performed using Clontech Universal Primer A Mix solution (Mountain View, CA, USA) (longer primer: 0.4 μM 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3', shorter primer: 2 μM 5'-CTAATACGACTCACTATAGGGC-3'), and mitfa3-out primer (Table 1), for 30 cycles. PCR products were re-amplified using 1 μL 10 μM mitfa3-in primer (Table 1) and Nested Universal Primer A (5'-AAGCAGTGGTATCAACGCAGAGT-3'). 5'-RACE was performed using the 5-RACE kit purchased from Clontech Laboratories, Inc. The PCR procedures were the same as those described above, using mitfa5$^{+/-}$ primers (Table 1) and Universal Primer A Mix solution. Amplified products were gel-purified and cloned into the PMD18-T vector (Takara, Shiga, Japan) for sequencing (Xiao et al., 2014).

Table 1. Oligo primers used for *mitfa* cDNA cloning or RT-PCR analysis from the Koi carp.

Primer name	Primer sequences (5'-3')
β-actin+	CCGTGACCTGACTACCCTC
β-actin-	ATACCGCAAGATTCCATACCC
mitfa-1+	ACAACTCCTGCCCGTCTAAC
mitfa-1-	CCGTTGTTGAGGTCCAGAGT
mitfa+	CTACAGTGATGACATTCTTGGGTTC
mitfa-	CCTTGTTTGGGCTGTCGTAG
mitfa3-out	GAAACTCCAGAAAGAGCAGCAA
mitfa3-in	AGCCTCACCCAGCCTTTATT
mitfa5-out	CTTTTACCAAGGCTCTGACCTCTGC
mitfa5-in	CGGTAGGTTAGACGGGCAGGAGTTGTT
NUP	AAGCAGTGGTATCAACGCAGAGT
3'-primer	AAGCAGTGGTATCAACGCAGAGTACT$_{16}$VN
UPM$_{(short)}$	CTAATACGACTCACTATAGGGC
UPM$_{(long)}$	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT

RT-PCR

Reverse transcription was conducted in a total reaction volume of 20 μL, which in-

cluded 2 μg total RNA and 2 μL RT reaction mixture. For PCR amplification, both specific primers and β-actin primers were added into the same reaction at the beginning of PCR and each PCR run for 30 cycles. The PCR products were separated by agarose gel electrophoresis and photographed under UV illumination. We list the primers used in the experiments in Table 1. For each batch, the experiment was repeated more than 3 times.

RESULTS

Molecular cloning and sequence analysis of the *mitfa* gene from the Koi carp

The full-length *mitfa* cDNA was cloned from the Koi carp (GenBank accession No. KC565527). The *mitfa* cDNA gene from the Koi carp was 1634 bp long and contained an open reading frame of 1236 bp, coding for 412 amino acids (Figure 1).

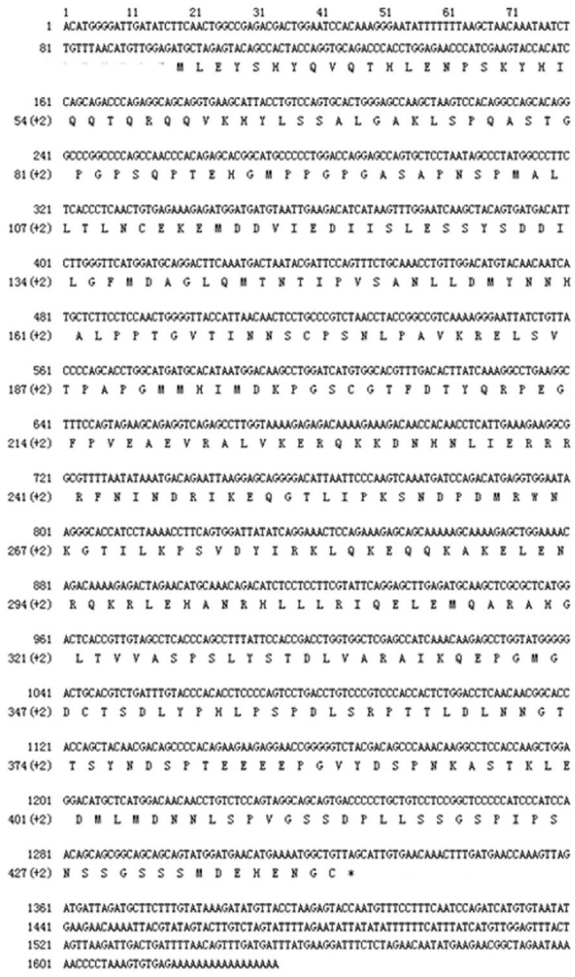

Figure 1. Multiple nucleotide sequence and amino acid sequence alignment of *mitfa* in Koi carp.

We compared the cDNA sequences of *mitfa* from Koi carp and 5 other fish, including *Amphilophus citrinellus* (GenBank accession No. AY206404), *Astatotilapia burtoni* (GenBank accession No. AY206400), *Maylandia zebra* (GenBank accession No. AY196318), zebrafish (GenBank accession No. NM130923), and *Paralichthys olivaceu* (GenBank accession No. AB457038) (Figure 2). This analysis revealed a high level of sequence similarity in the *mitfa* gene among these fish. The *mitfa* nucleotide and amino acid sequences from the Koi carp and zebrafish were 84 and 92.9% identical, respectively.

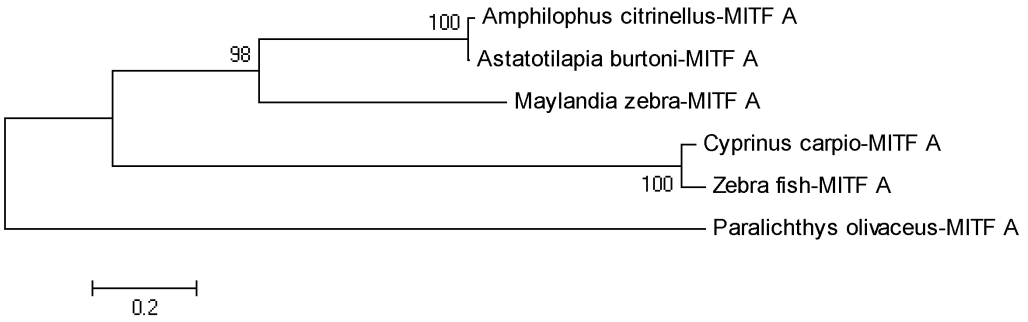

Figure 2. Phylogenetic tree of the *mitfa* gene in the 6 species (*Amphilophus citrinellus*; *Astatotilapia burtoni*; *Maylandia zebra*; *Cyprinus carpio*; zebrafish; *Paralichthys olivaceus*) from NCBI. This phylogenetic tree was generated by comparative analysis of the *mitfa* coding sequences and using unweighted pair group method with arithmetic mean calculation and the MEGA5 software.

Expression of the *mitfa* gene in skin of the Koi carp

The body color of *P. merus* was black or has white spots (Figure 3A). There were 3 types of the pigment cells present, including melanophores, erythrophores, and xanthophores, in the skin tissue, scales, or fins of *P. merus* (Figure 3B-D). The body color of *Taisho sanke* was white with red and black spots (Figure 3E) and contained melanophores, erythropores, and lipophores in the skin, scales, and fins (Figure 3F-H). *Kohaku* showed some red spots on their white bodies (Figure 3I), consisting of erythropores and xanthophores, while melanophores were not observed (Figure 3J-L). The whole body color *Yamabaki ogon* was yellow (Figure 3M), with an abundance of erythropores and xanthophores in the skin, scales, and fins (Figure 3N-P). Iridocytes, 1 type of pigment cells, was observed in the skin and scales of the Koi carp.

We conducted RT-PCR to explore whether the *mitfa* gene is differentially expressed in different varieties of Koi carp. The result (Figure 4) showed that higher levels of the *mitfa* mRNA are expressed in the skin of *P. merus* and *T. sanke*. Additionally, *mitfa* was expressed in the skin of *Kohaku* and *Y. ogon*, although *mitfa* mRNA levels were lower than in *P. merus* and *T. sanke*.

Expression of the *mitfa* gene during body color development in Koi carp

T. sanke, which has 4 types of pigment cells, was examined for body color change during development. The embryos of *T. sanke* were transparent, and no pigment cells were observed in its body except in the eyes. Xanthophores were observed on the first day after hatching. The number of xanthophores increased and gradually appeared from the head to tail in the fry of *T. sanke*. Iridocytes emerged on the belly after 7 days. Melanophores were first

observed after 12 days, and their number had increased by 24 days, when they were widely distributed throughout the body (Figure 5).

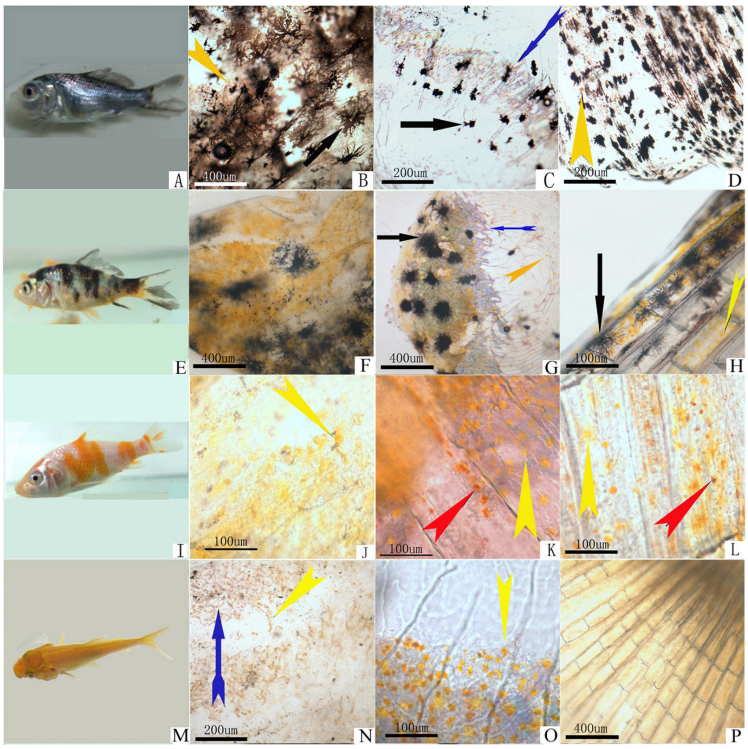

Figure 3. Four varieties of Koi carp and microscopic observation of their pigments. **A.-D.** *Procypris merus*. **E.-H.** *Taisho sanke*. **I.-L.** *Kohaku*. **M.-P.** *Yamabaki ogon*. The photos from the second to the fourth columns are derived from the tissues of the skin, scale, and fin, respectively. Simple arrow indicates the melanocyte; gray arrowhead indicates the xathophore; black arrowhead indicates the erythrophore; bifurcated tail end arrow indicates the iridocyte.

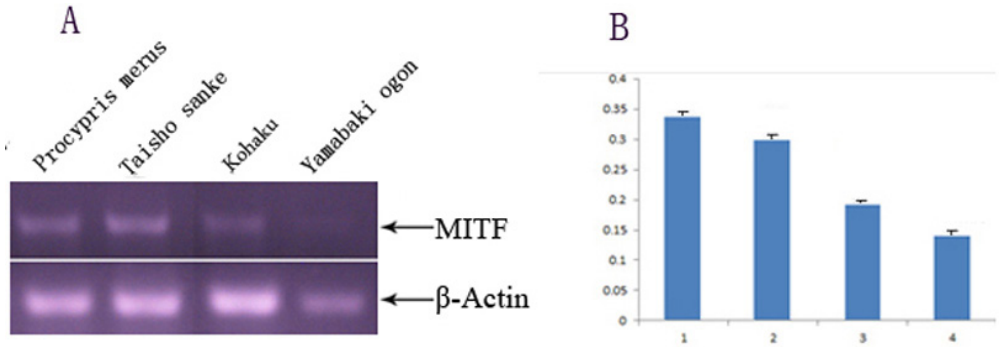

Figure 4. RT-PCR analysis revealed levels of the *mitfa* mRNA among of Koi carp, including *Procypris meru*, *Taisho sanke*, *Kohuku*, and *Yamabaki ogon* (**A**). The relative level of expression was calculated by dividing the total pixel from each *mitfa* mRNA band with the total pixel from the corresponding β-actin mRNA band. Column 1 indicates *P. merus*; 2 indicates *T. sanke*; 3 indicates *Kohaku*; 4 indicates *Y. ogon* (**B**). Quantitative results are from 3 independent experiments.

Figure 5. Early development of body pigment in *Taisho sanke*. **A.** 1 dph. **B.** and **C.** 3 dph. **D.** 7 dph. **E.** 10 dph. **F.** 14 dph. **G.** 16 dph. **H.** 18 dph. **I.** 24 dph. Gray arrowhead indicates the xathophore; black arrowhead indicates the erythrophore; bifurcated tail end arrow indicates the iridocyte, simple arrow shows the melanocyte. dph = days post-hatching.

Total RNA was extracted from the blastula, 1-day-old larvae, and 30-day-old fry of *T. sanke*. RT-PCR analysis showed that the level of the *mitfa* mRNA was lower at the blastula stage and in 1-day-old larvae of *T. sanke*, with high expression of the *mitfa* mRNA in 30-day-old *T. sanke.*

DISCUSSION

In mammals, MITF consists of 413-520 amino acids and is a member of the basic helix-loop-helix leucine zipper (bHLH-Zip) protein family (Pogenberg et al., 2012). In this study, we cloned the *mitfa* cDNA from Koi carp, which shows high homology in the nucleotide sequences of Koi carp and zebrafish. Based on our analysis of the amino acid sequence of MITFa in Koi carp using Smartprotein, we found 3 domains: the amino acids from 61-76 constitute the low abundance phenotype, the bHLH-Zip is formed by amino acids 205-258, and 285-409 constitutes an domain (DUF3371), which contains a set of bHLH transcription factors, the members in the family of MIT/TFE (MITF transcription factor). Analysis with EXPASY revealed that amino acids 199-252 form Myc bHLH-Zip in Koi carp. Compared with mammals, there are 2 amino acid mutations at sites of 222 and 244 in the bHLH of MITFa in zebrafish and Koi carp.

MITF is a biomarker of differentiation from neural crest cells to melanin in mammals (Goding, 2000). It can regulate the expression of tyrosinase, which is an enzyme important in melanin synthesis (Hodgkinson et al., 1993; Lister et al., 2001). The zebrafish MITFb is mainly expressed in retinal pigment epithelium, and its ectopic expression can rescue the pigment phenotype of the *mitfa* mutant (Lister et al., 1999). Therefore, in zebrafish, the 2 *mitf* genes have undergone subfunctionalization to provide complementary expression and functions in pigmentation (Lister et al., 2001; Altschmied et al., 2002). In this study, we examined whether in Koi carp with different body colors, or in the ontogenesis of Koi carp, expression of the *mitfa* gene was closely associated with melanin abundance in the cell. Notably, expression of the *mitfa* gene was also detected in Koi carp without melanocytes in the skin, including *Kohaku*, *Y. ogon*, and Koi embryos or hatched larvae.

Pigment cells are derived from the embryonic neural crest (Hultman and Johnson, 2010). Skin pigmentation is a complex process involving a series of cellular, genetic, and physiological factors (Colihueque, 2010). In response to external stimuli, multiple signals are generated by non-melanocytic cells in the epidermis, which communicate a pigmentation response to the basal melanocyte population. Alpha-melanocyte-stimulating hormone is one such peptide hormone that has been suggested to modulate the pigment response (Thody, 1999). Although cAMP triggers numerous downstream effects, an important target is the MITF gene, which is transcriptionally upregulated by cAMP signaling in a melanocyte-restricted fashion, thus linking extracellular signals to MITF expression and the transcriptional regulation of pigmentation (Park et al., 2006). Warrdenburg syndrome is caused by *mitf* gene mutations in human, which can result in skin pigment loss and the production of abnormal ididocytes (Pingault et al., 2010). Both melanocytes and the iris are thought to be derived from the same precursor cells. In the zebrafish containing the *mitf* gene mutant, the iris was found to be overexpressed, and the black patches disappeared (Rawls et al., 2001), whereas a reduction in melanophore number and stripe formation was caused by the absence of iridophores (Howe et al., 2013). Pnp4a is considered a marker of iridoblasts and co-regulates the differentiation and development of melanin cell lineage and iridescent cell lineage together with MITF and foxd3 (Curran et al., 2010). Curran et al. (2010) suggested that melanoblasts and iridoblasts originate from the same precursor cells. However, Tu and Johnson (2010) found that melanocytes and xanthophores originate from the same precursor stem cell, while iridocytes were produced by other cells in zebrafish. We found that the *mitfa* gene was involved in the regulation of fish body color, not only in melanocytes, but also in other pigment cells. Skin color determination is a very complex process in fish associated with a series of cellular, genetic, environmental, and physiological factors (Aspengren et al., 2009). Additional studies are needed to determine the strategy of pigment cell development in fish.

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