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FUNDAMENTAL STUDIES

Deficient melanin production contributes to the absence of melanophores in early development of red carp

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Red carp and red crucian carp are ornamental fish with a red body color. Unlike in red crucian carp, no melanophores are observed in red carp embryos or larvae. To explore the roles of the *mitfa* gene in body color formation in red carp, we investigated the structural characteristics and physicochemical properties of the *mitfa* gene in 16 kinds of fish. The *mitfa* amino acid sequence similarity between red carp and red crucian carp was 95.6%, and this was 91.5% similar between carp and zebrafish. Compared with red crucian carp, red carp showed lower *tyrp1* messenger ribonucleic acid (mRNA) expression but similar *mitfa* mRNA expression in the body pigment stage of the embryo. Moreover, *mitfa*$^{+}$ cells as well as melanocytes could be observed in cultured embryo cells derived from red carp and red crucian carp. Our data show that the absence of melanophores in red carp is not the result of *mitfa* gene deletion or mutation, increasing our understanding of the molecular and genetic mechanisms of coloration in cyprinid fish.

KEYWORDS

body color, melanophore, *mitfa*, red carp

1 | INTRODUCTION

A wide variety of fishes are rich in coloration, and some have become popular ornamental species for this reason. Crucian carp and carp, freshwater fishes that are widely bred in Asia, include ornamental varieties such as goldfish and koi carp produced by targeted cultivation over a long time period. In carp, a greater percentage of black color in parents is reported to reduce the percentage of red color in offspring. Conversely, in crucian carps, the percentage of black color in parents has less of an effect on red color in offspring (Liu et al., 2016). These results indicate that the genetic control of red phenotype differs between carps and crucian carps.

In red crucian carp, a monochromatic fish, melanocytes produce a gray body color in the embryo and larva, and as the melanocytes disappear, the body color changes from gray to red (Zhang et al., 2017). According to

transcriptome data, tyrosine metabolism pathways regulated by *mitfa* are involved in the downregulation of melanogenesis, which causes the gray-to-red/orange color transformation in red crucian carp (Zhang et al., 2017). Microphthalmia-associated transcription factor (MITF) plays an important regulatory role in the migration and survival of melanocytes. As a master regulator, *mitfa* genes have been carefully characterized at deoxyribonucleic acid (DNA) and protein levels across species, especially in mammals. Research on the *mitfa* gene in fish has been limited mainly to zebrafish, a significant model organism for the study pigment cell biology, especially in the fields of melanoma. It was reported that there are two subtypes of *mitf* genes in fish, *mitfa* and *mitfb* (Braasch, Brunet, Volff, & Manfred, 2009). In zebrafish, it has been shown that *mitfa* is closely related to the development of melanocytes, and *mitfb* is involved in retinal pigment epithelium development (Curran et al., 2010; Li, Zhu, Hong, Zhang, & Hong, 2014). As one of the earliest expressed genes, *mitfa* is a master regulation factor in the melanocyte lineage (Levy, Khaled, & Fisher, 2006; Lister, Close, & Raible, 2001; Lister, Robertson, Lepage, Johnson, & Raible, 1999; Steingrímsson et al., 2002; Zeng, Johnson, Lister, & Patton, 2014). In mammals, *mitfa* regulates the differentiation of melanin cells by controlling the expression of tyrosine gene families (Levy et al., 2006; Odenthal, Rossnagel, Haffter, Kelsh, & Vogelsang, 1996). Mutations of the *mitfa* gene cause pigmentation diseases such as deafness, dystopia canthorum, and melanomas (Levy, Khaled, Robinson, Vequilla, & Chen, 2010; Smith, Kelley, Kenyon, & Hoover, 2000; Tassabehji, Newton, & Read, 1994; Yokoyama, Woods, Boyle, Aoude, & Macgregor, 2011). The *mitfa* gene directly stimulates the expression of many genes encoding the effectors of melanin synthesis, including dopachrome tautomerase (*dct*), tyrosinase (*tyr*), and tyrosinase-related protein 1 (*tyrp1*) (Kevin et al., 2010; Lin et al., 2013) and controls the growth of melanocytes by regulating cell cycle regulatory factors such as T-box transcription factor 2, cyclin-dependent kinase 2, biomineralization protein spp16 (P16), and cyclin-dependent kinase inhibitor 1 A (Kelsh, 2004; Curran et al., 2010).

To further elucidate the role of the *mitfa* gene in body color formation in cyprinid fishes, we investigated the structural characteristics and physicochemical properties of the *mitfa* gene in 16 kinds of fish. We then measured *mitfa* mRNA expression in the embryos of the monochromatic red carp, a fish that has no melanocytes in their skin. The results will provide a better understanding of the molecular and genetic mechanisms of color formation in cyprinid fish.

2 | MATERIALS AND METHODS

2.1 | Materials

Red crucian carp (*Carassius auratus red var.*) and red carp (*C. carpio red var.*), were sourced from the Engineering Center for Fish Breeding of the National Education Ministry, Hunan Normal University, Changsha, China. Artificial breeding of fish was carried out in April, which is the natural reproductive season for carp in Southern China. Hybrids were derived by crossing red crucian carp with red carp. Embryos were raised in a laboratory at 23–24°C, and larvae were cultured in a separate cement pool. All sampling procedures were conducted according to the standards and ethical guidelines established by the Animal Ethical Review Committee of Hunan Normal University.

2.2 | Molecular cloning of full-length *mitfa* genes

Total RNA was extracted from the skin of red crucian carps. Reverse transcription polymerase chain reaction (RT-PCR) was conducted using *mitfa-mid*$^{+/-}$ primers (Table 1) for 35 cycles. Rapid amplification of cDNA 3′-ends (RACE) was performed using the Clontech Universal Primer A Mix solution (Mountain View, CA) and *mitfa* 3-out primers (Table 1) for 30 cycles. Polymerase chain reaction (PCR) products were reamplified using *mitfa* 3-in primers (Table 1) and Nested Universal Primer A (5′-AAGCAGTGGTATCAACGCAGAGT-3′); 5′-RACE was performed using a 5-RACE kit purchased from Clontech Laboratories, Inc (Mountain View, CA). PCR procedures were the same as those described above, using *mitfa* 5-out/-in 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.

TABLE 1 Primers used for cloning mitfa gene sequences

Primer names	Primer sequences
mitfa-mid+	5′-TTGTAGCCTCTCCCAGCCT-3′
mitfa-mid−	5′-CATGTTCATCCATACTGCTGCT-3′
3′-out	5′-GCAGAGGTCAGAGCCTTGGT-3′
3′-in	5′-TTCCTCCGACCTGGTTGCT-3′
5′-out	5′-CTGACCTCTGCTTCTACTGG-3′
5′-in	5′-TGCTTCACCTGCTGCCTC-3′
UPM	5′-CTAATACGACTCACTATAGGGCAAGCAGCAGTGGTATCAACGCAGAGT-3′
NUP	5′-AAGCAGTGGTATCAACGCAGAGT-3′

UPM: Universal Primer A Mix; NUP: Nested Universal Primer A.

2.3 | RT-PCR

Total RNA was extracted from somatic embryos, adult skin, and somatic embryo cells. Primers used for RT-PCR are listed in Table 2, and the reaction was performed for 30 cycles. The resultant PCR products were separated by agarose gel (1.5%) electrophoresis. For each sample, RT-PCR analysis was performed on three biological replicates.

2.4 | Cell culture

For primary cell culture in vitro, somatic embryo cells collected from red carp, red crucian carp, and their hybrids were sterilized in 75% ethanol for 30 s. The chorion and yolk were then removed, and the embryos were washed with phosphate-buffered saline. After scattering and placing in a dry dish for 15–30 min, embryo pieces were cultured in a complete growth medium composed of Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO), supplemented with 7.5% fetal bovine serum, 2.5% fish serum, 1% nonessential amino acids (Gibco, Langley, OK), 1 mM glutamine (Gibco), and 10 ng/mL bFGF (Peprotech, Rocky Hill, NJ). Cells were subcultured at 1:2 split ratios with 0.05% trypsin–ethylene diamine

TABLE 2 Primer sequences used for reverse transcription polymerase chain reaction analysis

Names of oligo primers	Sequences
mitfa-F1	AACAACTCCTGCCCGTCTA
mitfa-R1	AAGGCTCTGACCTCTGCTTCTAC
mitfa-F2	ACAACTCCTGCTCATCTAACCT
mitfa-R2	GCTCTGACCTCTGCTTCTACT
β-actin-F	CCATTGAGCACGGCATCATC
β-actin-R	TCGGTGAGCAGGGTTGGG
dct-F	GCCGACTAAGTTTTTCAATC
dct-R	GTCGTAGATGGAGATGTT
tyrp1-F	CGTTTGCCCTGCCATACTG
tyrp1-R	GGGTTTCTTCTGATCGGA
tyr-F	AGATGGACCGACGGGCAAA
tyr-R	CCGATGCGATTATTCCTGCTA
foxd3-F	AGCCGCCCTACTCCTACAT
foxd3-R	GGGTCCAGTAGTTGCCTT
fms-F	AACAAGCCACGGCAGAT
fms-R	TGGGGTCAAAGGTCACG
ltk-F	TCCCAGAACCCTTAGCG
ltk-R	TCCTGCGGCTCCATAAG

tetraacetie acid (EDTA) solution (Gibco) 4 days later. Thereafter, cells were passaged at 90% confluence, and the culture medium was changed every 2 days.

2.5 | Plasmid and transfection

In the pDestTol2-*mitfa*: green fluorescent protein (gfp) construct, the *gfp* sequence is driven by a 2.1 kb zebrafish mitfa promoter. Cells were transfected with pDestTol2-*mitfa: gfp* and pCS2FA-transposase at a 1:1 ratio using Lipofectamine 2000 (Invitrogen, Waltham, MA). For details on methods, please see Huang et al., (2017).

3 | RESULTS

3.1 | The *mitfa* cDNAs exhibit a high sequence similarity among 16 fish species

The full-length *mitfa* cDNA cloned from red crucian carp was 1,606 bp and contained an open reading frame of 1,236 bp, coding for 421 amino acids (GenBank accession No. KP757748). MEGA 7.0 was used to compare this red crucian carp *mitfa* cDNA sequence with those obtained from NCBI (http://www.ncbi.nlm.nih.gov/) for carp and 14 other species (Figure 1a). This analysis demonstrated a high level of sequence similarity in the *mitfa* gene among these fishes.

BioEdit software was then used to compare the amino acid similarity of *mitfa* between the three cyprinid fish and the 13 other fish (Figure 1b). The *mitfa* of zebrafish, carp, and red crucian carp coded for 412 amino acids, which may be related to that they all belong to *cyprinid* fish. The *mitfa* of *Ictalurus punctatus*, a fish closely related to these cyprinids, coded for 411 amino acids, while the *mitfa* of *Maylandia zebra*, *Amphilophus citrinellus*, *Melanochromis auratu*, and *Maylandia callaino* coded for 401 amino acids. Among the *cyprinid* fish, the amino acid homology of *mitfa* in carp was closely related to that of red crucian carp and zebrafish. The *mitfa* amino acid sequence similarity between carp and red crucian carp was 95.6% and was 91.5% similar between carp and zebrafish.

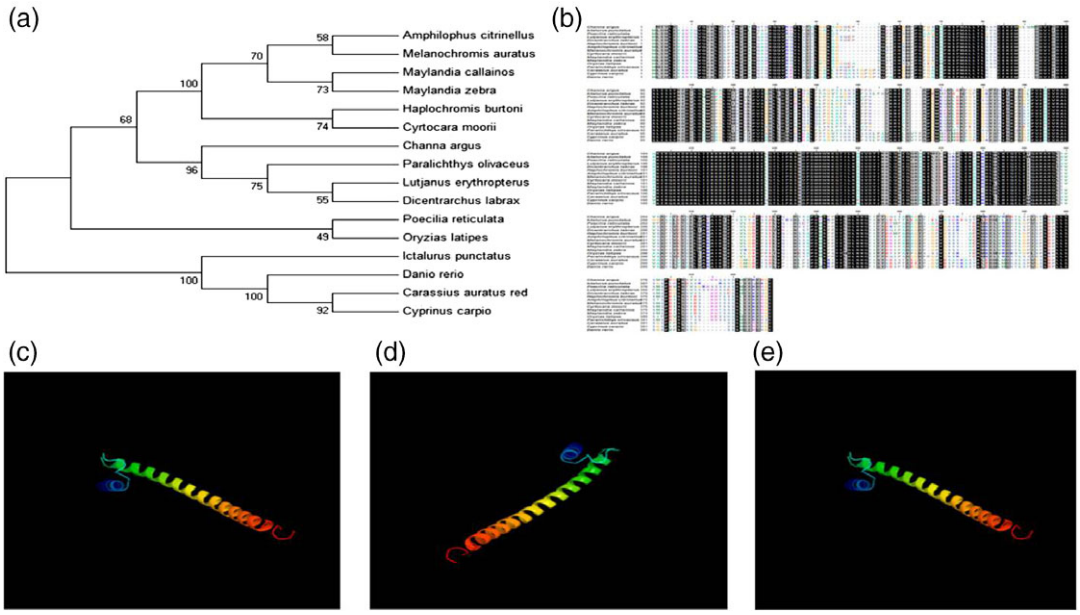

FIGURE 1 The structure and sequence of the *mitfa* gene. (a) Phylogenetic tree of the *mitfa* gene from 16 species of fish. (b) *mitfa* amino acid multiple sequence alignment from 16 species of fish. Predicted microphthalmia-associated transcription factor protein tertiary structure models for zebrafish (c), red common carp (d), and crucian carp (e)

TABLE 3 Basic physicochemical properties of microphthalmia-associated transcription factor proteins from16 species of fish

Latin name	Amino acid number	Protein molecular weight	Isoelectric point	Instability	Absorptivity	Lipophilic index
Channa argus	402	44,435.99	5.81	62.76	0.470	70.60
Ictalurus punctatus	411	45,700.09	5.68	65.05	0.520	75.01
Poecilia reticulata	404	44,716.37	5.93	69.14	0.467	71.96
Lutjanus erythropterus	408	45,443.13	5.81	65.67	0.682	72.18
Dicentrarchus labrax	405	45,030.57	5.93	65.54	0.533	73.19
Haplochromis burtoni	401	44,692.33	5.75	66.08	0.501	74.19
Amphilophus citrinellus	401	44,630.18	5.80	65.37	0.502	75.16
Melanochromis auratu	401	44,688.32	5.80	65.37	0.501	75.64
Cyrtocara moorii	401	44,717.36	5.75	64.16	0.501	76.13
Maylandia callaino	401	44,634.21	5.66	66.86	0.502	76.13
Maylandia zebra	400	44,595.15	5.84	66.79	0.502	73.65
Paralichthys olivaceus	407	45,310.76	5.97	61.56	0.681	70.44
Oryzias latipes	406	45,297.06	5.74	59.08	0.464	76.40
Danio rerio	412	45,207.80	5.65	63.88	0.523	72.48
Carassius auratus	412	45,443.96	5.50	63.93	0.520	73.64
Cyprinus carpio	412	45,580.12	5.42	64.12	0.518	71.26

3.2 | The physicochemical properties of MITF

The MITF molecular weight was predicted as 45,443.96, the isoelectric point was 5.50, the protein instability was 63.93, and the absorbance coefficient and fat-soluble index were 0.520 and 73.64, respectively, using the Protparam tool (http://web.expasy.org/proparam/), in red crucian carp. The protein physicochemical properties of MITF in the other 15 fish species were also analyzed (Table 3).

Phosphorylation refers to the amino acid residues (often serine, threonine, and tyrosine) covalently linked to a phosphate group so that the molecular conformation of the target protein changes, resulting in loss or access of enzyme activity. Using the NetPhos2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) to predict the MITF phosphorylation site, the results showed that there are potential phosphorylation sites in the MITF amino acid sequences of all 16 species of fish, with zebrafish showing the greatest number of potential phosphorylation sites (Table 4).

The Signal P 4.0 Server (http://cbs.dtu.dk/services/SignalP/) analysis indicated that no signal peptides existed in the *mitfa* of any of the 16 fish species. Moreover, there were no transmembrane domains in the MITF of any species (TMHMM Server v.2.0, http://www.cbs.dtu.dk/services/TMHMM-2.0/). Predict Protein (http://www. predictprotein. org/) showed that the MITF secondary structures were similar among the 16 fish species, with a predominance of α-helices (35.44–44.04%) followed by irregular curly (36.95–47.57%) and fewer β-sheets (4.62–9.11%). Prediction by Phyre2 of the tertiary structure model of the protein sequence translated from the coding sequence (CDS) region of the *mitfa* gene showed that the α-helix constituted a leucine zipper structure with a confidence level of 100%, and these models covered 19–24% of the amino acid sequence region in fish, including zebrafish, crucian carp, and carp (Figure 1ce).

3.3 | The embryonic expression of *mitfa* mRNA in red carp, red crucian carp, and their hybrids

Red carp, a variety of carp with a red/orange body color, show no melanophores in either the embryo or adult (Figure 2a1–8), although melanophores were observed in the embryo of red crucian carp (Figure 2b1–8). The

TABLE 4 Predicted microphthalmia-associated transcription factor phosphorylation sites

Phosphorylation sites prediction	Serine	Theronine	Tyrosine
Channa argus	28	11	7
Ictalurus punctatus	36	12	8
Poecilia reticulata	31	7	7
Lutjanus erythropterus	30	9	10
Dicentrarchus labrax	27	10	10
Haplochromis burtoni	26	9	9
Amphilophus citrinellus	26	9	8
Melanochromis auratus	26	9	10
Cyrtocara moorii	26	9	8
Maylandia callainos	27	8	10
Maylandia zebra	27	9	10
Paralichthys olivaceus	33	12	10
Oryzias latipes	28	9	7
Danio rerio	34	14	8
Carassius auratus	33	12	8
Cyprinus carpio	29	12	10

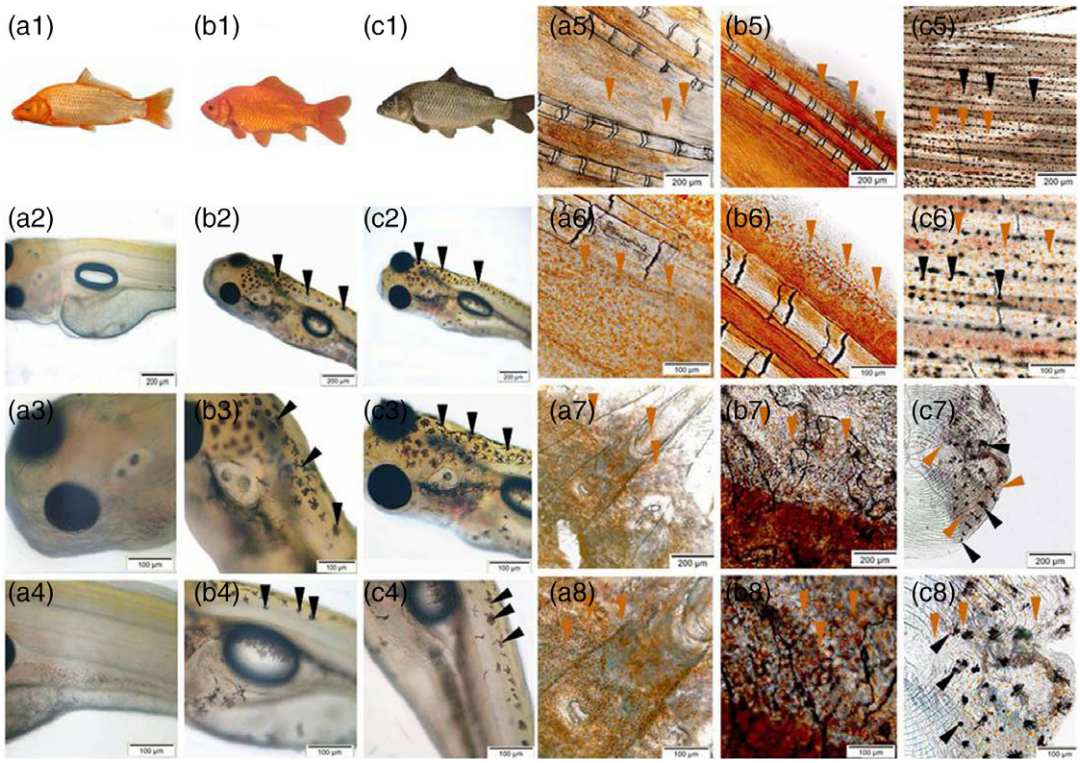

FIGURE 2 Observation of body color in red carp (a1–8), red crucian carp (b1–8), and their hybrids (c1–8). Panel 1 shows adult fish; 2, 3, and 4 show embryos; 5 and 6 show fins; and 7 and 8 show scales. Black arrows show melanophores; orange arrows show xanthophores and erythrophores

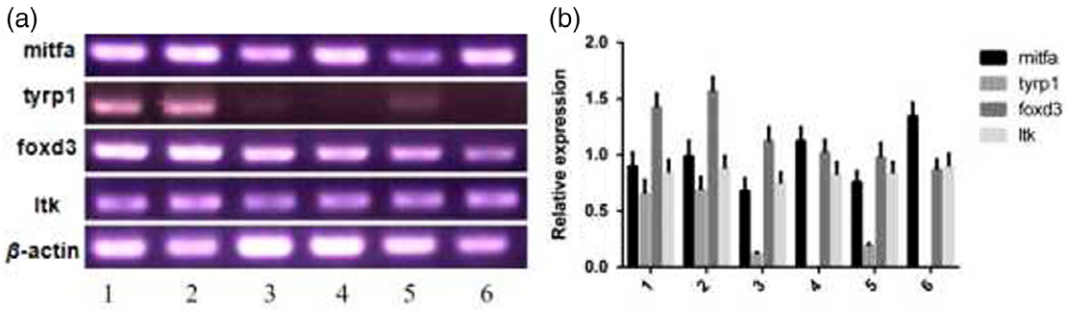

FIGURE 3 mRNA expression analysis of several pigment genes in the embryos of red carp, red crucian carp, and their hybrids. Body pigment stage embryo of red crucian carp (1), hybrids (2), and red carp (3). Adult skin of red crucian carp (4), hybrids (5), and red carp (6). (mean ± *SD* of relative expression; $n = 3$ per group)

hybrids of red carp and red crucian carp were a silver-gray color, in both the embryo and adult (Figure 2c1–8).

We measured the mRNA expression levels of *mitfa* and several *mitfa*-related genes, including those encoding Forkhead box D3 (FOXD3), TYRP1, and leukocyte tyrosine kinase (LTK), in the embryos of red carp, red crucian carp, and their hybrid. The results show that the mRNA expression levels of *mitfa*, *foxd3*, and *ltk* genes were high in body pigment stage embryos and adult skin of red carp, red crucian carp, and their hybrids. In contrast, the mRNA expression level of *tyrp1* was low in the body pigment stage embryo of red carp and high in the adult skin of the hybrid (Figure 3).

mitfa is a master regulation factor in the melanocyte lineage (Lister et al., 1999; Lister et al., 2001; Levy et al., 2006; Steingrimsson et al., 2004; Zeng et al., 2014). As melanin is not present in the body color of red carp embryos, the *mitfa* PCR product of red carp embryos was cloned and sequenced after recovery and purification to verify whether *mitfa* is expressed. As shown in Figure 4, the sequence of *mitfa* RNA from red carp embryos was consistent with that of koi carp (GenBank accession No. KC565527).

3.4 | The expression of *mitfa* mRNA in cultured embryonic cells of red carp, red crucian carp, and their hybrids

To further verify the expression of *mitfa* in red carp embryos, pDestTol2-*mitfa: gfp* and pCS2FA-transposase were transfected at passage 3 into cells derived from somite stage embryos of red carp, red crucian carp, and their hybrids and cultured without any cytokines to sustain pluripotency in vitro. GFP-positive cells (*mitfa*$^+$ cells) were observed in

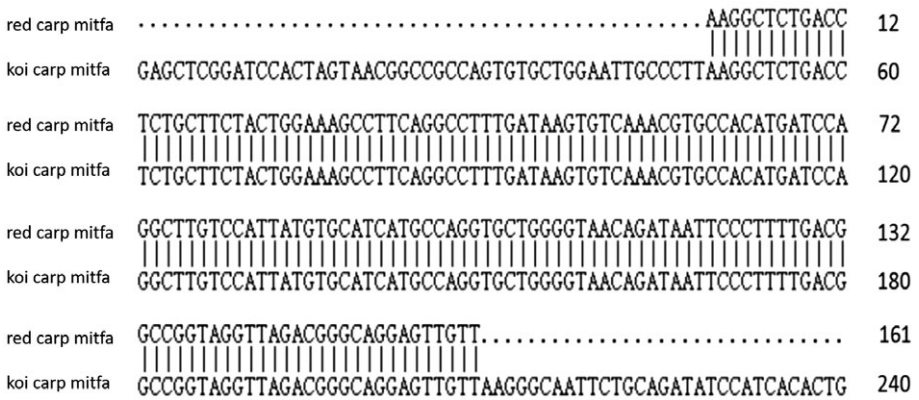

FIGURE 4 *mitfa* nucleotide sequence alignment of red carp and koi carp. The sequence of *mitfa* RNA from red carp embryos was consistent with that of koi carp

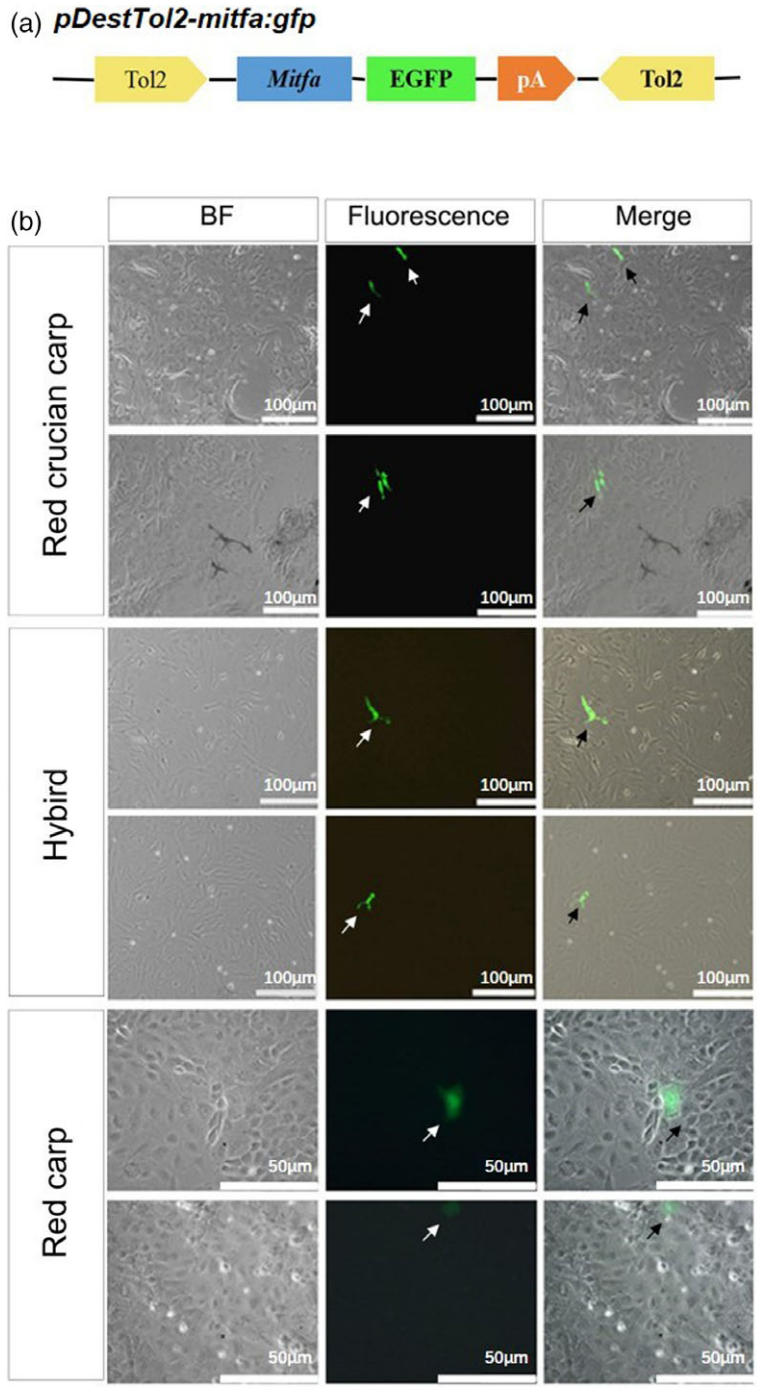

FIGURE 5 *mitfa* + cells derived from embryos of red crucian carp, red carp, and their hybrid (H) and transfected with *mitfa*-green fluorescent protein reporter. Upper panel shows the schematic diagram for the Tol2 transposon-base vector with *mitfa* promoter. Arrows show GFP-positive cells (*mitfa* + cells)

embryo cells from red carp, red crucian carp, and their hybrids (Figure 5). These *mitfa*$^{+}$ cells were able to proliferate and could be subcultured (Figure 6). Furthermore, melanocytes were observed in primary culture and subculture cells from the somite stage embryos of red carp, red crucian carp and their hybrid (Figure 7).

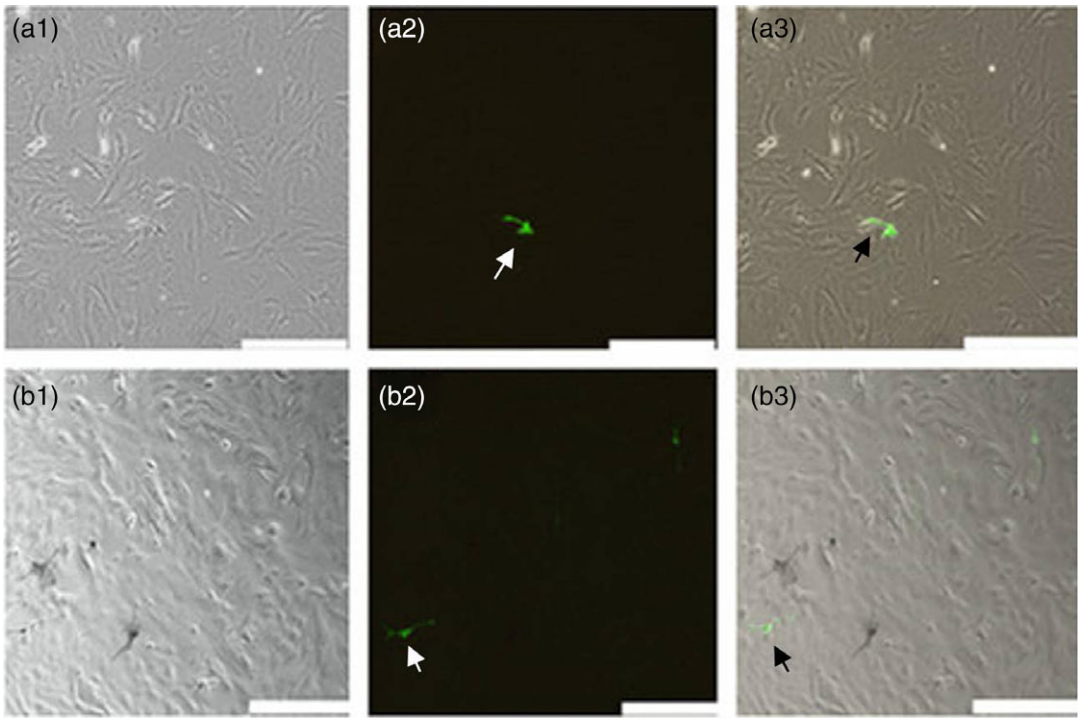

FIGURE 6 *mitfa* + cells proliferation status in vitro (a1–a3) and could be subcultured (b1–b3). Arrows show *mitfa* + cells

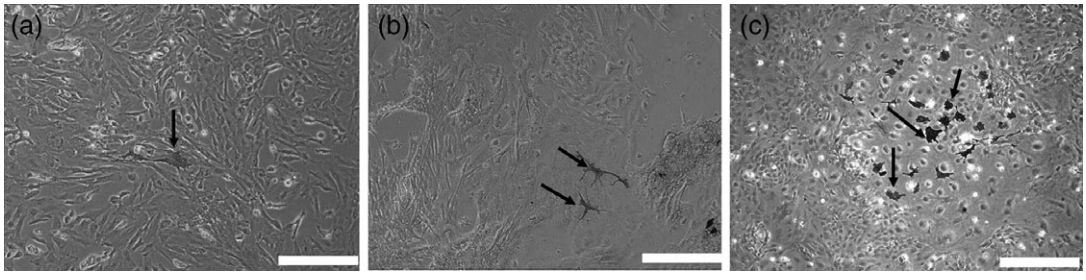

FIGURE 7 Melanocytes were observed in subculture cells from the somite stage embryos of red crucian carp, red carp, and their hybrids. (a) red crucian carp; (b) hybrid; (c) red carp. Arrows show melanocytes

To explore the molecular basis of pigment formation in embryonic cells in vitro, we measured the mRNA expression of *mitfa* and several pigment-related genes, including *tyr*, *dct*, t*yrp1*, *fms*, and *ltk*. As shown in Figure 8, the melanogenesis-related genes *mitfa* and *foxd3* and the melanocyte marker genes *tyr*, *dct*, and *tyrp1*, as well as the xanthophore marker gene *fms* and the iridocyte marker gene *ltk*, were expressed in vitro in the embryonic cells of red carp, red crucian carp, and their hybrids.

4 | DISCUSSION

Skin coloration is determined by pigment cells, which are classified according to the pigments they contain. Melanophores are a black color and contain the pigment melanin. Xanthophores and erythrophores are a yellow or

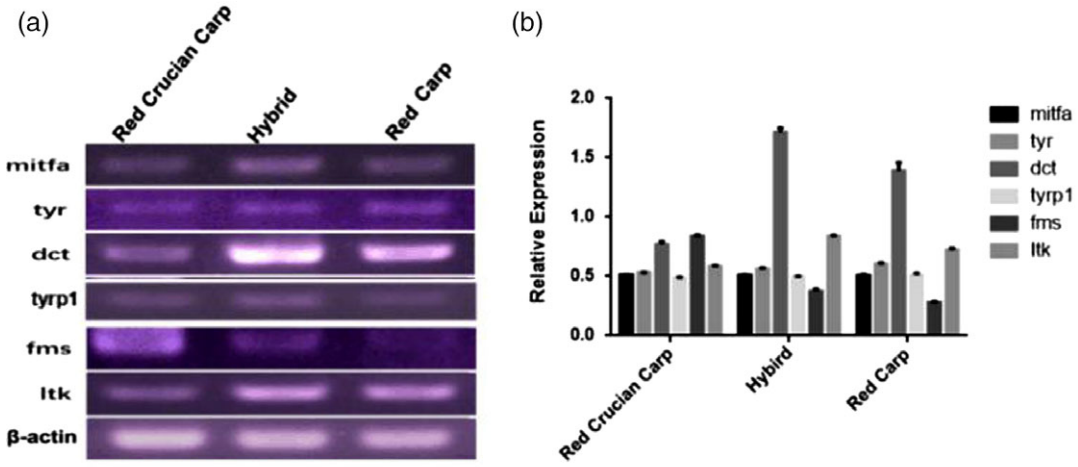

FIGURE 8 Expression analysis of several pigment genes, such as the melanogenesis-related genes (*mitfa*, *foxd3*), the melanoma marker genes (*tyr*, *dct*, *tyrp1*), the xanthophore marker gene *fms*, and the iridocyte marker gene *ltk*, in embryonic cells of red crucian carp, red carp, and their hybrid (H). (mean ± *SD* of relative expression; $n = 3$ per group)

red color, and both contain pteridines and carotenoids. Iridophores are silver-colored reflective platelets containing purine (Kelsh, 2004; Protas & Patel, 2008). (MITF is a member of the basic helix-loop-helix leucine zipper protein family [Hodgkinson et al., 1993]). The *mitf* gene in humans produces 18 transcripts, among which *mitfa*, *mitfd*, and *mitfh* are necessary for retinal pigment epithelium development, and *mitfm* plays a critical role in the development of melanocytes (Koludrovic & Davidson, 2013; Li, Zhu, & Hong, 2013; Oboki, Morii, Kataoka, Jippo, & Kitamura, 2002). *mitfa* and *mitfb* genes have also been reported in zebrafish, with *mitfa* closely related to the development of melanocytes and *mitfb* involved in the retinal pigment epithelium development in zebrafish (Curran et al., 2010; Li et al., 2014). In the present study, amino acid homology comparisons of *mitfa* between 3 cyprinid fish and 13 other fish showed that carp are more closely related to the other two cyprinids, red crucian carp, and zebrafish.

mitfa directly regulates the expression of multiple genes necessary for melanophore development, including *tyr*, *tyrp1*, and *dct* (Lister et al., 2001). It has also been reported that the *mitf* gene is directly regulated by *pax3* (Lacosta, Muniesa, Ruberte, Sarasa, & Domínguez, 2005a; Lacosta, Muniesa, Ruberte, Sarasa, & Domínguez, 2005b; Takeda et al., 2000; Widlund et al., 2002) and *foxd3* (Curran et al., 2009; Thomas et al., 2009). In red crucian carp, the mRNA and protein level of the *mitfa* gene in skin was significantly lower than those of white crucian carp (Zhang, Liu, Peng, et al., 2017). According to transcriptome analysis, the expression of *mitfa* mRNA was related to melanin synthesis or melanophore development, which are closely related to the gray-to-red body color transformation (Zhang, Liu, Fu, et al., 2017). In this study, we found *mitfa* mRNA expression in red carp (although their *tyrp1* mRNA expression was low), despite there being no melanocytes in the skin of embryos or adults. Notably, $mitfa^+$ cells, which acquire the expression of MITF, and melanocytes were observed in vitro in cells derived from red carp embryos. There are several cell types involved in the melanophores development, including *mitfa* ± cells (Huang et al., 2017). In vivo, most *mitfa* ± melanoblasts, the undifferentiated and unpigmented cells in which *mitfa* is highly expressed, differentiate into tyr^+ pigment melanocytes, which then become pigmented by synthesizing melanin (Cheli, Ohanna, Ballotti, & Bertolotto, 2010). Our results indicate that the absence of melanin in red carp is not caused by deletion or mutation of the *mitfa* gene. Regulation of melanin synthesis is related to the expression of some functional genes, such as those involved in methylation, which may lead to the absence of melanocytes in the body color formation of red carp.

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CONFLICTS OF INTEREST

The authors declare no potential conflict of interests.

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