Chinese Journal of Oceanology and Limnology
Vol. 30 No. 1, P. 80-91, 2012
DOI: http://dx.doi.org/10.1007/s00343-012-1043-9

Differences in MITF gene expression and histology between albino and normal sea cucumbers (*Apostichopus japonicus* Selenka)*

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Received Mar. 1, 2011; accepted in principle Apr. 3, 2011; accepted for publication May 21, 2011

Abstract Albino *Apostichopus japonicus* occur both in the wild and in captivity. The offspring of albino *A. japonicus* also suffer from albinism. The formation of melanin in the melanocytes is dependant on microphthalmia-associated transcription factor (MITF). To investigate the role of MITF in controlling albinism, we cloned the full-length MITF cDNA from *A. japonicus* and compared MITF mRNA expression in albino and normal *A. japonicus*. In addition, we used light and electron microscopy to compare histological samples of normal and albino *A. japonicus*. The body wall of albino adults was characterized by significantly lower levels of MITF expression and lower numbers of epidermal melanocytes, which also contained less melanin. In albino juvenile offspring, MITF expression levels were significantly lower 32 d after fertilization and there were fewer, and less developed, epidermal melanocytes. Thus, we conclude that albino *A. japonicus* have fewer melanocytes and a reduced ability to synthesize melanin, likely because of lower expression of MITF.

Keyword: microphthalmia-associated transcription factor (MITF); melanocyte; melanosome; melanin; *Apostichopus japonicus*

1 INTRODUCTION

The sea cucumber (*Apostichopus japonicus* Selenka) is an economically important echinoderm species that is cultured in Northern China (Yang, 2005). As is typical in echinoderms, coloration varies widely among *A. japonicus* individuals. In normal individuals, juveniles develop pigmentation on their body wall at ~1 cm in length. As individuals reach adulthood, their dorsal surfaces turn dull yellowish brown to maroon in color, and their ventral surfaces turn fawn to russet (Hyman, 1955). However, some proportion of both wild and captive population are albinos. These individuals hide among the benthic algae as they are conspicuous targets for predators. Furthermore, the offspring of albino *A. japonicus* are also albinos, and have little or no pigmentation on their body wall from the early juvenile stage.

Albinism is widespread in the animal kingdom, and is caused by the absence of melanocytes or melanin in the integument (Arthur et al., 2005; Nolan and Robert, 1990; Seldenrijk et al., 1982; Spritz et al., 2003; Wang et al., 2007). For example, the melanocytes in the skin of larval albino turbots (*Scophthalmus maximu*) contain fewer melanosomes, while the adults do not have melanocytes (Guo et al., 2007). In the periodic albino *Xenopus laevis*, the oocytes do not contain melanin and, though it is

* Supported by the National Natural Science Foundation of China (No. 40976089), the National Marine Public Welfare Research Project (No. 200805069), the National Science and Technology Support Program of China (No. 2011BAD13B02), and the Breeding Project of Shandong Province (China)

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present in skin melanocytes during the larval stages, it is absent in metamorphosed individuals (Fukuzawa and Ide, 1986).

To date, at least 12 different genes have been reported whose inactivation can lead to various forms of albinism (Colin, 2007). Of these, the tyrosinase (TYR) gene family encodes three functional enzymes involved in the melanin biosynthetic pathway, including TYR, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2). Inactivation in these three enzymes can lead to various forms of albinism (William, 2000). Recently, the microphthalmia-associated transcription factor (MITF) was implicated as an important regulator of expression of the TYR gene family. Furthermore, MITF is also an essential regulator for melanocyte development, proliferation, and survival (Jiri and Jan, 2010).

Research on the MITF gene has been limited primarily to vertebrates, particularly humans and mice. Nakayama et al. (1998) concluded that MITF gene expression is essential for melanocyte development in mice and that mutations in MITF are responsible for abnormalities in neuroepithelial and neural crest-derived melanocytes. Furthermore, mutations in the human MITF gene are associated with albinism-deafness (Tietz) syndrome (Amiel et al., 1998) and Waardenburg syndrome type 2 (WS2) (Tassabehji et al., 1994, 1995). This latter condition is characterized by varying degrees of deafness, minor defects in structures arising from the neural crest, and pigmentation anomalies. Despite its importance, little is known about the role of MITF in *A. japonicus*.

To evaluate which gene/s might be associated with albinism in *A. japonicus*, we sequenced the transcriptome of normal and albino *A. japonicus* using 454 sequencing technology. Sequencing analysis revealed that the frequency distribution of the MITF contig in the normal *A. japonicus* transcriptome contig library was several times higher than for the albino *A. japonicus*, suggesting that mRNA expression of the MITF gene is lower in albino *A. japonicus*. To investigate the correlation between MITF gene expression and albinism in *A. japonicus*, we cloned the MITF full-length cDNA of *A. japonicus* and quantified the differences in MITF mRNA expression in the adult body wall and juvenile offspring between albino and normal individuals. In addition, we evaluated histological differences between normal and albino individuals to determine the distribution and ultrastructure of epidermal melanocytes in the body wall.

2 MATERIAL AND METHOD

2.1 Animal culture and sampling

We collected normal and albino *A. japonicus* adults from the Yellow Sea, China in November 2009 and reared the two types separately in our laboratory. The animals were housed in aquaria (3 m×3 m×2 m) filled with aerated seawater ((15±1)°C) under a 14/10 h (light/dark) photoperiod and fed with commercial feed twice daily. After two weeks, we removed five normal and five albino adults (weight: 102.07±21.65 g (mean±standard deviation)) and collected a sample of their body wall tissue for MITF mRNA expression analysis and histology (Fig.1). The remaining adults were cultured for use as broodstock.

In June 2010, both normal and albino broodstock adults were artificially induced to spawn and produce gametes. Gamete production was immediately followed by external fertilization and the fertilized ova developed into juveniles within 25 d. There was no initial difference in appearance between juveniles produced by normal and albino broodstock individuals. We first observed pigmentation on the body wall of normal juveniles at ~1 cm in length, whereas the albino juveniles developed little or no pigment. We collected juveniles for MITF mRNA expression analysis 25, 32, 39, 46, 53, 60, 67, 74, 81, and 88 d after fertilization (Table 1). At the end of the experiment, we collected tissue samples of the remaining juveniles for histology (Fig.1).

2.2 Total RNA isolation

We isolated total RNA from the body wall of *A. japonicus* using an RNeasy Mini Kit (Qiagen,

Table 1 The body length of albino and normal juveniles

Sampling day	Juvenile length (mm)	
	Albino juvenile	Normal juvenile
Day 25	0.684±0.308	0.792±0.352
Day 32	1.821±1.021	2.106±1.165
Day 39	3.152±1.854	3.621±1.659
Day 46	4.361±2.291	4.742±2.499
Day 53	5.281±2.411	5.943±2.548
Day 60	6.318±2.832	7.074±2.731
Day 67	7.172±2.548	7.902±2.836
Day 74	8.076±2.445	8.953±2.855
Day 81	9.031±2.655	10.047±2.987
Day 88	10.111±2.932	10.922±3.183

Values indicate the mean±stand deviation (SD) (n=100).

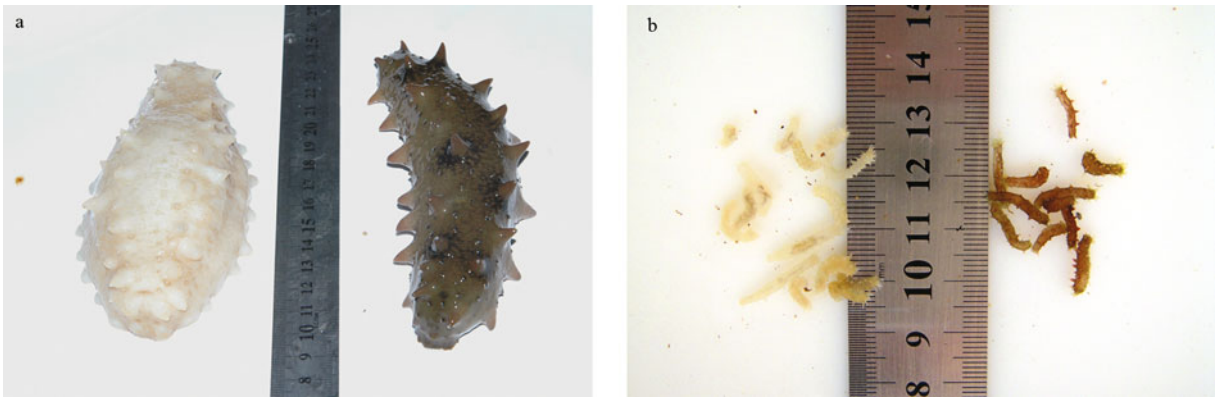

Fig.1 Normal and albino *A. japonicus* adults (a) and juveniles (b)

Texas, USA). The quality and quantity of RNA were determined by electrophoresis with ethidium bromide staining on a 1% agarose gel.

2.3 Cloning of *A. japonicus* MITF full-length cDNA

We obtained the sequence of an MITF cDNA fragment containing 505 nucleotides from a 454 sequencing contig library (NCBI/GenBank, accession number SRA020828.4). The sequence was deposited in GenBank under the accession number HQ401009. To obtain the full-length MITF cDNA, we conducted 5′ and 3′ rapid amplification of the cDNA ends by (RACE) PCR (SMARTer™ RACE cDNA Amplification Kit, Clontech, Mountain View, CA). We designed two gene-specific primers (GSP1 and GSP2; Table 2) based on the sequence of the MITF cDNA fragment. First-strand cDNA synthesis was carried out using a SMARTer RACE cDNA Amplification Kit, following the manufacturer's instructions. We used DNase-treated (RNase-Free DNase Set, Qiagen) total RNA as a template. The 5′-RACE cDNA was synthesized using 5′-CDS Primer A and SMARTer II A Oligonucleotide in 10 μL containing 2 μL 5×First-Strand Buffer, 1 μL DTT (20 mmol/L), 1 μL dNTP Mix (10 mmol/L), 0.25 μL RNase Inhibitor (40 U/μL), and 1 μL Reverse Transcriptase (100 U/μL). The 3′-RACE cDNA was synthesized using 3′-CDS Primer A in the same reaction system. The reaction mixture was incubated at 42°C for 90 min then at 70°C for 10 min. For 5′-RACE/3′-RACE PCR amplification, we used 5′-RACE/3′-RACE cDNA as template, primers GSP2 and UPM (Table 2) for cloning the 3′ end of the MITF sequence, and primers GSP1 and UPM (Table 2) to clone the 5′ end of the MITF sequence. The total volume of the RACE PCR amplification mixture was 50 μL, containing 34.5 μL PCR-Grade water, 5 μL 10× Advantage 2 PCR Buffer, 1 μL dNTP Mix (10 mmol/L), 1 μL 50× Advantage 2 Polymerase Mix, 2.5 μL 5′-RACE cDNA/3′-RACE cDNA, 5 μL UPM Primer, and 1 μL GSP1/GSP2 primer. The RACE PCR program consisted of 30 cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 3 min. We used a GenElute™ PCR Clean-Up Kit (Sigma, California, USA) to purify the PCR products. The products were ligated and transformed using the pMD-18 T vector (TaKaRa, Shiga, Japan) and *Escherichia coli* competent cells DH5α (TaKaRa, Shiga, Japan). To identify the positive recombinants, we used ampicillin for blue/white selection on LB plates. The positive recombinants were sequenced and analyzed using DNASTAR (Madison, WI).

2.4 Sequence alignment and phylogenetic analysis of *A. japonicus* MITF protein

The MITF protein sequence was translated from MITF full-length cDNA using the ExPASy translate

Table 2 Sequences of RACE PCR, RT-PCR and real-time PCR primers (N = A, C, G, or T; V = A, G, or C)

Primer	Sequence
ActinF	5′(47)-CATTCA ACCCTAAAGCCAACA-(67)3′
ActinR	5′(227)-TGGCGTGAGGAAGAGCAT-(244)3′
MitfF	5′(1221)-CCTTACTGCGTCTTCTGC-(1238)3′
MitfR	5′(1362)-GTTCCTGCTTGATGGTCG-(1379)3′
UPM	Long 5′-CTAATACGACTCACTATAGG GCAAGCAGTGGTATCAACGCAGAGT-3′ Short 5′-CTAATACGACTCACTATAGGGC-3′
GSP1	5′-GCTGTTCCTCTGTCAGGGTAATCGG-3′
GSP2	5′-GATGCCCACTGTCTCTCAGGGTTTC-3′
5′-CDS Primer A	5′-(T)25V N-3′
3′-CDS Primer A	5′-AAGCAGTGGTATCAACGCAGAGTAC(T) 30VN-3′

tool (www.expasy.ch). We performed sequence alignment and phylogenetic analysis using animal MITF protein sequences deposited in NCBI, including *Saccoglossus kowalevskii* MITF (NCBI/GenBank, accession number ACY92571), *Xenopus* (*Silurana*) *tropicalis* MITF (accession number AAI35969), *Gallus gallus* MITF (accession number BAA25648), *Homo sapiens* MITF (accession number AAH65243), *Equus caballus* MITF (accession number NP_001157346), *Canis lupus familiaris* MITF (accession number NP_001003337), *Mus musculus* MITF (accession number AAF81266), *Sus scrofa* MITF (accession number NP_001033090), *Macaca mulatta* MITF (accession number XP_002802781), *Danio rerio* MITF (accession number NP_571922), *Drosophila melanogaster* MITF (accession number AAQ01726), *Ciona intestinalis* MITF (accession number NP_001087207), *Mesocricetus auratus* MITF (accession number CAD30262), *Poecilia reticulata* MITF (accession number ABI64148), *Xenopus laevis* MITF (accession number NP_001165646), and *Rattus norvegicus* MITF (accession number NP_001178018). We used CLUSTALW (www.ebi.ac.uk) for sequence alignment and ClustalX and MEGA3 for phylogenetic analysis.

2.5 Quantitative analysis of *A. japonicus* MITF mRNA expression

Using real-time RT-PCR, we analyzed the mRNA expression of MITF in the body wall of *A. japonicus* adults and their juvenile offspring at times during melanogenesis. Total RNA isolation was carried out using the methods described above. To synthesize the first-strand cDNA, we used DNase-treated (RNase-Free DNase Set, Qiagen) total RNA as the template and M-MLV reverse transcriptase (Promega, Madison, WI). The total reaction volume for RT-PCR amplification was 25 μL, containing 4 μL DNase-treated RNA, 1 μL M-MLV reverse transcriptase, 5 μL M-MLV buffer (25 mmol/L KCl, 10 mmol/L Tris-HCl, 0.6 mmol/L $MgCl_2$, and 2 nmol/L DTT, pH 8.3), 1 μL AP-oligodT18, 5 μL dNTP, 1 μL ribonuclease inhibitor (Promega), and 8 μL RNase-free water. The RT-PCR thermal conditions consisted of 1 cycle at 70°C for 5 min and 42°C for 1 h. Real-time PCR was performed in a Mastercycler eppendorf realplex real-time PCR system (Eppendorf, Hamburg, Germany). The total volume of the real-time PCR amplification was 25 μL, containing 2 μL cDNA, 0.5 μL of each primer (MITFF and MITFR; Table 2), 12.5 μL SYBR Green PCR master Mix (TakaRa, Japan), and 9.5 μL RNase-free water. The real-time PCR program consisted of 1 cycle at 95°C for 5 s followed by 40 cycles at 95°C for 5 s, 59°C for 20 s, and 72°C for 30 s, and a final melt curve step. We used realplex software version 2.2 (Eppendorf, Hamburg, Germany) for data analysis using the comparative CT method. We used β-actin (NCBI/GenBank, accession number EU668024, primers ActinF and ActinR; Table 2) as the inference control. ΔCTs, denoting the target CTs minus the internal control CTs, were measured to normalize the efficiency of RT-PCR. The calibrator ΔCT, which is the group with the highest ΔCT value, was set as the reference sample. Then, ΔΔCTs were calculated by subtracting the ΔCTs for each sample from the calibrator ΔCT. The relative abundance of mRNA MITF was measured by $2^{-\Delta\Delta CT}$. The values of the relative mRNA abundance represent the mean ± standard deviation (SD). We tested for differences in the relative abundance of mRNA MITF between albino and normal individuals at a given sampling time using one-way analysis of variance (ANOVA) followed by Tukey's test. The tests were performed in SPSS (Version 13.0; SPSS Inc., Chicago, IL, USA) with a significance level of $P<0.05$.

2.6 Light microscope observations

The body wall samples taken from adults and the entire bodies of juveniles were fixed in Bouin-Hollande solution, dehydrated in ethanol, cleared in xylene, then embedded in paraffin. The tissue was then sectioned (7 μm) and stained with H&E stain to display the tissue structure. In addition, we stained 5 μm sections with Masson Fontana stain to visualize the distribution of melanocytes (Barbosa et al., 1984; Gaitanis et al., 2005).

2.7 Electron microscope observations

Small epidermis fragments taken from adults and juveniles were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde, buffered at pH 7.4 in 0.1 mol/L phosphate buffer solution, then dehydrated and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, then observed with a Hitachi H-7000 transmission electron microscope operating at 80 kV.

3 RESULT

3.1 Isolation and characterization of *A. japonicus* MITF full-length cDNA

The full-length cDNA of *A. japonicus* MITF contained 3104 nucleotides (Fig.2). It consisted of a

Fig.2 The full cDNA and deduced amino acid sequence of *A. japonicus* MITF

The nucleotides and amino acid residues are numbered on the left. The start and stop codons are included in a box; the classical polyadenylation signal in the 3′-UTR is underlined; and the highly conserved bHLH domain of *A. japonicus* MITF is shaded.

3′ un-translated region (UTR) of 1 021 bp, a 5′ UTR of 586 bp, and an open-reading frame (ORF) of 1 497 nucleotides (position 587–2 083). The cDNA sequence of the MITF gene was deposited in NCBI/GenBank under accession number HQ606465. We found no evidence of nucleotide mutation in the cDNA of albino *A. japonicus* MITF.

3.2 Sequence alignment and phylogenetic analysis of *A. japonicus* MITF protein

The translated *A. japonicus* MITF protein contained 499 amino acids, including a basic helix loop helix (bHLH) domain located at amino acids 279–340 (Fig.2). Protein alignments with all known animal MITFs revealed that the *A. japonicus* MITF bHLH domain shared 67% identity with *Ciona intestinalis* MITF bHLH domain; 74% identity with *Drosophila melanogaster* MITF bHLH domain; 75% identity with *Saccoglossus kowalevskii* MITF bHLH domain; 82% identity with the MITF bHLH domains of *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Canis lupus familiaris*, *Sus scrofa*, *Mesocricetus auratus*, *Macaca mulatta*, *Equus caballus*, *Gallus gallus*, *Xenopus laevis*, *Xenopus* (*Silurana*) *tropicalis*, and *Danio rerio*; and 83% identity with the *Poecilia reticulata* MITF bHLH domain. Comparison of *A. japonicus* with other animals confirmed that bHLH domains are highly conserved in vertebrates. Furthermore, the *A. japonicus* MITF bHLH domain was closely related to vertebrate MITF bHLH domains (Fig.3).

3.3 Quantitative analysis of MITF mRNA expression in albino and normal *A. japonicus*

The relative abundance of mRNA MITF in the body wall of normal *A. japonicus* adults was significantly higher than in adult albinos ($P<0.05$). This was consistent with the 454 sequencing analysis. In juvenile offspring, during the process of melanogenesis, MITF mRNA expression in normal juveniles increased significantly during the 32 d following fertilization ($P<0.05$) and peaked 46 d

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Mu. musculus      309 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 370
Ra. norvegicus    309 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 370
Ho. sapiens       202 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 263
Ca. familiaris    202 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 263
Su. scrofa        196 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 257
Me.auratus        196 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 257
Ma. mulatta       272 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 333
Eq. caballus      302 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 363
Ga. gallus        251 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 312
Xe. laevis        269 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 330
Xe. tropicalis    269 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQQR 330
Da. rerio         293 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQHR 354
Po. reticulata    184 ERQKKDNHNLIERRRRFNINDRIKELGTLIPKSNDP------DMRWNKGTILKASVDYIRKLQREQER 245
Ci. intestinalis  211 DRIKKDNHNIIERRRRYNINDRIRELGHLVPKSSDP------ELRWNKGSILKAAVDYIQHLQNDQQK 272
Sa. kowalevskii   288 DRQKKDNHNMIERRRRFNINDRIKELGTLLPTNGDP------DQRINKGTILKSSVDYIRRLRKDASK 349
Dr. melanogaster  398 DRQKKDNHNMIERRRRFNINDRIKELGTLLPKGSDAFYEVVRDIRPNKGTILKSSVDYIKCLKHEVTR 465
Ap. japonicus     279 DRQKKDNHNMIERRRRFNINDRIKELGTLIPKSPDP------DQRQDKGSILKMSVDYIRKLQREREQ 340
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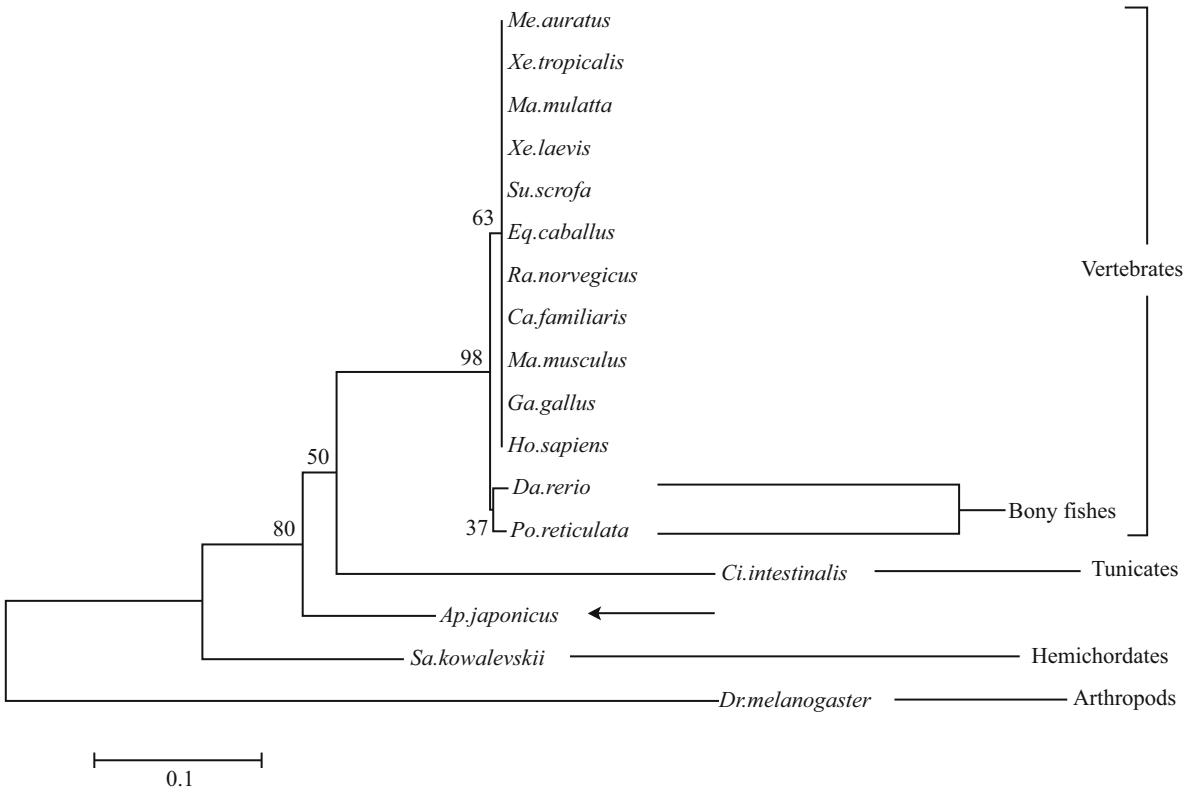

Fig.3 Protein alignment and phylogenetic analysis of the *A. japonicus* MITF bHLH domain with other known animal MITF bHLH domains

The GenBank accession numbers for these species are: *Saccoglossus kowalevskii* MITF (GenBank accession No. ACY92571.1), *Xenopus* (*Silurana*) *tropicalis* MITF (Gen-Bank accession No. AAI35969.1), *Gallus gallus* MITF (GenBank accession No. BAA25648.1), *Homo sapiens* MITF (GenBank accession No. AAH65243.1), *Equus caballus* MITF (GenBank accession No. NP_001157346.1), *Canis lupus familiaris* MITF (GenBank accession No. NP_001003337.1), *Mus musculus* MITF (GenBank accession No. AAF81266.2), *Sus scrofa* MITF (GenBank accession No. NP_001033090.1), *Macaca mulatta* MITF (GenBank accession No. XP_002802781.1), *Danio rerio* MITF (GenBank accession No. NP_571922.1), *Drosophila melanogaster* MITF (GenBank accession No. AAQ01726.1), *Ciona intestinalis* MITF (GenBank accession No. NP_001087207.1), *Mesocricetus auratus* MITF (GenBank accession No. CAD30262.1), *Poecilia reticulata* MITF (GenBank accession No. ABI64148.1), *Xenopus laevis* MITF (GenBank accession No. NP_001165646.1), and *Rattus norvegicus* MITF (GenBank accession No. NP_001178018.1). During protein alignment, we used the deduced protein sequences in CLUSTALW. The asterisk indicates identical amino acids. During the phylogenetic analysis, the tree topology was evaluated by 1 000 replication bootstraps; numbers on each branch of the tree represent the bootstrap support value; and the arrow denotes the *A. japonicus* MITF bHLH domain.

after fertilization. Thereafter, MITF expression levels decreased steadily before stabilizing. During the same developmental period, the levels of MITF expression rose steadily, but not significantly, in juvenile albinos. Compared with normal juveniles, the MITF expression levels in albino juveniles were significantly lower 32 d after fertilization ($P<0.05$) (Fig.4).

3.4 Histological observations of albino and normal *A. japonicus*

H&E staining discriminated the cuticle, epidermis, and dermis tissue, with no clear boundary between the epidermal and dermal layers in the adult body wall. The epidermal layer of albino adults was thinner relative to normal adults. Staining of *A. japonicus* adults with Masson Fontana revealed that the melanocytes were primarily distributed in the epidermal layer. Albino adults had fewer epidermal melanocytes compared with normal adults, which may be related to the thinner epidermal layer (Fig.5). Electron microscope observations of normal adult epidermis revealed the ultrastructure of the mature melanocytes, which consisted primarily of developed melanosomes. Melanosomes were less dense in the melanocytes of albino adults compared with those of normal adults. Furthermore, the melanocytes of albino adults also contained non-pigmented vacuoles (Fig.6).

The structure of the body wall of *A. japonicus* juveniles (cuticle, epidermis, and dermis) was already clearly developed. The body wall of juveniles sampled 88 d after fertilization had a more open structure compared with adults. Normal juveniles had already accumulated a number of melanocytes in the base layer of the epidermis, exhibiting incipient melanogenesis of the epidermal layer. However, albino juveniles had very few epidermal melanocytes during the same developmental phase (Fig.7). The melanocytes of normal juveniles contained both developing and developed melanosomes, whereas the melanocytes of albino juveniles did not contain any developed melanosomes. Furthermore, the melanocytes of albino juveniles contained many non-pigmented vacuoles (Fig.8).

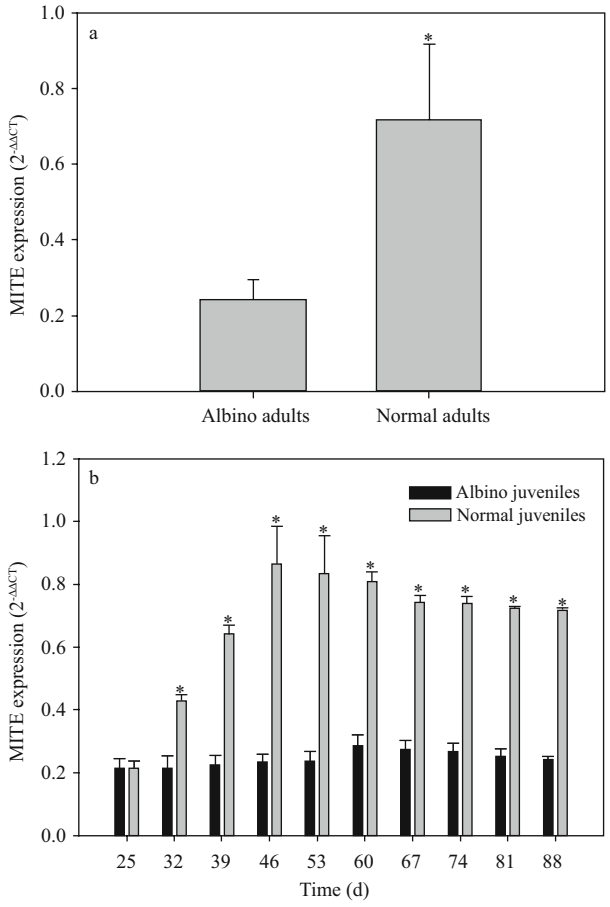

Fig.4 Real-time RT-PCR analysis of *Apostichopus japonicus* MITF expression

a. relative mRNA abundance of MITF in the body wall of albino and normal adults; b. relative mRNA abundance of MITF in *A. japonicus* juveniles at various sampling times. The asterisk indicates significant differences ($P<0.05$) between albino and normal individuals at a given sampling point. Each bar represents the mean±standard deviation (SD) ($n=5$).

4 DISCUSSION

We isolated and characterized the full-length cDNA of *A. japonicus* MITF, consisting of 3 104 nucleotides. The translated *A. japonicus* MITF protein contained 499 amino acids, including a basic helix loop helix (bHLH) domain located at amino acids 279–340. Protein alignments with other known animal MITFs revealed that the *A. japonicus* MITF bHLH domain was most homologous with vertebrate MITF bHLH domains, sharing at least 82% amino acid identity. Phylogenetic analysis also confirmed that the *A. japonicus* MITF bHLH domain is closely related to vertebrate MITF bHLH domains. Mutations of MITF in albino humans or mice frequently exhibit a compromised region encoding the basic helix loop helix leucine zipper (bHLHZip) domain (Kazuhisa et al., 2000). However, we did not find evidence of nucleotide mutation in the homologous domain of albino *A. japonicus* MITF.

Our analysis of *A. japonicus* MITF mRNA expression suggested that the level of expression was significantly lower in the body wall of albino

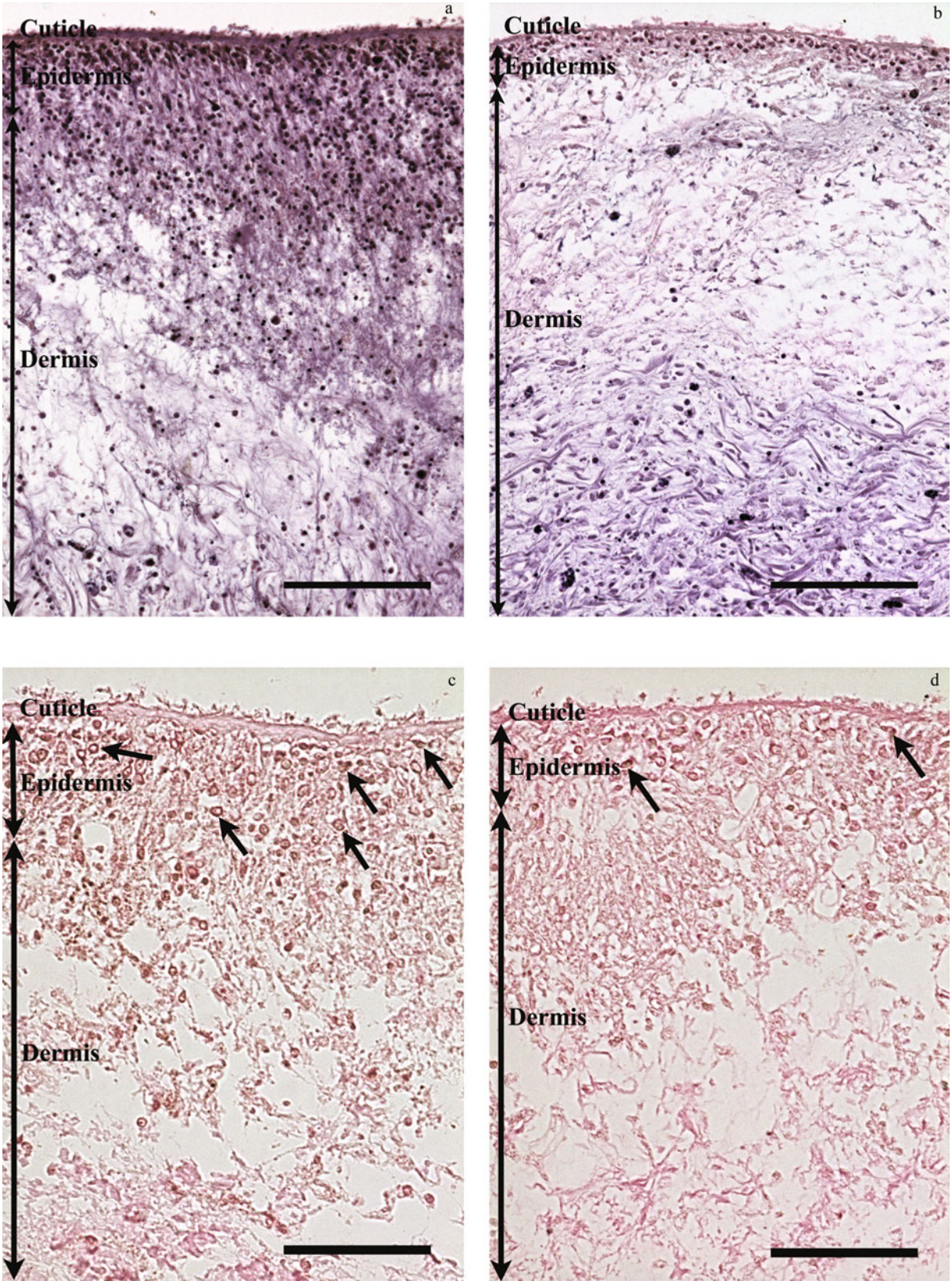

Fig.5 Histological cross section of the body wall of a normal (a, c) and albino (b, d) *Apostichopus japonicus* adult with melanocytes (arrows) stained with H&E stain (a, b) and Masson Fontana stain (c, d)

Scale bar (a, b)=100 μm, (c, d)=50 μm.

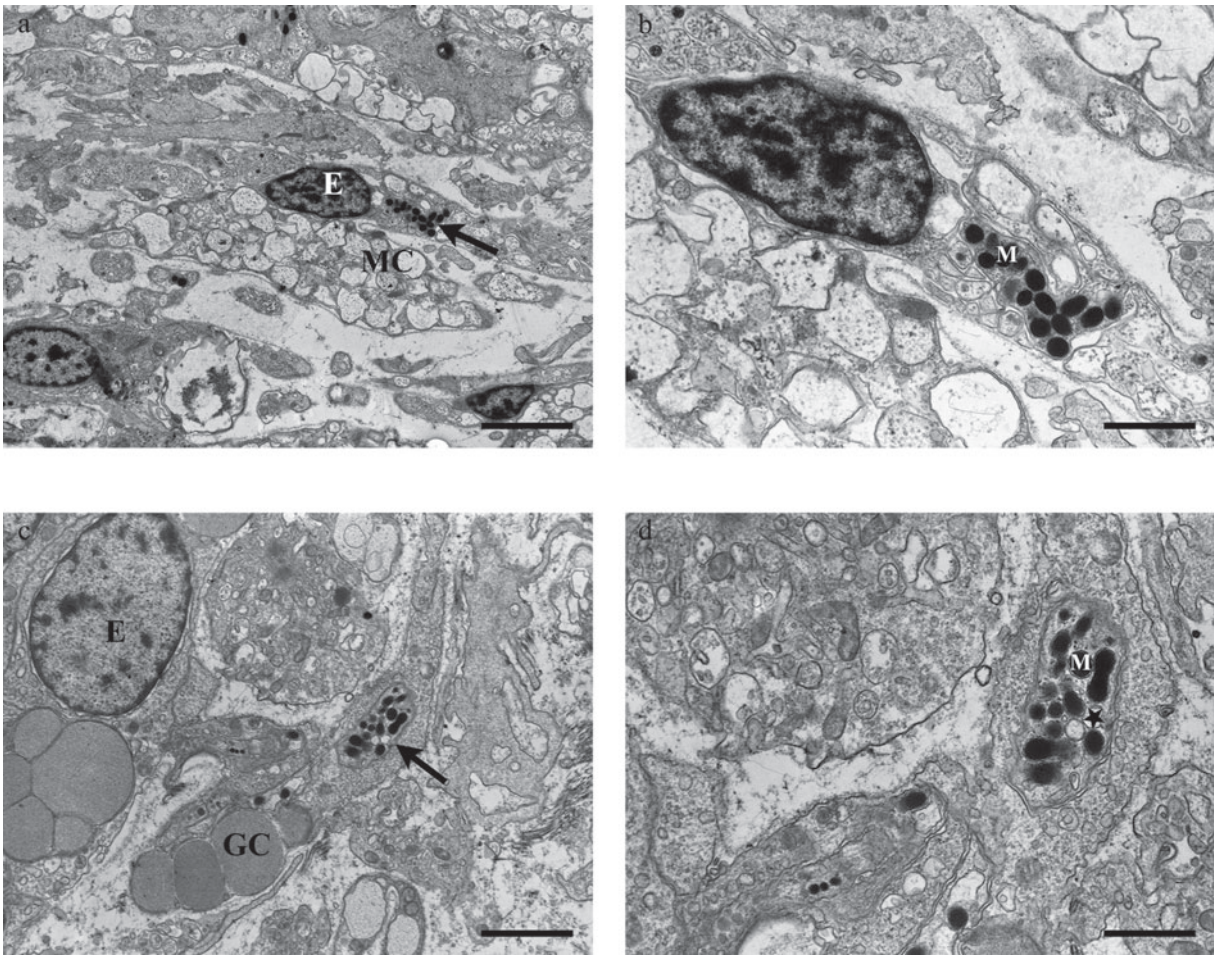

Fig.6 Electron micrograph of epidermal melanocyte (arrow) of normal (a, b) and albino (c, d) *Apostichopus japonicus* adult

M: melanosomes; E: epithelial cell; MC: mucous cell; GC: granule-containing cell; ★: melanosome vacuoles. Scale bar (a, c)=0.2 μm, (b, d)=0.1 μm.

adults, which also contained fewer epidermal melanocytes than normal adults. Because MITF plays an important role in the survival and proliferation of melanocytes in adult humans (Levy et al., 2006), it is reasonable to conclude that the reduced number of epidermal melanocytes in albino *A. japonicus* is due to the decreased expression of MITF. Furthermore, in the melanocytes of albino adults, the melanosomes contained less melanin as well as non-pigmented vacuoles. According to Wasmeier et al. (2008), non-pigmented melanosome vacuoles represent pre-melanosomes in which melanins are not yet deposited. MITF regulates the expression of the tyrosinase (TYR) gene family through a CATGTG promoter sequence (Jiri and Jan, 2010). Therefore, in albino *A. japonicus* adults, the decrease in expression of the MITF gene likely down-regulated the expression of the TYR gene family, leading to a reduction in the ability of albino individuals to synthesize melanin.

The expression of MITF mRNA was significant lower in juvenile albino *A. japonicus* than in normal juveniles 32 d after fertilization. By 88 d after fertilization, we observed fewer melanocytes in the epidermis of these albinos. MITF is essential for the development of embryonic melanocytes in the mouse (Opdecamp et al., 1997). The slow development of melanocytes during the early stages of melanogenesis in albino *A. japonicus* juveniles is likely associated with the decrease in MITF expression. In addition, electron microscope observations of albino juveniles revealed that their melanocytes contained less developed melanosomes than those in normal juveniles. We also observed very little deposition of melanin in the melanosomes of albino juveniles. As the development and melanization of melanocytes is

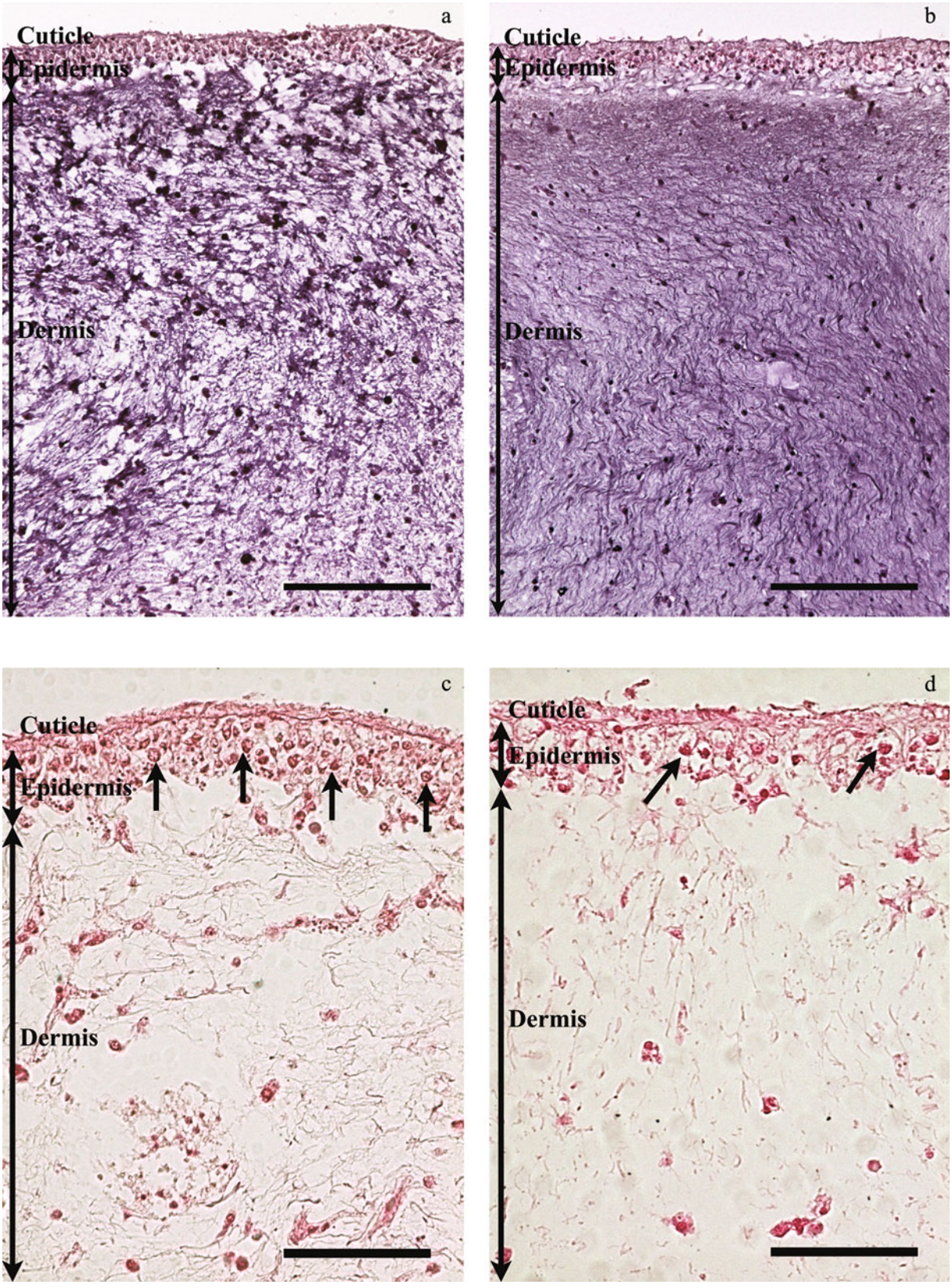

Fig.7 Histological cross section of the body wall of normal (a, c) and albino (b, d) *Apostichopus*

A. japonicus juvenile with melanocytes (arrows) stained with H&E stain (a, b) and Masson Fontana stain (c, d). Scale bar (a, b)=100 μm, (c, d)=50 μm.

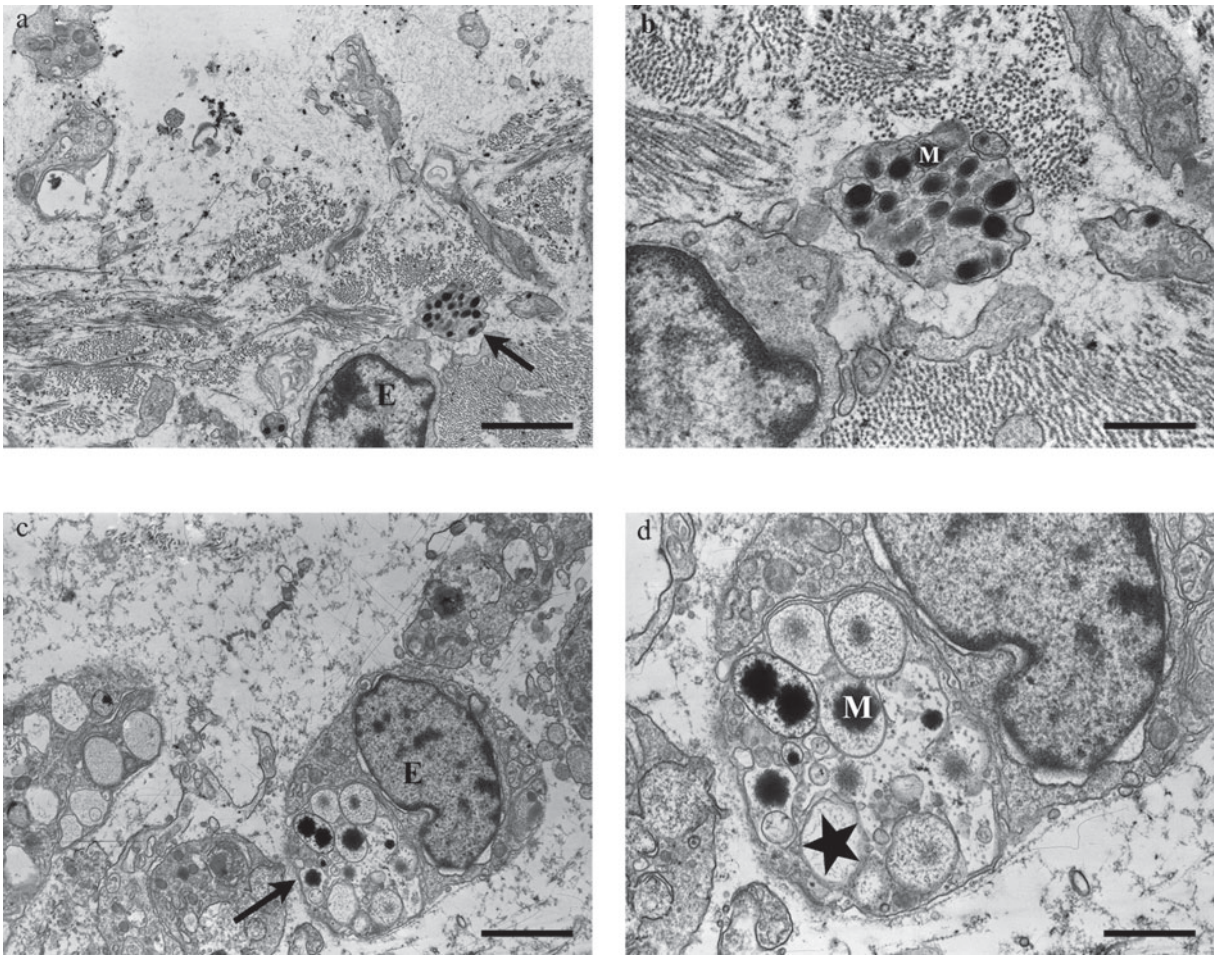

Fig.8 Electron micrograph of epidermal melanocyte (arrow) of normal (a, b) and albino (c, d) *Apostichopus japonicus* juvenile

M: melanosomes; E: epithelial cell; ★: melanosome vacuoles. Scale bar (a, c)=0.2 μm, (b, d)=0.1 μm.

dependant on activation of the MITF gene (Ichiro et al., 2003), the lack of melanocytes and melanin in albino juvenile offspring is likely caused by lower MITF expression from an early stage of melanogenesis.

A number of signaling molecules are thought to be involved in the regulation of MITF expression. For example, α-MSH (α-Melanocyte Stimulating Hormone) up-regulates the transcriptional activity of MITF via a cAMP dependent pathway whereas Asp (Agouti signal protein) down-regulates the MITF expression and inhibits melanoblast differentiation (Aberdam et al., 1998). Similarly, WNT (Wingless in *Drosophila*, Int in mouse) is an essential signaling molecule that regulates the derivation of melanocytes from neural crest cells and can induce MITF expression (Takeda et al., 2000). Previous 454 sequencing analysis suggested that the frequency distribution of the WNT contig was higher in the normal *A. japonicus* transcriptome contig library than in that of albino *A. japonicus*, suggesting that WNT mRNA expression is lower in albino *A. japonicus*. Therefore, the down-regulation of MITF in albino *A. japonicus* is likely caused by decreased expression of the WNT gene. However, the regulatory pathway/s for MITF gene expression remain poorly understood in *A. japonicus*, and so deserve further attention.

5 CONCLUSION

We isolated and characterized the full-length MITF cDNA from *A. japonicus*. Compared with normal adults, albino adults exhibited: (1) significantly lower expression MITF in the body wall; (2) fewer melanocytes in the body wall; and (3) melanocytes containing melanosomes with less or no melanin. Compared with normal juveniles, albino juveniles exhibited: (1) significantly lower

MITF expression 32 d after fertilization; (2) fewer epidermal melanocytes; and (3) melanocytes containing less developed melanosomes. We conclude that albino individuals have fewer melanocytes and a poor ability to synthesize melanin, likely because of the significantly lower expression of MITF.

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