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Genetics and evolution of plumage color in Crested Ibis: Analysis of the melanocortin-1 receptor (*MC1R*)

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Abstract

The melanocortin-1-recepter gene (*MC1R*), an important regulator in melanin synthesis, may cause different plumage color patterns in birds: gain-of- function mutations lead to the synthesis of eumelanin, whereas loss-of-function mutations help to generate pheomelanin synthesis. We had chosen *MC1R* as a candidate gene for the depigmentation of crested ibis, cloned and sequenced the crested ibis *MC1R* gene the first time. The crested ibis *MC1R* sequence, highly conserved with other birds during evolution, had seven transmembrane domains which played an indispensable function through evolution. We did not found any substitution on this sequence among all the sample individuals. The phylogenetic tree showed that crested ibis separated early in the evolution of birds. *TYR*, *TYRP1*, *TYRP2* and *MC1R* were expressed in blood and the expression of the four genes showed no significant difference ($p>0.05$) between normal and albinism individuals, and this result demonstrated that melanic pigments are not involved in the production of red pigmentation in birds. Further study of the crested ibis albinism should focus on analyzing carotenoid-based genes.

Key words: Cloning, Molecular characterization, Phylogenetic tree, Expression pattern.

Introduction

Crested ibis (*Nipponia nippon*), one of the most endangered birds in the world, has an important ornamental value and nests in a small part of China now. The distinctive crested ibis has red facial skin and legs. Non-breeding adults are white while breeding adults have grey head, neck, mantle and scapulars. Plumage phenotype plays significant roles in specific signaling (1, 2), speciation, camouflage and sexual selection. However, albino phenomenon has appeared in a small group of crested ibis in Yangxian (China) in recent years. The color of crested ibis's cheek and shins skin turned red to white, along with the same change of feathers' color below the flight plumage. The genetic changes underlying phenotypic evolution are noticeable in this regard, and ecologists and biologists have been extensively studied avian plumage polymorphisms, the units at which natural selection can act. Crested ibis could benefit from research into its genetic diversity as a tool for conservation in the future.

Abundant avian research achievements lay the foundation and provide opportunities for representing evolution mechanism of birds either at the macroevolution or microevolution levels. Several candidate loci which cause phenotypic coloration have been identified in mammal and avian species (3-14). Avian phenotypic color of integument and plumage is determined by the level and ratio of two types of melanin- pheomelanogenesis (yellow-red pigments), and eumelanins (black-brown pigments) (15). The melanocortin-1-recepter gene (*MC1R*), a seven transmenbrane G-protein coupled receptor, located on the Extension (*E*) locus as a candidate gene in vertebrate. *MC1R* is an important regulator in melanin synthesis, and it regulates the expression of eumelanin or pheomelanin with melanocyte-stimulating hormone (*MSH*) in feather bud and hair follicle melanocytes (4, 7, 16, and 17). *ASIP*, encoded by the Agouti (*A*) locus, acts as an antagonist of *MC1R* by invalidating the activity of *MSH* (18-20). Eumelanin synthesis is due to the stimulation of *MC1R* by *MSH*, while pheomelanin synthesis is caused by the absence of *MSH* or inhibition of *MSH* (17). After Takeuchi found mutations of *MC1R* may cause different plumage color patterns in 1996, multiple researches have widely characterized *MC1R* in birds from that time on. According to these studies, sequence variants in *MC1R* are associated with plumage phenotypic coloration in several species: bananaquit (6), Lesser Snow Geese, Arctic Skua (8), Red-footed Booby (9), Swans (21), Flycatchers (22), Eleonora's Falcon (23), and Gyrfalcon (24). These *MC1R* mutations have shown to determine the synthesis of two types of melanin: gain-of-function mutations which lead to the synthesis of eumelanin, whereas loss-of-function mutations help to generate pheomelanin synthesis. Previous study indicated that the function of *MC1R* had been conserved among different vertebrate taxa during evolution (6), so amino acid mutations in *MC1R* may also have conserved influence in distantly species. The Glu92Lys mutation, a mutation associated with generating eumelanin synthesis in mice, is also relevant to increase the production of eumelanin in chickens and bananaquits (5, 6, and 25).

Albinism is caused by a lack of pigmentation in eye, hair, and skin (26). In birds, the role of *MC1R* has been associated with melanism. The alternative phenotype (not melanic) has sometimes been called white (24). It has been indicated that albinism and melanism in

most avian species were caused by *MC1R* mutations (9, 27). There are some examples of MC1R being related to some degrees of depigmentation in beach mice and zebrafish (28, 29). We were interested in the depigmentation of crested ibis, and we choose *MC1R* as a candidate gene in this study to find out whether sequence variations exists in ibis' *MC1R* and whether these variations correspond to the ibis' albinism. We also checked the different expressions of several genes which may involve in melanin synthesis among crested ibis with different phenotype. The data regarding the *MC1R* of crested ibis has not been reported so far. In the present study we gained full *MC1R* coding sequence (CDS) of crested ibis, and did corresponding structural analysis research and phylogenetic study. This study would represent a basic step to understand the molecular bases for the avian plumage and skin tinctorial evolution.

Materials and methods

Animal Source and DNA/RNA Preparation

The blood samples were collected by random selection from 40 crested ibises (33 normal morphs, 7 albinism morphs) reared in Yangxian Shaanxi, China. Blood samples were collected in tubes containing acid citrate dextrose (ACD) (30mg/ml), with the volume proportion of 1: 9 (ACD: Blood samples). Genomic DNA was isolated from blood samples and stored at -80°C, following standard procedures (30). Total RNA was extracted from 6 blood samples (3 from red ventral plumage individuals and the other 3 from white ventral plumage individuals) with Trizol Reagent (Invitrogen) and further treated with RNase-free DNaseI (Promega, USA). The quality of RNA samples was assessed by 1% agarose gel electrophoresis based on the integrity of 28S and 18S RNA bands and the spectrophotometric method with an A260 nm / A280 nm ratio from 1.8 to 2.0.

Primers and PCR Amplification Conditions

Three pairs of primers (Table 1) for amplifying MC1R entire coding region of crested ibis were designed from the genomic DNA of chicken MC1R (GenBank gene ID: AB201629). Amplification was performed in a 12 μL reaction volume as follows: 25 ng genomic DNA, 0.25 μM of each primer, 1 × Buffer (including 1.5 mM $MgCl_2$), 200μM dNTPs and 0. 25 units of Taq DNA polymerase (MBI). The first cycling protocol was 2 min at 95°C, followed by 36 cycles of 94°C for 30s, annealing temperature (Table 1) for 30s, 72°C for 40s, and ending with a final extension at 72°C for 10 min.

The expression of Tyrosinase (*TYR*), tyrosinase-related protein 1 (*TYRP1*), tyrosinase-related protein 2 (*TYRP2*), *MC1R* were measured by Real-time Quantitative PCR because they play essential roles in melanin biosynthesis. PrimeScript RT reagent Kit (TaKaRa) was used to invert total RNA to cDNA according to manufacturer's protocols. All the gene-specific primers were designed from the mRNA of chicken and their position were shown in Table. qPCR analysis was performed on GenAmp7300 (Applied Biosystems) thermocycler with SYBR Green PCR kit (TaKaRa), using β-actin gene as endogenous control. Each sample reaction was performed in triplicate with the same cycling condition: first denaturation at 95°C for 2 min, followed by 40 cycles of 95°C denaturation for 15s, 60°C annealing for 20s.

The relative changes of *TYR*, *TYRP1*, *TYRP2* and *MC1R* genes in two different phenotypes were calculated using $2^{-\Delta\Delta Ct}$ method (31). Real- time PCR data were obtained as C_t value. The amounts of these four mRNAs were normalized to the endogenous control β-actin. The data were expressed as mean ±SEM. The differences were considered significant at $P < 0.05$.

Sequence and Statistical Analysis

The ibis *MC1R* sequence was analyzed using the TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/, 32) and other programs provided by NetNGlyc 1.0, NetOGlyc 3.1 (33), and NetPhos 2.0 (34).

The similarity of MC1R gene was searched using BLAST program at web server of NCBI. Multiple alignments were performed using the MegAlign program of Lasergene. The diverse avian *MC1R* genes were aligned using amino acid sequences by Clustal X sequences alignment program (35). Neighbor-joining algorithms method of MEGA 4.0 (Molecular Evolutionary Genetic

Table 1. Primers used for *MC1R* gene cloning and quantitative RT-PCRs of *TYR*, *TYRP1*, *TYRP2* and *MC1R* in crested ibis.

Primer		Sequence (5'-3')	Position (nt)	Reference Sequence	Annealing temp. (°C)	Fragment size (bp)	Remark
MC1R-1	F	CGGGGCCATGTCGATGCTG	41-59	AB201629	64	83	MC1R cloning
	R	CCGTGGCGTTGCTCTGGTT	105-123				
MC1R-2	F	ATGCCAGTGAGGGCAACCA	91-109	AB201629	55.4	857	MC1R cloning
	R	CTGGCTCCGGAAGGCATAGAT	927-947				
MC1R-3	F	GGCCCTTCTTCTTCCACCTC	802-821	AB201629	61	191	MC1R cloning
	R	CTACCAGGAGCACAGCACC	974-992				
TYR-QRT	F	TGGGGAGTGCAAGTTTG	408-424	NM_204160	60	191	TYR qRT-PCT
	R	TGGAGCCGTTGTTCATCT	581-598				
TYRP1-QRT	F	CCTGGATGGACTGGACCTACCT	405-426	NM_205045	60	126	TYRP1 qRT-PCT
	R	GTGGATTGTCACCTTGGCTTGG	510-530				
TYRP2-QRT	F	GAAGCCACCAGTTGTCAGGAAG	554-575	NM_204935	60	143	TYRP2 qRT-PCT
	R	GACCAAGCAGACTCATCCAGTG	675-696				
MC1R-QRT	F	GTGAGGGCAACCAGAGCAA	50-68	NM_001031462	60	173	MC1R qRT-PCT
	R	GAAGTAGTACATGGGCGAGTG	202-222				
BCDO2-QRT	F	TGGTCCTGGGAAGTTTGAGTT	282-302	KF206120	60	155	BCDO2 qRT-PCT
	R	GGTTGTGCTGGCTGTTAGTCA	416-436				
β-actin	F	ACGTCGCACTGGATTTCGAG	721-740	NM_205518	60	282	qRT-PCT
	R	TGTCAGCAATGCCAGGGTAC	983-1002				

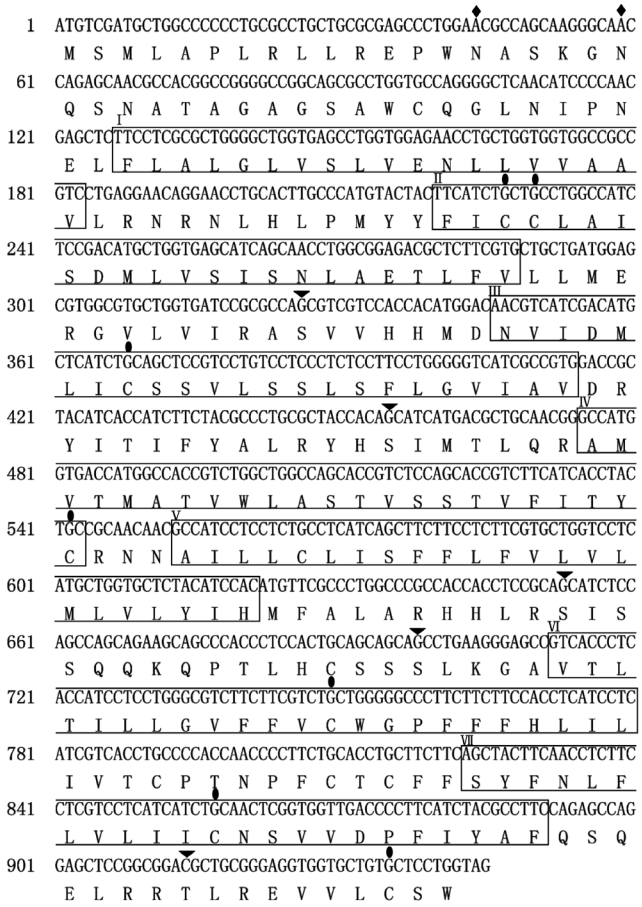

Figure 1. Sequence of the crested ibis melanocortin receptor 1 gene. The seven transmembrane domains are enclosed by solid lines. The potential N-glycosylation consensus sites, phosphorylation sites and palmitoylation sites are marked with diamonds, triangles and ovals respectively.

Analysis) program (36) was used to generate a phylogenic tree. Bootstrap analyses were conducted using 1,000 replicates. Except the crested ibis *MC1R*, all *MC1R* sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Their GenBank accession numbers are: *Coturnix japonica* (AB201633), *Meleagris gallopavo* (GU905062), *Gallus gallus* (AB201629), *Coereba flaveola* (AF362604), *Anas platyrhynchos* (HQ190952), *Malurus leucopterus* (AY614610), *Anser canagica* (FJ170062), *Babax lanceolatus* (JN635728), *Zosterops japonicus* (JN635726), *Pellorneum ruficeps* (JN635724), *Cairina moschata* (EU877265), *Falco rusticolus* (JQ290361), *Buteo jamaicensis* (HM454192), *Catharacta skua* (AY521219), *Stercorarius parasiticus* (AY521216). MC1R genes of *Mus musculus* (NM_008559), *Homo sapiens* (NM_002386), *Sus scrofa* (NM_001008690), and *Bos taurus* (JQ004019) served as outgroup to root the tree.

The expression of *TYR*, *TYRP1*, *TYRP2* and *MC1R* results of different groups are tested for significance by the Pair Wise Fixed Reallocation Randomisation Test© (37).

Results

Cloning and molecular characterization of MC1R in the Crested Ibis

Sequences were compared and matched to other *MC1R* sequences in GenBank and finally edited in Seqman. A 945 bp long coding region, encoding a protein of 314 amino acids, was obtained after our analysis (Fig. 1). There was no variable nucleotide site in crested ibis *MC1R* gene among all samples. The Melanocortin-1 Receptor of the mallard is the most similar (95%) to the Melanocortin-1 Receptor of the crested ibis, followed by that of the cape barren goose, the black swan and the quail (each 94%) then the chicken (93%), the bananaquit (92%) and the lesser Antillean tanager (91%).

The ibis *MC1R* coding showed seven hydrophobic domains that can be treated as the transmembrance parts in its cellular structure. Analysis of the amino acid sequences indicates that all these transmembrance domains are conserved during evolution, especially the sixth region. Using NetNGlyc 1.0, we identified two potential N-glycosylation consensus sites in the N-terminal extracellular region of the crested ibis *MC1R*: N15 and N20 (Fig. 1). We found five phosphorylation sites in the crested ibis amino acid sequence (S^{109}, S^{152}, S^{218}, S^{233} and T^{305}) using the NetPhos 2.0 server and all of these sites were in intracellular domains. Seven potential sites were found for palmitoylation (C^{76}, C^{77}, C^{123}, C^{181}, C^{250}, C^{286} and C^{312}) by CSS-Palm 3.0.

Homology and phylogenetic analysis of putative amino acid sequence of MC1R

The ibis *MC1R* coding sequence showed high sequence identity with the homologous sequences in a variety of birds (Fig. 2). The transmembrane were highly conserved in the avian species that we compared. The amino acid sequence of crested ibis *MC1R* was aligned with *MC1R*s of other birds by BLASTP search (http://www.ncbi.nlm.nih.gov/blast/), revealing the same highest homology among quail, turkey, chicken with the

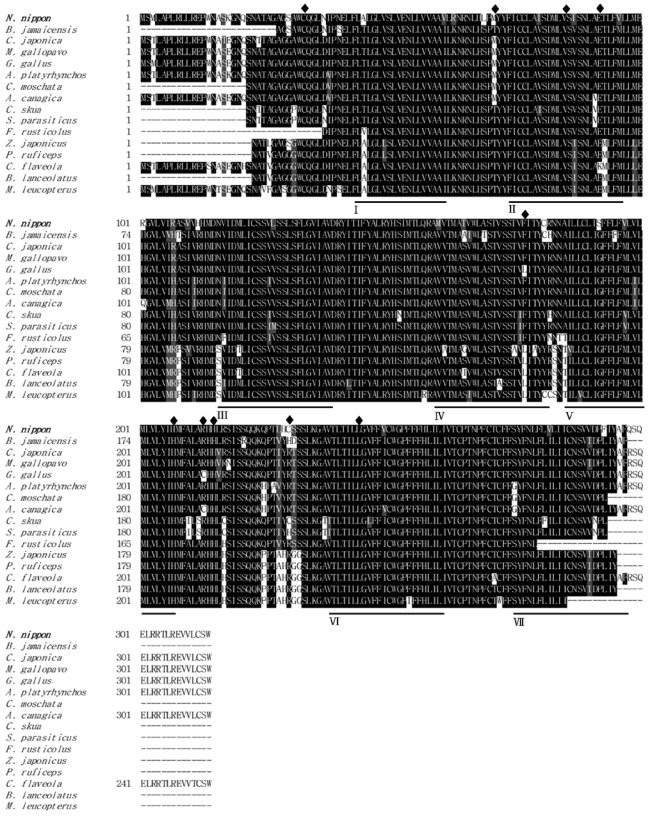

Figure 2. Comparison of the crested ibis (*N. nippon*) melanocortin 1-receptor amino acid sequence with several avian sequences. The putative transmembrane domains are marked by underlines and Roman numerals. Amino acid residues which have essentially functional roles are marked by diamonds.

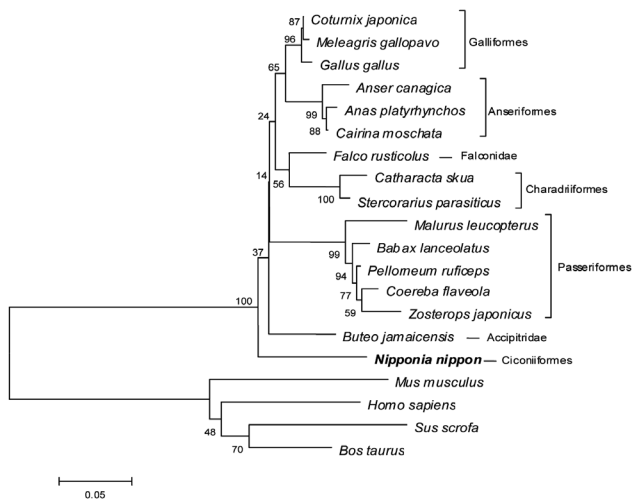

Figure 3. Phylogenetic tree of *MC1R* in birds. Numbers in the branches represent the bootstrap values (%) from 100 replicates. *MC1R* genes from *Mus musculus*, *Homo sapiens*, *Sus scrofa*, *Bos taurus* served as outgroup to root the tree. For GenBank accession numbers, refer to "Materials and methods".

identity of 90%, followed by mallard (89%) and Muscovy duck (89%). Crested ibis *MC1R* was 88, 88, 88, 87, 87, 86, 85, 85, 85, and 85% identical to red-tailed hawk, emperor goose, gyrfalcon, great skua, arctic skua, puff-throated babbler, Japanese white-eye, bananaquit, babax, and white-winged fairywren *MC1R*s at the amino acid level, respectively (Fig. 2).Based on an alignment of amino acid sequence of crested ibis *MC1R* and those of other birds and mammals, a phylogenetic tree of *MC1R* was constructed using the neighbor-joining method. (Fig. 3) The tree indicated that crested ibis's *MC1R* fitted in the subgroup of birds, and crested ibis separated early in the evolution of birds.

Expression pattern of TYR, TYRP1, TYRP2 and MC1R in normal morphs and albinism morphs

Real-time quantitative PCR was performed to examine gene expression of *TYR*, *TYRP1*, *TYRP2*, and *MC1R* in blood of two phenotypes of crested ibis. The result shows that all 4 genes were expressed in blood. *TYRP1* and *MC1R* were higher expressed in albinism group while *TYR* and *TYRP2* were slightly higher expressed in normal group. The expression of *TYR*, *TYRP1*, *TYRP2* and *MC1R* results of different groups are tested for significance by the Pair Wise Fixed Reallocation Randomisation Test© and all of these four genes showed no significant difference ($p>0.05$) between normal and albinism individuals (Fig. 4), indicating that the expression of these four genes may has no direct relation to the changing of ibis plumage color.

Discussion

In our comparisons of various sequences, the *MC1R* sequence of crested ibis was most similar to that of mallard (95%), followed by cape barren goose (94%), black swan (94%), quail (94%) and chicken (93%). Multi-species sequence comparisons show that the seven transmembrane domains in the crested ibis *MC1R* coding sequences are highly conserved among birds (Fig. 2), indicating that they may have conserved and indispensable functions through evolution. Previous

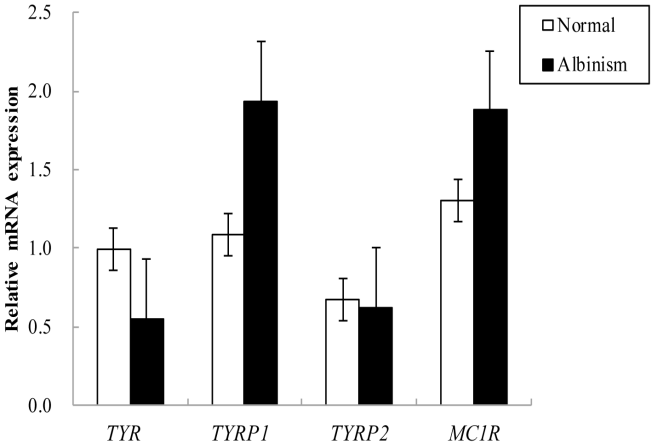

Figure 4. The expression of *TYR*, *TYRP1*, *TYRP2* and *MC1R* genes in blood samples from different phenotypes.

relevant studies have proved that several amino acid residues (C^{33}, M^{71}, V^{85}, E^{92}, F^{177}, H^{207}, C^{213}, H^{215}, R^{230}, and L^{244}) in avian *MC1R*s have essentially functional roles in variation of plumage and skin color (6, 8, 9, 21-24). The comparison of multi-sequences showed that five of these important sites were completely conserved in the avian evolution. One of these residues occurred in the extracellular region which acts an important role in ligand binding and regulation of *MC1R* activity; four of these residues occurred in the intracellular regions which are essential in protein signal transduction whereas five of these residues occurred in the transmembrane domains which maintain the structural integrity of the protein (10) (Fig. 2).

MC1R is one of the crucial regulators of the ratio of eumelanin and pheomelanin content in hairs and feathers: gain-of-function mutations lead to black coat color while loss-of-function mutations associate with yellow or red color (25, 38, and 39). Previous studies of *Chaetodipus intermedius*, *Phylloscopus*, and *Suliformes* have found that melanism has became independently through variation at *MC1R* and other loci such as *Agouti*, and *MC1R* is not related to color variation in these species (10, 18, 40-43). In this study, we did not find any substitution in crested ibis *MC1R* gene, indicating that there was no evident genetic exchange during ibis evolution. Based on our finding and previous studies, we conclude that *MC1R* is not correlated with the two phenotypic patterns of plumage and skin coloration among crested ibis.

The evolutionary relationship of avian groups remains contentious as there might be a rapid divergence in their early evolutionary period (44, 45). Some studies suggested that Galliformes and Anseriformes have the same ancestor and the Galliformes separated early in the evolution of birds (46). In this study, the phylogenetic tree of *MC1R*s obviously indicates that Galliformes and Anseriformes actually are sister taxa and had closely relationship, but it seemed that the divergence of crested ibis predated that of chicken (*Gallus gallus*) in our phylogenetic tree (Fig. 3). *Buteo jamaicensis* (Accipitridae) and *Falco rusticolus* (Falconidae) formed distinct clades in our analysis (Fig. 3) which was consistent with a former theory that Falconidae and Accipitridae are not stemmed from a monophyletic Falconiformes (47),

Two types of melanins, pheomelanins (yellow–red pigments) and eumelanins (black–brown pigments),

play vital roles in the pigmentation of plumage and pelage (48, 49, and 50). *TYR*, *TYRP1*, and *TYRP2* were directly involved in the process of melanogenic pathway (51). The partial coding sequence of crested ibis has been cloned in our previous research in which nine sequence variants were found (52), but there was no correlation between these substitutions and the two phenotypes - normal and albinism during the next analysis. In this study, the expression of *TYR*, *TYRP1*, and *TYRP2* showed no significant difference (analysis of variance, $p > 0.05$) in normal and albinism individuals, indicating that all the three genes did not play obvious roles in this kind of albinistic phenomenon. The result is completely in line with the current understanding that melanic pigments are not involved in the production of red pigmentation in birds generally as red integumentary colors are caused by carotenoid pigments (35, 53-56). The albino ibis is also not caused by the content of *MC1R* in their body since no significantly different expression of this receptor was observed in two groups in this study (Fig.4).

In conclusion, the crested ibis *MC1R* sequence is highly conserved during evolution as it showed high similarity with other birds. All the seven transmembrane domains in *MC1R* coding sequences are highly conserved among birds, indicating that they may have indispensable functions through evolution. The divergence of crested ibis predated that of other birds in our phylogenetic tree, and this species might separate early in the evolution of birds. *TYR*, *TYRP1*, *TYRP2* and *MC1R*, indispensable elements in the synthetization of melanic pigments, were not included in the process of crested ibis albinism, and it needs to do further study to find out the main cause of this phenomenon, such as analyzing carotenoid-based genes.

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