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Molecular Cloning, Characterization and Expression Analysis of Duck Tyrosinase-Related Protein-1

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Abstract: The tyrosinase family is known to be crucial in the melanin biosynthetic pathway and is responsible for the rate limiting step. In the present study, the complementary DNA (cDNA) of TYP1 was cloned from the eye of duck by homology cloning and rapid amplification of cDNA ends (RACE) approaches. The full-length cDNA of TYP1 consisted of 2123 nucleotides, containing an Open Reading Frame (ORF) of 1608 bp that encoding a 536 amino-acid peptide, a 5'-terminal Untranslated Region (UTR) of 255 bp and a 3'-terminal UTR of 260 bp with two canonical polyadenylation signal sequence (AATAAA) and a poly(A) tail. The phylogenetic tree display that TYP1 protein is highly conserved and the deduced peptide shares 70.9-93.7% similarity with quail, chicken and mammalian TYP1 proteins. The Semiquantitative RT-PCR analysis indicated that the transcripts of TYP1 mRNA had the highest expression in eyes and black hair follicle, intermediate in white hair follicle and negligible or absent in skin, muscle, heart, liver, kidneys, spleen, lungs, brain and intestine. The study may useful for the further study on polymorphism and correlation on duck feather color and the function of TYP1 of birds.

Key words: Cloning, tyrosinase-related protein-1, duck, expression analysis, skin, China

INTRODUCTION

Melanin is a crucial pigment of the animal eye, skin and coat visible color formation and its synthesis is catalyzed by the enzymes of tyrosinase family. In vertebrates, the Tyrosinase-related Protein (TYP) gene family encompasses three members, Tyrosinase (TYR), Tyrosinase-related Protein-1 (TYP1) and TYP2, identified as Dopachrome Tautomerase (DCT). The three proteins are expressed by three distinct genes, share remarkable sequence homology but evolved different function in the regulation of melanin synthesis. TYR is the critical, rate-limiting enzyme of melanogenesis and its activity affects the type and quantity of melanin production. TYP2 is catalyses the non-decarboxylative tautomerization of l-dopachrome to 5, 6-Dihydroxyindole-2-Carboxylic Acid (DHICA) in the melanin biosynthetic pathway (Del Marmol and Beermen, 1996).

TYP1 is a protein within the melanocyte that modifies the color of the skin and hair of animals (Jackson, 1988). Recently, most researchers are focused on the association between different mutations of TYP1 and coat or skin color. The loss or reduction of function mutations of TYP1 have been identified in many mammal species. In human, a point mutation in the coding region of TYP1 gene is responsible for one genetic type of human oculocutaneous albinism (OCA3) (Boissy *et al*., 1996). In mouse, TYP1 was proved to be the product of the mouse brown locus (Jackson, 1988) and exhibits a novel DHICA oxidase activity (Kobayashi *et al*., 1994a, b). Similarly, mutation in TYP1 also has association with brownish coat color in dog, cat, cattle and Soay sheep (Schmutz *et al*., 2002; Berryere *et al*., 2003; Schmidt-Kuntzel *et al*., 2005; Gratten *et al*., 2007). In birds, a single nucleotide substitution in TYP1 has a perfect association with sex-linked roux phenotype in Japanese quail (Nadeau *et al*., 2007; Minvielle *et al*., 2009).

TYP1 is actually the first cloned color gene (Shibahara *et al*., 1986; Jackson, 1988) and the sequence of TYP1 has been partially or totally cloned and sequenced in several vertebrates. Although, chicken TYP1 has been cloned and sequenced (April *et al*., 1998), the report on the cloning of duck plumage color related gene is limited. Recently, in duck breeding, a huge variety of distinct plumage color patterns have been observed (Gong *et al*., 2010) Interestingly, when pure white plumage female Liancheng ducks cross with white male Baigai

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ducks (a kind of crossbreeding offspring between Peking duck and White Tsaiya) the plumage of their offspring appeared the phenotype of black back and white abdomen. But the formation mechanism of plumage color remain poorly understood. In this study, we present the full-length TYP1 cDNA from duck and its evolutionary relationship among other vertebrates. Furthermore, the expression pattern of TYP1 gene in various tissues was investigated which may provide information on further study of the function of TYP1 and the plumage color formation of ducks.

MATERIALS AND METHODS

Animals and tissue collection: Three healthy black back and white abdomen ducks (16 weeks) were selected from a cross-population of white female Liancheng ducks and white male Baigai ducks which were provided by the Huang pi Limited liability company. All the ducks were provided normal management and natural daylight. The ducks were anesthetized with ether and killed by the blooding of jugular veins. Various tissues including heart, liver, spleen, lung, kidney, brain, skin, muscle, intestine, eye, white hair follicle and black hair follicle were surgically removed, immediately frozen in liquid nitrogen and stored at -80°C until total RNA extraction.

RNA extraction and cDNA synthesis: The total RNA were isolated from heart, liver, spleen, lung, kidney, brain, skin, muscle, intestine, eye, white hair follicle and black hair follicle of three ducks by using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol.

The amount of total RNA was estimated by Spectrophotometer ND-1000 (Nano-Drop, USA). The first-strand cDNA was synthesized from 1 μg of DNase-treated (TOYOBO CO., DNaseI) total RNA according to M-MLV reverse transcriptase kit (TOYOBO, Japan) at 42°C. The cDNA was used as the template for PCR reactions in gene cloning and expression profile analysis.

Cloning and sequencing of TYP1 cDNA fragment: Based on conserved regions in other TYP1 sequences, including Gallus gallus (NM-205045), Mus musculus (NM-031202), human (NM-000550) and quail (AB005228), primers were designed using the primer design procedure, Oligo 6.0 and Primer premier 5.0 to amplify duck TYP1 cDNA fragment from eye (Table 1). The PCR was performed in a final volume of 15 μL, containing 50-300 ng cDNA come from duck eye, 30 μM of each primer, 0.1 mM deoxynucleoside triphosphate, 2.5 mM MgC_{12}, 10 x buffer, 0.5 U of DNA polymerase (TransGen Biotechnology Company, Beijing, P.R. China) on an thermal cycler (Applied Biosystems, Foster City, CA). The PCR reaction mixtures were subjected to 35 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 30 sec. After a 5 min final extension at 72°C, the products were visualized on a 1.2% agarose gel using ethidium bromide staining.

PCR products were carefully excised from the agarose gels, followed by purification with a TransGen gel extraction kit (TransGen Biotechnology Company, Beijing, P.R. China). The PCR purified products were ligated and subcloned into the PEASY-T1 plasmid vector (TransGen Biotech) according to manufacturer's protocol. Clones were selected by blue-white screening, DNA sequencing was performed in Augct Company (Beijing, China) using an automated ABI3730 analyzer (Applied Biosystems, Foster City, CA, USA).

Rapid amplification of 3'and 5'cDNA ends (RACE): Two pairs of Gene-Sequence Primers (GSP) and Nested Gene-Sequence Primers (NGSP) were designed based on the above PCR product sequences which were subsequently used to design primers for 5'-RACE and 3'-RACE to obtain the entire TYP1 cDNA sequence. For 3'-RACE and 5'-RACE PCR, 10 mg of RNA isolated from eye was used and the RACE reactions were performed by using SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, CA, USA) according to the manufacturer's protocols. About 10 pmol of 10x Universal Primer A Mix

Table 1: Primers used for RT-PCR, RACE and semi-quantitative RT-PCR

Primer name	Primer sequence (5'-3')	Temperature (°C)	Function
TYP1-1F	AATGAGATGTTTGTTACTG	-	RT-PCR
TYP1-1R	ACTGATCAGTGAGAAGAGG	-	
NUP	AAGCAGTGGTATCAACGCAGAGT	-	RACE
UPM	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT	-	RACE
GSP1	CAGAAAACCTGGGATACAGCTATGA	68	3'-RACE
GSP2	TTGATTCGTTGGCTACAGGTAGG	-	5'-RACE
NGSP1	CCAGGGGGCTCTCCATGTAA	60	3'-RACE
NGSP2	CGCGCAATGATAACCAGAGA	-	5'-RACE
TYP1-2F	AATGAGATGTTTGTTACTG	53	sqRT-PCR
TYP1-2R	ACTGATCAGTGAGAAGAGG	-	
β-actin-F	AACTGGGATGACATGGAGAAGA	60	sqRT-PCR
β-actin-R	ATGGCTGGGGTGTTGAAGGT		

Table 2: The TYP1 mRNA and protein GenBank accession numbers of different species

Species	mRNA	Protein
Quail	AB005228	BAA89535
Chicken	NM-205045	NP-990376
Human	NM-000550	AAC15468
Mouse	NM-031202	AAH76598
Cattle	AF400250	AF445638
Sheep	NM-001130023	ACF21681
Pig	AB207240	ADB96155
Horse	NM-001081840	NP-001075309

(UPM) and the GSP1 and GSP2 were used in the first 3'-RACE and 5'-RACE PCR, respectively. PCR cycling parameters were: 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 3 and 5 min at 72°C for the final extension. About 1 μL of PCR products from the first run were used as template in the second nests PCR run with NUP and NGSP as primers. The temperature program included: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 35 sec, annealing at 60°C for 35 sec and extension at 72°C for 1.5 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels. 3'-RACE and 5'-RACE PCR products were gel-purified and sequenced as described earlier.

Cloning and sequencing of PCR products: The nucleotide and deduced amino acid sequences of TYP1were analyzed using BioEdit (version 7.0.1) software package and EXPASY search program. The sequences of different species were compared with the NCBI BLAST search program (Table 2). The Phylograms were created by MEGA4.0 Neighbor-Joining (NJ) software with 1000 bootstrap trials after multiple alignment of sequence data by CLUSTALW (Thompson *et al*., 1994; Edgar, 2004; Tamura *et al*., 2007). In addition, signal peptide and transmembrane sequences were predicted using Phobius.

RT-PCR analysis of TYP1 from different tissues: To determine the distribution of duck TYP1 in various tissues, semi-quantitative RT-PCR was conducted for expression analysis. The TYP1 gene-specific primers (TYP1-F2, TYP1-R2) were designed based on the obtained cDNA sequence (Table 1). The conditions for PCR were: denature at 94°C for 5 min, followed by 25-36 cycles of 30 sec at 94°C, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The control reactions using the gene-specific primers to duck β-actin (GenBank accession no: EF667345) were conducted with 38 cycles for PCR amplification from the same cDNA samples. All experiments were repeated three times.

The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide and visualized with ultraviolet light and band intensity was analyzed by using Quantity one software (Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

Sequence analysis of TYP1: Using consecutive techniques of RT-PCR and RACE, a full length of TYP1 cDNA is 2123 bp, containing an Open Reading Frame (ORF) of 1608 bp which encoding a 536 amino-acid peptide with a predicted molecular mass of approximately 60.62 kDa and theoretical isoelectric point of 5.66. The full-length nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1. The cDNA contained a 5'-terminal Untranslated Region (UTR) of 255 bp nucleotides, a 3'-terminal UTR of 260 bp nucleotides including a TGA termination codon (nucleotides 1865-1867) and two putative polyadenylation consensus signals (AATAAA) and a poly(A) tail. The Prosite software analysis indicated that there existed a putative signal peptide of 23 amino acids (position 1-23 aa) and a predicted mature protein of 512 amino acids (position 24-536 aa). Furthermore, there are six potential N-glycosylation sites (N-X-S/T) and a possible transmembrane region (454-476 bp) in the amino acid sequence of the mature protein (Fig. 1).

Multiple sequence alignments and phylogenetic relationship: The alignment results showed that duck TYP1 shares a high identity with the nucleotide sequences of chicken (90.9%), quail (89.2%), human (76.1%), pig (76.1%),cattle (75.7%), sheep (75.6%), horse (74.9%) and mouse (74.5%).

The deduced amino acid sequence of duck TYP1 indicated significant sequence identities to TYP1 of other species, including quail (93.7%), chicken (93.3%), panda (77.1%), human (76.6%), pig (76.1%), cattle (75.9%), sheep (75.9%), mouse (75.8%) and horse (70.9%). A amino acid sequence alignment of the duck TYP1 with other species is shown in Fig. 2 showing the sequence identities ranged from 70.9-93.7%. Based on the phylogenetic analysis, the duck TYP1 appears to be closely related to that of quail and chicken which is similar with the result of the BLAST.

Expression of TYP1 mRNAs in tissues: To determine the TYP1 gene expression levels in different tissues, simi-quantitative RT-PCR method was performed. The agarose gel electrophoresis of the PCR products for TYP1 and β-actin from individual samples showed that fragments of 208 and 164 bp were obtained, respectively (Fig. 3a, b).

The Simi-quantitative RT-PCR results showed highest expression of TYP1 gene in eye and black hair follicle whereas expression was lower in white hair follicle. Negligible or no expression of TYP1gene was observed in skin, muscle, heart, liver, kidney, spleen, lung, brain and intestine (Fig. 3c).

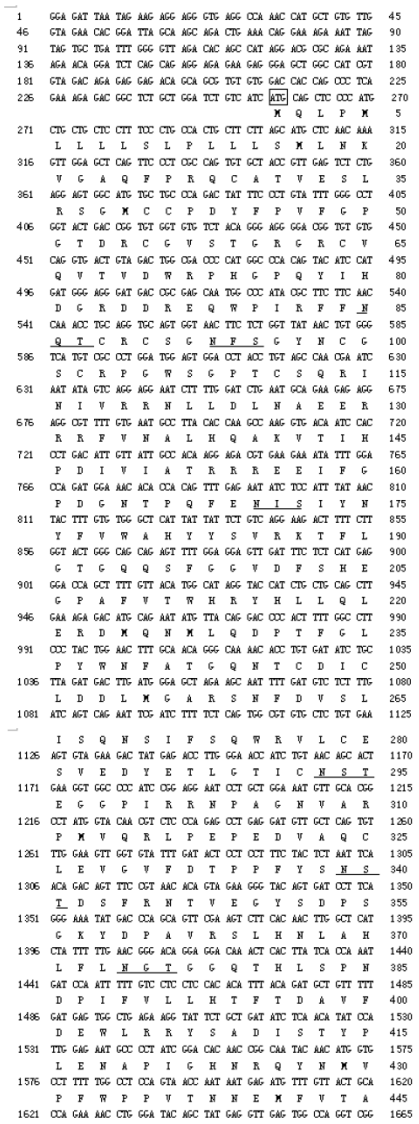

Fig. 1: Full-length nucleotide sequence and deduced amino acid sequence of duck TYP1 gene.The letter in the box indicates the start codon (ATG), the stop codon (TGA) is indicated with an asterisk, the potential N-glycosylation sites are underline and the polyadenylation signal sequence (AATAAA) are underlined and in bold. The transmembrane region is shaded

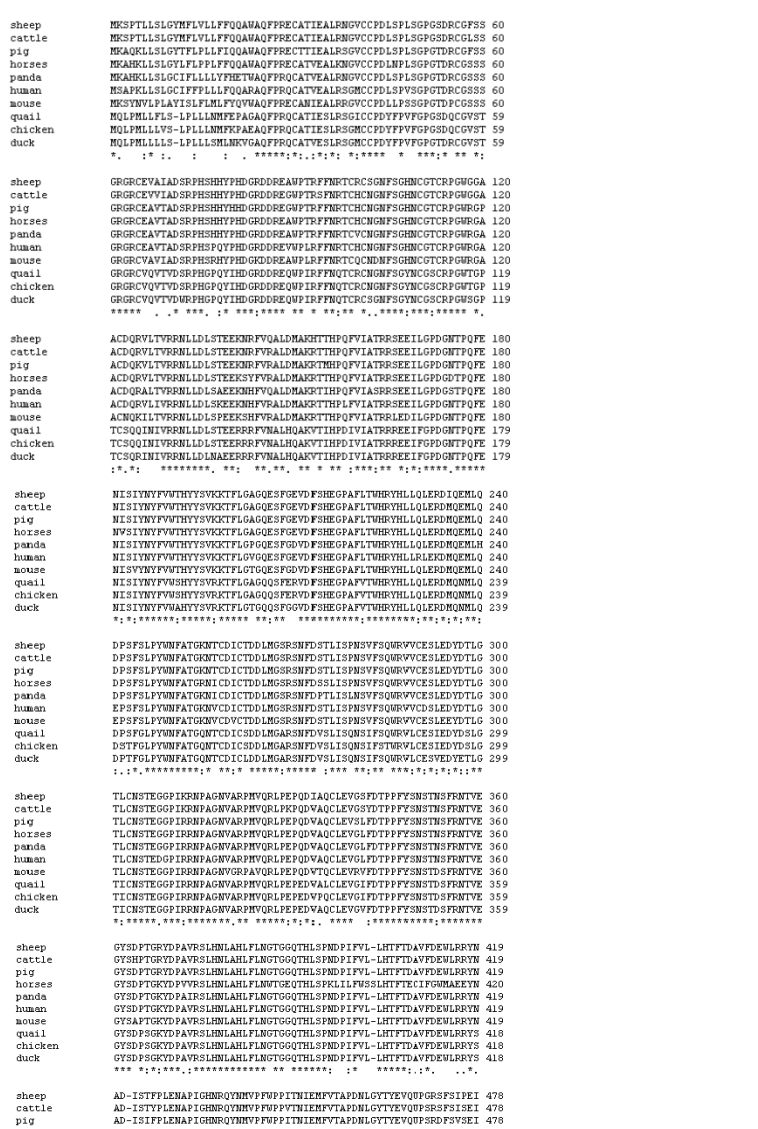

Fig. 2: Amino acid sequence alignment of the predicted duck TYP1 with those of others species. The TYP1 proteins sequence of other species were derived from the NCBI GenBank

Coloration and color patterning belong to the most diverse phenotypic traits in animals. They are available genetic markers and play important roles on the breeding and product quality evaluation. In human and mouse,

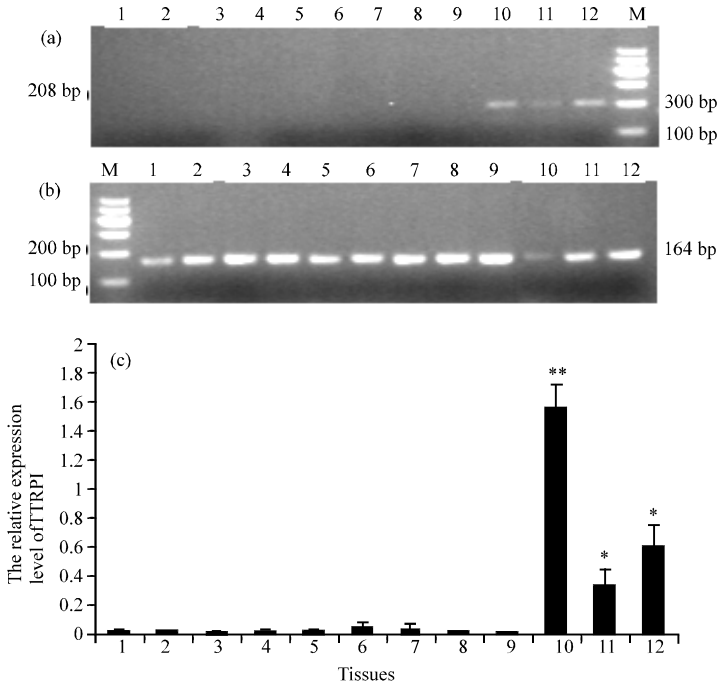

Fig. 3: Phylogenetic analysis of TYP1. Accession numbers for TYP1 proteins are shown in Table 1. The phylogenetic tree was performed by the Neighbor-Joining (NJ) method of MEGA 4.0. The bootstrap percentage from 1000 replicates is indicated at each node

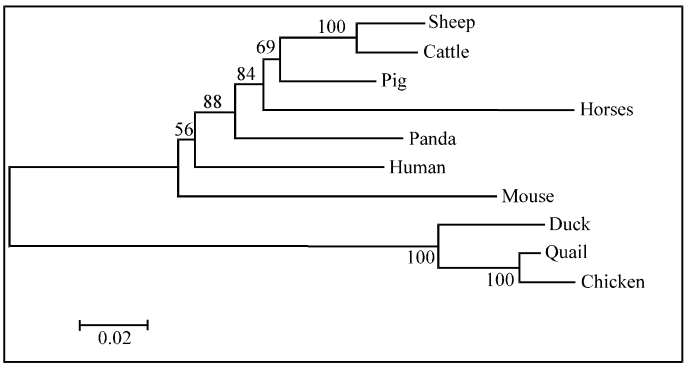

Fig. 4: Expression of duck TYP1 mRNA in different tissues; (a) The expression profile of TYP1 in duck; (b) The expression profile of β-actin in duck; (c) The expression level of TYP1 transcript in different tissues Lane 1-12 delegate heart, liver, spleen, lungs, kidneys, muscle, skin, brain, lintestine, eye, white hair follicle and black hair follicle, respectively. M, marker1. Significant differences were indicated with an asterisk at $p<0.05$ and with two asterisks at $p<0.01$

some color gene including MCIR, TYR, TYP1, TYP2, KIT, MITF, etc. have been cloned and the associations between color and gene mutation have been studied. In birds, TYP1 is the first sex-linked pigmentation gene to be identified.

To date, the full-length chick TYP1 cDNA was isolated (April *et al*., 1998) and an associations of a Phe282 Ser mutation of TYP1 with roux quail was revealed, however, the study on pigmentation gene of duck is lagging behind. In the present study, the full-length cDNA encoding of TYP1 was successfully cloned from duck.

Alignment analysis indicated that duck TYP1protein has high homology to those of other vertebrates, the same protein length and the highest amino acid sequence identity with quail TYP1 protein. Whilst the full coding

sequence of TYP1 in duck is 3 bp longer than in chicken (1611bp) and 90.9% similar to the chicken sequence at the nucleotide level (Fig. 4). On the other hand, duck TYP1 amino acid shares 93.3% identities to chicken TYP1 amino acid, furthermore, they have the same potential N-glycosylation sites, splice site and transmembrane region (April *et al*., 1998). The phylogenetic tree displayed that the duck TYP1 protein is highly conserved and has closely evolutionary relationships with that of quail and chicken. Therefore, cloning of TYP1 gene could be useful for the further study on polymorphism and correlation on duck feather color.

In mammalians, TYP1 is one of melanocyte-specific gene that is expressed in both melanocytes and the retinal epithelium (RPE), where it is involved in the distal eumelanic pathway (Murisier *et al*., 2006, 2007). In human cell lines, TYP1 was only detectable in cells containing eumelanin (Del Marmol and Beermen, 1996). In birds, plumage melanin is synthesised in the melanocyte of hair follicle. Study has demonstrated that there are two duplicates of TYP1 gene in medaka, the expression of the two duplicates mainly detected in the retinal pigment epithelium and in melanophores of the body and have time and space differences (Braasch *et al*., 2006). In this study, we initially detected the expression of TYP1 mRNA in different tissues of adult duck.

Simi-quantitative RT-PCR result indicated that there is high relative expression of duck TYP1 in the retinal pigment epithelium and melanophores, primarily in eyes and black hair follicle, weak expression in white hair follicle.

This is similar to the detecting result of the relative TYP1 expression in dark and light sheep which showed that TYP1 was downregulated in light sheep (Gratten *et al*., 2007). There was a very low level expression in skin, muscle, heart, liver, kidney, spleen, lung, brain and intestine. To the knowledge, the expression difference may lie in the absence of melanocyte or relate to the development of melanocyte in different period.

CONCLUSION

In this study, this is the first report on cloning of duck TYP1 gene. The data indicated TYP1 play an important role on the process of duck plumage pigementation.

Therefore, the study may high light on the further study of the function of TYP1 and the plumage color formation of birds.

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