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Sequencing and Homological Analysis of Silver Fox *TYR* Gene

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ABSTRACT

To explore the sequence structure of fox *TYR* gene and it's mechanism to regulate the pelage color's dividing. In this study, the partial DNA sequence (1370 bp) of silver fox *TYR* gene was obtained by PCR amplification and direct sequencing. This sequence contained the intron 1 of 368 bp, the complete exon 2 of 217 bp and the intron 2 of 785 bp and GC content was 40.5%. Based on the sequences of *TYR* gene exon 2, the homological analysis and the construction of un-rooted phylogenetic tree were carried out among silver fox and the other 16 species by using DNAMAN software and Clustalx (1.83) software, respectively. The homological analysis showed that silver fox had the closest genetic relationship with dog and the closer genetic relationship with domestic cat and domestic ferret. The cluster result of un-rooted phylogenetic tree was similar to that of the homological analysis. The length of *TYR* gene exon 2 were 217 bp for all analyzed species, which indicated that *TYR* gene exon 2 were highly conservative in phylogeny. The above results can provide the important biological information for further researching the development and regulation mechanism of fox coat color which result from *TYR* gene.

Key words: Silver fox, *TYR* gene, species, homological analysis, cluster analysis

INTRODUCTION

Tyrosinase is the key regulatory enzyme of melanogenesis and it can catalyzes the rate-limiting steps of melanin biosynthetic pathway and plays a major role in animal coat color (Del Marmol and Beermen, 1996; Aigner *et al*., 2000; Chen *et al*., 2012). The *TYR* gene belongs to the *TYR* gene family, the members of which (tyr, typ-1 and typ-2) encode the tyrosinase, tyrosinase related protein 1 and 2, respectively (Garcia-Borron and Solano, 2002). The mammalian *TYR* gene is composed of 5 exons and 4 introns and coding sequence has a length of 1.6 kb (Aigner *et al*., 2000). The function mutations of the *TYR* gene were shown to result in mammalian albinism phenotype, which were confirmed in human (Spritz *et al*., 1990; Oetting and King, 1999), cattle (Schmutz *et al*., 2004), domestic cat (Schmidt-Kuntzel *et al*., 2005) and rhesus monkey (Zhang, 2000; Ding *et al*., 2000) and so on.

The silver fox, a variant of the red fox (*Vulpes vulpes*) (Kukekova *et al*., 2004) originated from Alaska of North America and the eastern region of Siberia and it has become a major breed of valuable fur animals through the artificial breeding and training of more than 100 years. The hair coat of silver fox is mixed black and white and have a layer guard hair like fog. Silver-blue fox and

blue-silver fox are the hybrids of silver fox and blue fox. Crystal fox, golden island fox and agate fox are the hybrids of silver fox and red fox. All of the hybrids above have concentrated the fine qualities of silver fox. For example, there are thin, unbending, short and smooth guard hairs and uniform distributed some silvery hair in the hybrids coat. The fur of fox are one of the major ornament of clothes for modern people in winter.

In recent years, some reports have focused on structure of the *TYR* gene and more attention has been focused on expression, polymorphism analysis of the *TYR* gene in many species. Yang *et al.* (2006) studied the correlation between *TYR* gene polymorphism and coat color of sheep, suggesting that *TYR* gene could be a functional gene affecting the synthesis of coat color of sheep. Gao *et al.* (2008) reported that *TYR* expression level might be related to coat color of alpaca. Chen *et al.* (2012) reported that *TYR* expression level was positively correlated with the degree of gray in Jining gray goat. Previous investigations of the *TYR* gene focused on its variation in sheep, goat and alpaca and little attention was paid to other special economic animals, especially to fox.

In the study, the partial DNA sequence of silver fox *TYR* gene was obtained by PCR amplification, sequencing and sequences alignment and this sequence was further analyzed. The results could provide the biological information to further study the *TYR* gene expression, corresponding protein structure and regulation mechanism of fox coat color.

MATERIALS AND METHODS

Sample preparation and extraction of genomic DNA: Skin samples were collected from silver fox by biopsy at the Golden Island fox breeding farms, Changli County, Qinhuangdao City, Hebei Province, samples were stored in liquid nitrogen.

Genomic DNA was isolated from each 100 mg skin sample of silver fox according to the standard phenol: chloroform extraction method and stored at -20°C.

Primer design, PCR amplification and sequencing: The fragment (1405 bp) of silver fox *TYR* gene containing partial intron 1, complete exon 2 and partial intron 2 was amplified using a pair primer designed by Primer 3 based on dog sequence from GenBank (NW_876273). The forward and reverse primers were 5'- TGTGAGAGCCAAAAGAGAAGA -3' and 5'- CTGAAGGAAAGAGCAAGTAGGA -3'), respectively and they were synthesized by Shanghai Sangon Biological Engineering and Technology services Co. Ltd (Shanghai, China).

PCR amplification was carried out in a Biometra personal PCR instrument with a total volume of 50 μL reaction containing 1 μL (75 ng μL^{-1}) of silver fox genomic DNA, 5 μL of 10×PCR standard reaction buffer (Mg^{2+} Plus), 4 μL deoxynucleoside triphosphates (2.5 pmol L^{-1} of each deoxynucleotide), 2 μL (10 pmol L^{-1}) of each forward and reverse primer, 0.5 μL (5 U μL^{-1}) of Taq DNA polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China) and 35.5 μL of distilled water. After predenaturation for 5 min at 94°C, the PCR profile consisted of a denaturation step at 94°C for 45 sec, an annealing step at 58°C for 45 sec and an elongation step at 72°C for 1 min for a total of 34 cycles, followed by a final extension of 10 min at 72°C. The PCR products were detected on 1.5% agarose gel including 0.5 μg mL^{-1} of ethidium bromide, photographed under UV light and sequenced by Shanghai Sangon Biological Engineering Technology, Biological and Technology and Service Co., Ltd. (Shanghai, China).

Sequences analysis and database search of *TYR* gene: Sequence of the silver fox *TYR* gene was examined and edited using the BioEdit version 7.0.5.2 (Hall, 1999). Searches for the other sequence similarity were performed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). A total of 16 sequences with the exon 2 of the *TYR* gene belonging to 16 species were searched from GenBank. Alignment and homological analysis of sequences were performed using DNAMAN software. The un-rooted phylogenetic tree among species was constructed using Clustalx (1.83) software.

RESULTS

PCR amplification, sequencing and identification of silver fox *TYR* gene: The 1405 bp fragment of silver fox *TYR* gene was obtained by PCR amplifying (Fig. 1). By alignment of sequences and detecting chromatogram of nucleotide sequences, we were able to analyze 1370 bp bases because sequencing of bases from 10145 to 10184 were not successful, according to the reference sequence (NW_876273).

The alignment result revealed that the obtained sequence had 92.3% identity and the corresponding region of dog *TYR* gene (NW_876273), which suggested that the sequence was the partial sequence of silver fox *TYR* gene. Further analysis showed that the sequence contained the intron 1 of 368 bp, the complete exon 2 of 217 bp and the intron 2 of 785 bp and the content of A, C, G and T were 29.3, 22.8, 17.7 and 30.2%, respectively.

Homology analysis of *TYR* gene exon 2 among species: Based on the sequences of *TYR* gene exon 2, the homological analysis were carried out between silver fox and the other 16 species respectively by using DNAMAN software. Results revealed that the sequence similarity between silver fox and dog, domestic cat, domestic ferret, pig, cattle, sheep, goat, human, gorilla, baboon, gibbon, monkey, house mouse, Norway rat, chicken and goldfish were 98.6, 93.6, 92.6, 92.2, 89.5, 88.9, 88.5, 89.4, 89.4, 88.9, 88.5, 88.0, 82.0, 81.1, 76.0 and 67.3%, respectively (Table 1).

Phylogenetic analysis: Based on the sequences of *TYR* gene exon 2, the un-rooted phylogenetic tree among silver fox and other 16 species was constructed by using Clustalx (1.83) software (Fig. 2). According to Fig. 2, the phylogenetic tree was significantly divided into three major clades. The mammalian animals (including silver fox, dog, domestic ferret, domestic cat, sheep, goat, cattle, monkey, gibbon, human, gorilla, baboon, house mouse and Norway rat), goldfish and chicken were fall into a clade, respectively. Further observation found that the mammalian animals were again divided into four small clade. Silver fox, dog, domestic ferret and domestic cat fall into one small

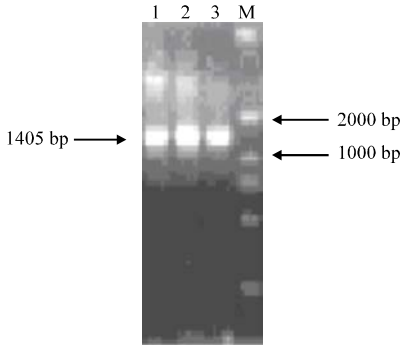

Fig. 1: PCR products (1405bp) of silver fox *TYR* gene (1-3: PCR products; M: DNA Marker DL 2000)

Table 1: Homology analysis of silver fox to other species in *TYR* gene exon 2

Species name in English	Species name	Length (bp)	Sequence similarity (%)	GenBank No.
Dog	*Canis lupus familiaris*	217	98.6	NM_001002941
Domestic cat	*Felis catus*	217	93.6	AY959315
Domestic ferret	*Mustela putorius furo*	217	92.6	EF405957
Pig	*Sus scrofa*	217	92.2	GU376733
Cattle	*Bos taurus*	217	89.5	AY162287
Sheep	*Ovis aries*	217	88.9	EU0283NO.
Goat	*Capra hircus*	217	88.5	JN862823
Human	*Homo sapiens*	217	89.4	NM_000372
Gorilla	*Gorilla gorilla*	217	89.4	AF237797S2
Baboon	*Papio hamadryas*	217	88.9	AF183584
Gibbon	*Nomascus leucogenys*	217	88.5	AF183610
Monkey	*Rhinopithecus avunculus*	217	88.0	AF183635
House mouse	*Mus musculus*	217	82.0	NM_011661
Norway rat	*Rattus norvegicus*	217	81.1	NM_001107535
Chicken	*Gallus gallus*	217	76.0	AB023291
Goldfish	*Carassius auratus*	217	67.3	DQ870906

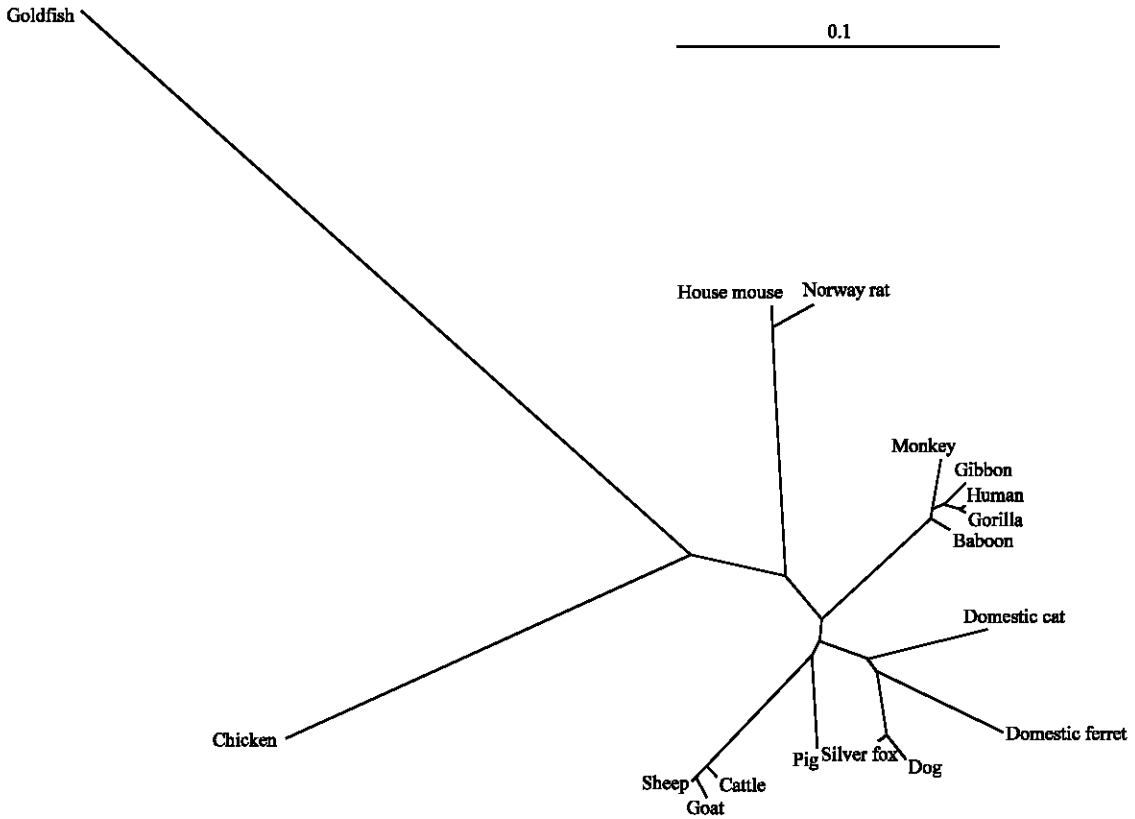

Fig. 2: Un-rooted phylogenetic tree of silver fox and other species based on the nucleotide sequence of *TYR* gene exon 2

clade. Sheep, goat, cattle and pig fall into another small clade. Monkey, gibbon, human, gorilla and baboon fall into the third small clade. House mouse and Norway rat fall into the fourth small clade.

DISCUSSION

According to *TYR* gene sequence of dog (NW_876273), PCR amplification was completed using silver fox genomic DNA as template. The obtained nucleotide sequence had highly identity to that of dog *TYR* gene (NW_876273) and the similarity was 92.3%. The alignment results preliminary showed that the obtained nucleotide sequence was the partial sequence of silver fox *TYR* gene. Further analysis indicated that the fragment contained the partial sequence of intron 1 (368 bp), the complete exon 2 (217 bp) and the partial sequence of intron 2 (785 bp) and GC content was 40.5%. In usual condition, GC content is about 40 to 45% in the vertebrate genome. Whereas, GC content was not uniform distributed in the genome, the content reached 67% in some DNA fragment, it was only about 33% in another DNA fragment (Sueoka, 1962). The genomes of warm-blooded vertebrates were a mosaic of alternating fragments, isochores, with low and high GC contents and embedded genes (Bernardi *et al*., 1985; Jaksik and Rzeszowska-Wolny, 2012). The difference of GC content in genomic, might play an important role in the regulation of gene expression and gene mutation (Bernardi *et al*., 1988). In this study, GC content (40.5%) of the obtained sequence for silver fox *TYR* gene was in the range of 40~45%. However, whether there was a close contact between the obtained sequence (particular exon 2) and functions of silver fox *TYR* gene, then need to carry out in further research. After comparing DNA sequence of exon 2 of silver fox *TYR* gene and the corresponding sequences of 16 species published in GenBank, the results showed that the similarity between silver fox and dog was the highest (98.6%), the next was domestic cat (93.6%) and domestic ferret (92.6%) and it also had higher similarities (92.2~81.1%) with pig, cattle, sheep, goat, human, gorilla, baboon, monkey, house mouse and Norway rat, etc. It is suggested that silver fox had the closest genetic relationship with dog and the closer relationship with domestic cat and domestic ferret. In addition, the length of *TYR* gene exon 2 were 217 bp for all of the species listed in Table 1, which suggested that *TYR* gene exon 2 was highly conserved. The un-rooted phylogenetic tree of silver fox and 16 other species also showed that silver fox had the closest genetic relationship with dog, then with domestic cat and domestic ferret. It is well known that silver fox and dog belong to the *Vulpes* species of canidae. Domestic cat belongs to the felis of felidae. Domestic ferret is a member of the weasel family and a descendant of the European Polecat. But the three species are all carnivores. The another small clade of un-rooted phylogenetic tree was sheep and goat clustered first and then cattle and pig were added successively. The third small clade from the mammalian animals were human and gorilla clustered first and then gibbon, monkey and baboon were added successively. The fourth clade from the mammalian animals only included two species: house mouse and Norway rat. Sheep, goat and cattle belong to ruminants. Human, gorilla, gibbon, monkey and baboon belong to primates. House mouse and Norway rat belong to rodent. Obviously, the cluster result was similar with the traditional classification in general and the species of each clade accorded with their physiological characteristics. These results were basically consistent with those of Liu *et al*. (2010, 2012) and Kang *et al*. (2008). Liu *et al*. (2012) reported that the closest relationship existing among silver fox, red fox, dog and domestic cat, between sheep and cattle, among human, chimpanzee, common gibbon and olive baboon and between house mouse and oriental house rat by constructing phylogenetic tree of MC1R gene for 19 species. In addition, Liu *et al*. (2010) drew the same conclusion that the closest relationship existing among sheep, goat and cattle, between human and rhesus monkey, between house mouse and Norway rat by constructing phylogenetic tree of MyoG gene for 12 species. Kang *et al*. (2008) also reported that the closest relationship existing among sheep, goat and cattle, between human and chimpanzee and between mouse and rat by constructing phylogenetic tree of *LF* gene for 10 species.

CONCLUSION

In this study, the partial DNA sequence (1370 bp) of silver fox *TYR* gene was obtained by PCR amplification and direct sequencing. This sequence contained the intron 1 of 368 bp, the complete exon 2 of 217 bp and the intron 2 of 785 bp and GC content was 40.5%. According to the sequence of *TYR* gene exon 2, the results of homology analysis and un-rooted phylogenetic tree both showed silver fox had the closest genetic relationship with dog. These can provide the important biological information for further researching the development and regulation mechanism of fox coat color which result from *TYR* gene.

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