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Sequencing and Homology Analysis of Intron 2 in Silver Fox Agouti Gene

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ABSTRACT

In order to explore the sequence structure of fox Agouti gene and it's mechanism to regulate the pelage color's dividing. In this study, the major part of intron 2 sequence (1038 bp) of Agouti gene from the silver fox were obtained by PCR amplification and direct sequencing. This sequence was aligned with red fox, giant panda, horse, pig, goat, cattle, sheep, domestic cat and rabbit and the sequence similarities were 100, 85.82, 75.75, 73.31, 66.22, 65.98, 65.77, 60.45 and 58.82%, respectively. The result of the homology analysis showed that the genetic relationship between silver fox and red fox was the highest, which was consistent with that they belong to *Vulpes* of the Canidae animal in traditional classification. Based on the sequence of Agouti gene intron 2, the phylogenetic tree was constructed for silver fox and the other 9 species using Clustalx (1.83) software. The cluster result of phylogenetic tree of all species was basically consistent with the taxonomy of NCBI and was similar to the physiological characteristics of the species and their traditional classification. The above results provide the important biological information for researching the mechanism of the formation mechanism of the coat color and artificially improving the coat color quality of fox and so on.

Key words: Silver fox, Agouti gene, sequencing, genetic relationship

INTRODUCTION

Coat color genetics of animal has been the subject of a large number of studies. For example, in mice, currently 378 (including 171 cloned genes and 207 uncloned genes) loci may affect pigmentation (Montoliu *et al*., 2012). Among these, Agouti gene is an important candidate gene and plays the important role in the pigment synthesis and color pattern evolution of domestic animals (Argeson *et al*., 1996; Vage *et al*., 1997; Fontanesi *et al*., 2010; Manceau *et al*., 2011). Agouti gene encodes the Agouti signalling protein (ASIP) which is involved in determining the switch from eumelanin to pheomelanin synthesis in melanocytes (Fontanesi *et al*., 2010) and it contains four exons and two introns, among which exons 2, 3 and 4 can translate 131-133 amino acids, but exon 1 can not be translated corresponding amino acids. The mRNA splicing mechanism of Agouti gene is very complex, there are different transcripts for different domestic animal (Girardot *et al*., 2005; Drogemuller *et al*., 2006; Fontanesi *et al*., 2010). It is known that the light-bellied-Agouti A^w

phenotype in mice is established by differential expression of ventral specific transcripts (1A, 1A' and 1AA'), as well as dorsal specific transcripts (1B and 1C) that differ only by their 5' UTR (Vrieling *et al*., 1994). Transcription analysis in wild type Agouti rabbits of revealed the presence of two major transcripts with different 5'-untranslated regions having ventral or dorsal skin specific expression (Fontanesi *et al*., 2010).

In recent years, some reports have focused on structure of the Agouti gene and more attention has been focused on expression, polymorphism analysis of the Agouti gene and the interactions between Agouti gene and the other coat color related gene (MC1R gene) in many species. Vage *et al*. (1997) reported that a deletion in the coding exon of the fox Agouti gene was found associated with the proposed recessive allele of Agouti in the darkly pigmented Standard Silver fox. In the fox the proposed extension locus is not epistatic to the Agouti locus. Vage *et al*. (2005) suggested that the MC1R/Agouti regulatory system was involved in the seasonal changes of coat color found in arctic fox. Argeson *et al*. (1996) showed that Agouti expression levels were positively correlated with the degree of yellow pigmentation in individual A^{hvy} mice.

The silver fox, a variant of the red fox (*Vulpes vulpes*), is a close relative of the dog (*Canis familiaris*) (Kukekova *et al*., 2004). The second intron sequences of red fox have issued in GenBank database (GenBank accession No. AJ250364), while that of silver fox have not been reported so far.

In this study, the major part of intron 2 sequence of the silver fox Agouti gene was obtained by PCR, direct sequencing and aligning. The total number of 10 Agouti gene sequences with the intron 2 from 10 species were studied to investigate its evolution and genetic relationship among species.

MATERIALS AND METHODS

Extracting genomic DNA: Genomic DNA from spleen samples of the silver fox was isolated according to the standard phenol: chloroform extraction method and stored at -20°C.

Primer design and PCR amplification: The 1088 bp fragment of Agouti gene intron 2 was amplified using forward primer: 5'- TCAAACATGCTCTCCAGGCT -3' and reverse primer: 5'-GATAAGAGGGGTTGGCTGGA-3', in 50 μL reaction mixture containing 1 μL (75 ng μL) of silver fox genomic DNA, 5 μL of 10×LA PCR Buffer II (Mg^{2+} Plus), 8 μL deoxynucleoside triphosphates (2.5 pmol L^{-1} of each deoxynucleotide), 1.0 μL (20 pmol L^{-1}) of each forward and reverse primer, 0.5 μL (5 U μL) of TaKaRa LA Taq® DNA polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China) and 33.5 μL of distilled water. Amplification was carried out with denaturing at 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 15 sec and 72°C for 1 min and ended with extension incubation at 72°C for 10 min. The amplified fragment spanned bases from 91 to 1178 including the most of the sequence of Agouti gene intron 2 (according to AJ250364). PCR products were detected on 1.5% agarose gel including 0.5 μg mL^{-1} of ethidium bromide, photographed under UV light.

PCR products were purified using TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (Code No. DV805A; TaKaRa Biotechnology Co. Ltd., Dalian, China) and sequenced directly with two PCR primers and one inner primer: 5'-CAAGGCGGACATTACAGGAC-3'. The amplification primers and one inner primer were designed based on red fox Agouti gene sequence (AJ250364) with Oligo 6 software (Molecular Biology Insights, Inc., Cascade, Colo.).

Sequence analysis and database search of Agouti gene: Sequence of the silver fox Agouti gene was examined and edited using the BioEdit version 7.0.5.2 (Hall, 1999) and DNAMAN software. Searches for the other sequence similarity were performed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). A total of 9 sequences with the intron 2 of the Agouti gene belonging to 9 species were searched from GenBank. All the sequences were aligned using the ClustalW program implemented in BioEdit version 7.0.5.2. The phylogenetic tree among species was constructed by Clustalx (1.83) software.

RESULTS

PCR amplification, sequencing and identification of silver fox Agouti gene: The 1088 bp fragment of silver fox Agouti gene was obtained by PCR amplifying (Fig. 1). By alignment of sequences and detecting chromatogram of nucleotide sequences, we were able to analyze 1038 bp bases only from 141 to 1178 because sequencing of bases from 91 to 140 was not successful, according to the reference sequence (AJ250364).

The alignment result revealed that the obtained sequence had 100% identity and the corresponding region of red fox intron 2 (AJ250364) (Fig. 2), which suggested that the obtained sequence was the sequence of intron 2 of silver fox Agouti gene.

Homology analysis of Agouti gene intron 2 among species: Homology analysis of Agouti gene intron 2 were carried among silver fox and the other species using DNAMAN software. Result revealed that the sequence similarity between the silver fox and the red fox, the giant panda, the horse, the pig, the goat, the cattle, the sheep, the domestic cat and the rabbit were 100, 85.82, 75.75, 73.31, 66.22, 65.98, 65.77, 60.45 and 58.82%, respectively (Table 1).

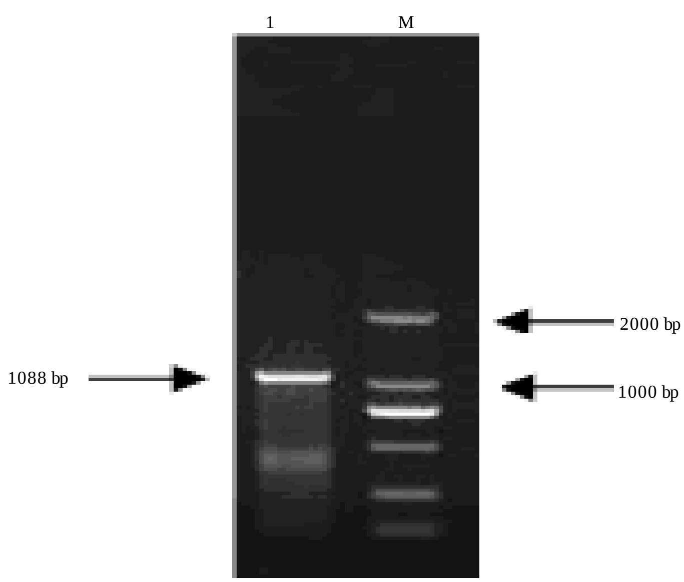

Fig. 1: PCR product (1088 bp) of fox Agouti gene, 1: PCR product, M: DNA marker DL 2000

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>emb|AJ250364.1|  Vulpes vulpes partial agouti gene for Agouti protein, exons
Length=1305

 Score = 1914 bits (1036),  Expect = 0.0
 Identities = 1038/1038 (100%), Gaps = 0/1038 (0%)
 Strand=Plus/Plus

Query  1     CTCCTTTGTGCTTATTCTTCAAAGTAATCCTACATGAATAAAGTATTCTTTGACTCATAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  141   CTCCTTTGTGCTTATTCTTCAAAGTAATCCTACATGAATAAAGTATTCTTTGACTCATAT  200

Query  61    GGTTTGGAAAAGCCCTGCATACCACCAATCTAATATGCAGCTTCACATGTTAGAGGCTCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201   GGTTTGGAAAAGCCCTGCATACCACCAATCTAATATGCAGCTTCACATGTTAGAGGCTCT  260

Query  121   GAGAAATCCAGAAGTCAAGACCCTCTGTGAATTTGGTTTAACTTAGCATTTCTGAAACTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261   GAGAAATCCAGAAGTCAAGACCCTCTGTGAATTTGGTTTAACTTAGCATTTCTGAAACTT  320

Query  181   ACTGAATCACAGAACTGTCAACAGTAAAAATTTTTGAAAATGCTGCAGAATATAAACAAG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321   ACTGAATCACAGAACTGTCAACAGTAAAAATTTTTGAAAATGCTGCAGAATATAAACAAG  380

Query  241   TGTGGGATGAATTAGTCTTTTCAGTGTTGAGGACTGAGAATGACTGCTCAGGGGAAAACA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381   TGTGGGATGAATTAGTCTTTTCAGTGTTGAGGACTGAGAATGACTGCTCAGGGGAAAACA  440

Query  301   TCAGGCACATTAAAGCCTGGCATTAAACATCTTCTAGTTCAACTTCATCTGATAAAAATA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441   TCAGGCACATTAAAGCCTGGCATTAAACATCTTCTAGTTCAACTTCATCTGATAAAAATA  500

Query  361   ATAGTAAGAGATATACTCTTTGTGTGCTGGGCCTGTGGAAGTGAGTACTATTTTTATTCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501   ATAGTAAGAGATATACTCTTTGTGTGCTGGGCCTGTGGAAGTGAGTACTATTTTTATTCC  560

Query  421   CTAGTTNACCAGTGAGGGAAATTGAGGCTGAGCCAAGTTAACTGACTAACCATGAGATCA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561   CTAGTTNACCAGTGAGGGAAATTGAGGCTGAGCCAAGTTAACTGACTAACCATGAGATCA  620

Query  481   CACAGTCTTAACTGGCCGGGCCCAGAATTCAACCCTGGGTGTTTTAAATTCCCAGAATCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  621   CACAGTCTTAACTGGCCGGGCCCAGAATTCAACCCTGGGTGTTTTAAATTCCCAGAATCC  680

Query  541   AAATTCAAAACTAATATCCCTCACTGCTTTGCCTCTCACTAGGAAAAGATAATATTCTAG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  681   AAATTCAAAACTAATATCCCTCACTGCTTTGCCTCTCACTAGGAAAAGATAATATTCTAG  740

Query  601   ATGTATCGGGAAGTAACTATCACCTTTGATCAACCTTGATGTGCCAGACTTCTCACTTTA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  741   ATGTATCGGGAAGTAACTATCACCTTTGATCAACCTTGATGTGCCAGACTTCTCACTTTA  800

Query  661   TAAACATACCTTGTTTATCCTCAAGACAACCCTGCAAGGCGGACATTACAGGACCCTATT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  801   TAAACATACCTTGTTTATCCTCAAGACAACCCTGCAAGGCGGACATTACAGGACCCTATT  860

Query  721   TGCAGATGAGAAAACTGGGCTTAAGGATATTAAGTAAGTTGCCTAAGCAAACACAGCTGT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  861   TGCAGATGAGAAAACTGGGCTTAAGGATATTAAGTAAGTTGCCTAAGCAAACACAGCTGT  920

Query  781   TATATGGCAGAGTCAGGATTCAAATCAGGTGCCTGGCTCCATAGGTCTGACAAATGAAAA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  921   TATATGGCAGAGTCAGGATTCAAATCAGGTGCCTGGCTCCATAGGTCTGACAAATGAAAA  980

Query  841   ATAGGAATATAGAAAACACAGGTCCCTTCATGTACATGATACTGAGTCCATGTTTCAGGG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  981   ATAGGAATATAGAAAACACAGGTCCCTTCATGTACATGATACTGAGTCCATGTTTCAGGG  1040

Query  901   CCTTCTTGGATTTCCTCTTTGCCCTCCTTCAGAGGACATTTTTCTTTCAGTCCCTGGCTC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041  CCTTCTTGGATTTCCTCTTTGCCCTCCTTCAGAGGACATTTTTCTTTCAGTCCCTGGCTC  1100

Query  961   CCAGTCAGCAGCCAGACTAGTAAGAACCTGTTGGCCTAAGTTCTCAAGTTGTGATTCATC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1101  CCAGTCAGCAGCCAGACTAGTAAGAACCTGTTGGCCTAAGTTCTCAAGTTGTGATTCATC  1160

Query  1021  CAGCCAACCCCTCTTATC  1038
             ||||||||||||||||||
Sbjct  1161  CAGCCAACCCCTCTTATC  1178
```

Fig. 2: BLAST result of Agouti gene intron 2 for silver fox (Query) and red fox (AJ250364) (Sbjct)

Table 1: Homology analysis of silver fox to other species in intron 2 of Agouti gene

Species name			
English	Latin	Nucleotide similarity (%)	GenBank No.
Red fox	*Vulpes vulpes*	100.00	AJ250364
Giant panda	*Ailuropoda melanoleuca*	85.82	DQ321816
Horse	*Equus caballus*	75.75	AF288358
Pig	*Sus scrofa*	73.31	AY916525
Goat	*Capra hircus*	66.22	EF587236
Cattle	*Bos taurus*	65.98	X99691
Sheep	*Ovis aries*	65.77	EU420022
Domestic cat	*Felis catus*	60.45	AY237394
Rabbit	*Oryctolagus cuniculus*	58.82	AM748788

Phylogenetic analysis: Based on the sequence of Agouti gene intron 2, the phylogenetic tree was constructed for silver fox and the other 9 species using Clustalx (1.83) software. The silver fox, the red fox, the giant panda and the domestic cat, fall into one small clade. The goat, the sheep, the cattle, the pig and the horse, fall into another small clade. The rabbit alone was a clade (Fig. 3).

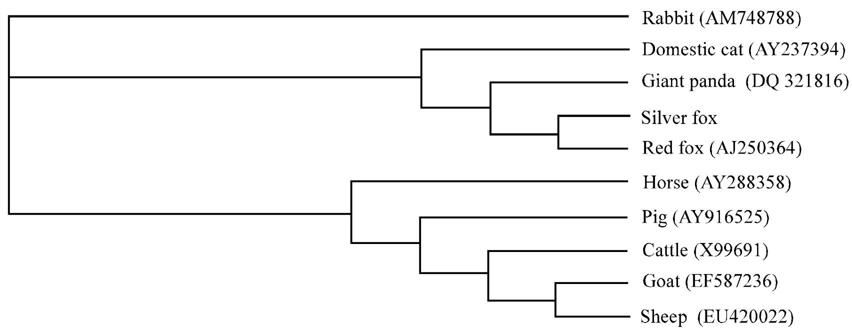

Fig. 3: Phylogenetic tree of silver fox and other species based on the partial sequence of intron 2 Agouti gene

DISCUSSION

Results in comparison with the corresponding sequences in GenBank, the similarities of intron 2 of the Agouti gene between the silver fox and the other species were 58.82-100%, which suggested that Agouti gene intron 2 was highly conserved in terms of evolution in mammal. The similarity between the silver fox and the red fox was the highest (100%), which showed their genetic relationship was the closest. This was consistent with that they belong to *Vulpes* of the Canidae animal in traditional classification (Kukekova *et al*., 2004; Tong and Zhang, 2009; Wang and Liu, 2009).

The phylogenetic tree of 10 species was basically consistent with the taxonomy of NCBI. Cluster result suggested that the silver fox, the red fox, the giant panda and the domestic cat had close relationship, which basically consistent with that of Zhong *et al*. (2010). Zhong *et al*. (2010) analyzed 12 concatenated heavy-strand protein-coding genes and discovered that arctic fox was the sister group of red fox and they both belong to the red fox-like clade in family Canidae. The silver fox is a variant of the red fox (*Vulpes vulpes*) in the wild environment (Kukekova *et al*., 2004; Hua and Hua, 2005), both of them belong to the *Vulpes* species of canidae (Tong and Zhang, 2009; Wang and Liu, 2009). The domestic cat (*Felis catus*) belong to the *Felis* of Felidae. The three species are all carnivores. The giant panda (*Ailuropoda melanoleuca*) is well known for dietary oddities: A bamboo specialist within the mammalian order Carnivora possessing a gastrointestinal tract typical of carnivores (Zhu *et al*., 2011). Cluster result of the four species accorded with their physiological characteristics. The another clade was the goat and the sheep clustered first and then the cattle, the pig and the horse was added successively. This result was basically consistent with those of Kang *et al*. (2008) and Liu *et al*. (2010). Kang *et al*. (2008) reported that the closest relationship existing among the goat, the sheep and the cattle by constructing phylogenetic tree of lactoferrin (LF) gene for 10 animal species. Liu *et al*. (2010) reported that the closest relationship existing among the goat, the sheep and the cattle by constructing phylogenetic tree of MyoG gene for 12 animal species.

CONCLUSION

In the study, the major part of intron 2 sequence of Agouti gene from the silver fox were obtained and the length was 1038 bp. The homology analysis and the phylogenic analysis based on the nucleotide sequences of Agouti gene intron 2 that silver fox has the nearest genetic relationship with red fox. The reconstructed phylogenetic among species tree was basically consistent with the taxonomy in the National Center for Biotechnology Information. The findings provide the important biological information for researching the mechanism of the formation mechanism of the coat color and artificially improving the coat color quality of fox and so on.

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