Identification of cDNA sequences and alternative splicing patterns of canine *AMEL* genes (*AMELX* and *AMELY*)

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ABSTRACT. Amelogenin is a major protein of the developing enamel matrix. There are two amelogenin genes (*AMELX* and *AMELY*) located on the X and Y chromosomes, respectively, in dogs. In the present study, we characterized full-length cDNAs and alternative splicing patterns of the *AMEL* genes in the tooth tissue of a dog by 5'- and 3'-rapid amplification of cDNA ends and AMEL-specific RT-PCR. Sequence analysis revealed that the coding regions of *AMELX* and *AMELY* were 579 and 576 bp (accession Nos. KP244310 and KP244311), respectively. The coding sequence of *AMELX* had 95.1% identity to that of *AMELY*. The *AMEL* genes on X and Y chromosomes were both expressed in developing tooth tissue. Eight different alternatively spliced transcripts were identified, five from *AMELX* and three from *AMELY*.

Key words: Amelogenin; Alternative splicing; Dog

INTRODUCTION

Dental enamel is a highly mineralized tissue that covers the crown of the tooth and is secreted by ameloblasts. There are two classes of proteins comprising the extracellular matrix in developing enamel: amelogenins and enamelins (Termine et al., 1980). Amelogenin, the major enamel matrix protein, contains high concentrations of proline, glutamine, leucine, and histidine and plays an important role in enamel formation. It is thought to function by regulating mineral crystal growth and organization. The amelogenin gene (*AMEL*) was shown to be located on the sex chromosomes of mammals. In some species, there are two *AMEL* genes (*AMELX* and *AMELY*) located on X and Y chromosomes, respectively (Lau et al., 1989; Nakahori et al., 1991). Other species, however, have only one *AMEL*, which is always located on the X chromosome (Lau et al., 1989).

Previous studies have demonstrated that both *AMEL* genes are transcribed in the developing tooth tissue in cattle (Gibson et al., 1992), pigs (Hu et al., 1996; Ikawa et al., 2005), and humans (Salido et al., 1992). In addition, alternative splicing has also been identified as a mechanism for generation of heterogeneous amelogenin products; all proteins derived from alternatively spliced transcripts have homologous 5' and 3' sequences within the coding regions (Gibson et al., 1992; Lau et al., 1992; Salido et al., 1992; Simmer et al., 1994; Li et al., 1995). The canine *AMEL* gene has been found to be located on both X and Y chromosomes (Yan et al., 2013). To date, only partial cDNA sequences from canine *AMEL* have been obtained (Yuasa et al., 1998; Haze et al., 2007). Furthermore, *AMEL* mRNA has not been detected in canine tooth tissue, the major location of amelogenin protein synthesis. In the present study, the complete cDNA sequence and alternative splicing patterns of the canine *AMEL* genes in developing tooth tissue were characterized by 5'- and 3'-rapid amplification of cDNA ends (RACE) and AMEL-specific RT-PCR amplification.

MATERIAL AND METHODS

Molars and incisors were dissected from a one-month-old male dog, and then the bones and the covering soft tissues were carefully removed. The animal experiments described here were performed in accordance with the guidelines on animal care, established by the Jilin University Animal Care and Use Committee. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer instructions. First strand cDNA was synthesized using the PrimeScript First Strand cDNA Synthesis Kit (Takara, Otsu, Japan) following the manufacturer instructions.

To obtain the full-length cDNA sequence of *AMELX* and *AMELY*, the 5' and 3' portions were first amplified using the method of Cosentino et al. (2010). Primers (Table 1) were designed using the canine *AMELX* and *AMELY* genomic sequences (accession Nos. NC_006621 and KC763835) or information in the literature (Cosentino et al., 2010). Briefly, cDNA was synthesized using primer 3-AP. For 3'-RACE, PCR was carried out with AL-X-1F (or AL-Y-1F) and 3-AP1 as primers for the amplification of the 3'-end of the *AMELX* and *AMELY* cDNA, respectively. The amplification was performed in 20 µL volume containing 1 µL cDNA, 0.5 µM each primer, 0.2 mM dNTPs, and 0.5 U *Ex Taq* polymerase (Takara). The amplification conditions were 95°C for 2 min, 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a 10-min extension at 72°C.

Table 1. Sequences of primers used in this study.

Primer name	Sequence (5'→3')
3-AP	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT
3-AP1	GGCCACGCGTCGACTAGTAC
AL-X-1F	ATCCAGCCCCAGCCACCCGT
AL-Y-1F	CATTCAGCCACAGCCACCTA
5-AP	GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGGG
AL-X-1R	AGGGGCTGGATGGGGTGCACG
AL-Y-1R	AGGGGCTGGATGGGGTGCATA
5-AP1	GGCCACGCGTCGACTAGTAC
AL-R	ATAACCAGGGTGCCCAGGATGA
AL-X-2F	GAAACTTCCTCTGAATACGCAT
AL-X-2R	TTACTAAGTGAAGTGATGGCAGG
AL-Y-2F	GTTTCAGAGGAAAACTTACTCCTG
AL-Y-2R	GGAACTGAAATATCTACTCCTGTC

For 5'-RACE, a poly (dC) tail was added using TdT terminal transferase to the 3' end of cDNA that had been treated with RNase H. PCR was performed with 5-AP and AL-X-1R (or AL-Y-1R) as primers and then nested PCR was carried out using primers AL-R and 5-AP1 for the amplification of the 5'-end of *AMELX* and *AMELY*. Except for the primers, the PCR components and amplification conditions were the same as those described for 3' RACE above. The PCR products were purified with TIANgel Midi Purification Kit (Tiangen, Beijing, China), and cloned into a pMD18-T vector (Takara) and sequenced. Sequence analysis was performed using DNAMAN software (Lynnon Biosoft, Vaudreuil, Quebec, Canada).

To obtain the complete coding sequences and potential alternative splice transcripts for canine *AMELX* and *AMELY*, two sets of chromosome-specific primers, AL-X-2F/2R and AL-Y-2F/2R (Table 1), were designed from the cDNA sequences obtained by 5'- and 3'-RACE, respectively. RT-PCR, cloning and sequencing were carried out as described above.

RESULTS AND DISCUSSION

3'-RACE identified a 311-bp fragment (excluding the poly(A) tail) for *AMELX*, and 302- and 616-bp fragments for *AMELY* from the template canine cDNA. Sequence alignment revealed that the difference in length of the two Y chromosome fragments was due to the selection of either of two polyadenylation signals (AATAAA) for *AMELY*. 5' RACE produced 157- and 185-bp fragments for *AMELX* and *AMELY*, respectively. The sequences have been deposited in GenBank with accession Nos. KP244310, KP244311, and KP244312.

PCR products were amplified using primer pairs AL-X-2F/2R or AL-Y-2F/2R and cloned. Fifty clones containing *AMELX* inserts were sequenced and five transcripts of different sizes were detected (Figure 1). These transcripts were named AMELX-a (719 bp), AMELX-b (671 bp), AMELX-c (623 bp), AMELX-d (368 bp), and AMELX-e (320 bp). However, only three transcripts of different lengths were identified from 50 clones containing *AMELY* inserts; these transcripts were named AMELY-a (660 bp), AMELY-b (612 bp), and AMELY-c (570 bp) (Figure 2). Sequence alignments showed that the different transcripts resulted from alternative splicing of the primary transcripts of *AMELX* and *AMELY*.

Based on genomic information (accession No. NC_006621) and mRNA data obtained in this study, we conclude that canine *AMELX* and *AMELY* genes contain 6 exons and 5 introns. Each intron shows a typical splice motif at the 5' (GT) and 3' (AG). The full-length cDNA of *AMELX*

(accession No. KP244310) contains a 73-bp 5'-untranslated region (UTR), a 579-bp open reading frame (ORF) including start and stop codons, and a 152-bp 3'-UTR excluding the poly(A) tail. The complete cDNA of *AMELY* contains a 101 bp 5'-UTR, a 576-bp ORF, and a 142 or 456 bp 3'-UTR excluding the poly(A) tail (accession Nos. KP244311 and KP244312).The ORFs of *AMELX* and *AMELY* encode putative proteins of 192 and 191 amino acids, respectively. Sequence identity of the coding sequences of *AMELX* and *AMELY* was 95.1% at the nucleotide level and 96.9% at the acid amino level. There are 28 single nucleotide differences and a 3 bp deletion resulting in 6 amino acid substitutions and one deletion between the deduced proteins encoded by *AMELX* and *AMELY* (AKD44024 and AKD44025).

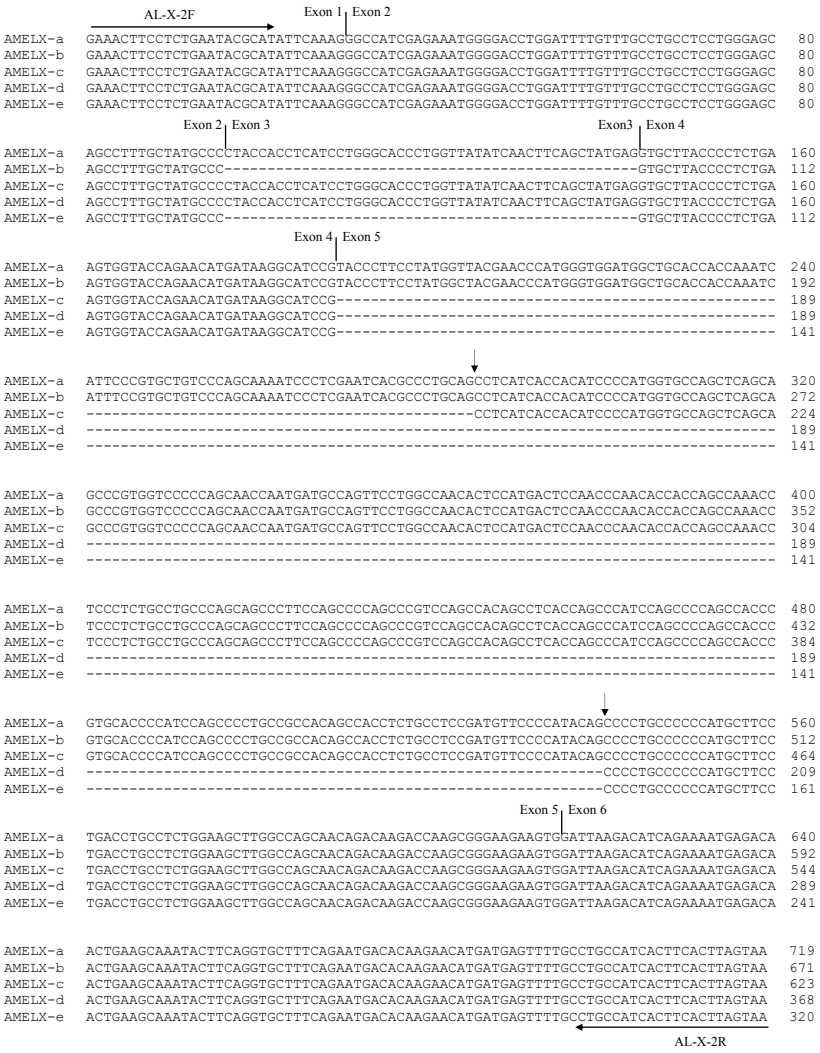

Figure 1. Alternative splicing patterns of canine *AMELX*. Vertical lines represent exon boundaries. Two downward arrows mark the internal splicing sites within exon 5. The positions of the primers are shown by solid lines with an arrow indicating the direction of extension.

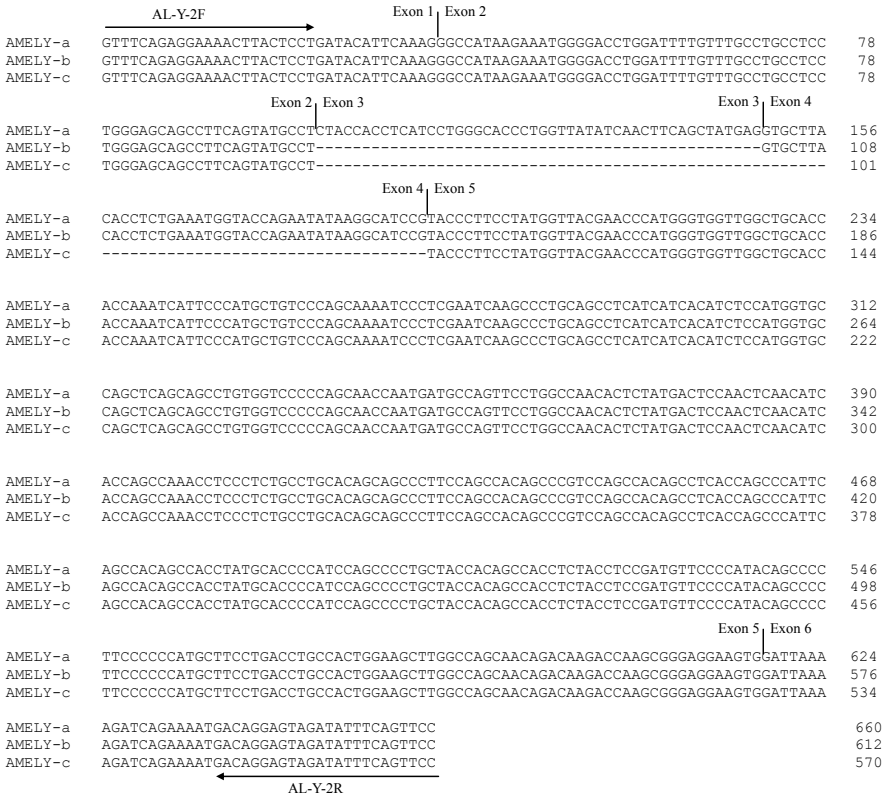

Figure 2. Alternative splicing patterns of canine *AMELY*. Vertical lines represent exon boundaries. The positions of the primers are shown by solid lines with an arrow indicating the direction of extension.

In summary, the canine *AMELX* and *AMELY* genes are both transcribed in developing tooth tissue. Alternative splicing generates at least eight messages, five from the *AMELX*, and three from *AMELY*.

Conflicts of interest

The authors declare no conflict of interest.

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