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Novel alternative splicing variants of *ACOX1* and their differential expression patterns in goats

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Abstract. As the first and rate-limiting enzyme of the peroxisomal β-oxidation pathway, acyl-coenzyme A oxidase 1 (ACOX1), which is regulated by peroxisome proliferator-activated alfa (PPARα), is vital for fatty acid oxidation and deposition, especially in the lipid metabolism of very long-chain fatty acids. Alternative splicing events of ACOX1 have been detected in rodents, Nile tilapia, zebra fish and humans but not in goats. Herein, we identified a novel splice variant of the *ACOX1* gene, which was designated as ACOX1-SV1, in addition to the complete transcript, ACOX1, in goats. The length of the ACOX1-SV1 coding sequence was 1983 bp, which presented a novel exon 2 variation owing to alternative 5′-splice site selection in exon 2 and partial intron 1, compared to that in ACOX1. The protein sequence analysis indicated that ACOX1-SV1 was conserved across different species. Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) analysis showed that these two isoforms were expressed spatially and differently in different tissue types. ACOX1 and ACOX1-SV1 were expressed at high levels in liver, spleen, brain and adipose tissue in kid goats, and they were abundantly expressed in the fat, liver and spleen of adults. Interestingly, whether in kids or in adults, in fat, the mRNA level of ACOX1 was considerably higher than that of ACOX1-SV1. In contrast, in the liver, the expression of ACOX1-SV1 was considerably higher than that of ACOX1. This differential expression patterns showed the existence of a tissue-dependent splice regulation. These novel findings for ACOX1 should provide new insights for further studies on the function of ACOX1 and its variants that should aid in the breeding of goats with improved meat quality.

1 Introduction

Goat meat is expected to assume greater importance in the future owing to the rapidly growing world population and a steady increase in the consumption of this meat (Henchion et al., 2014); moreover, meat with better qualities and improved flavor is desirable and demanded by consumers. The Fuqing goat breed is one of the finest local breeds found on the east coast of China and provides high-quality meat that is tender and has only a slight odor. Nonetheless, research on goat breeding for improving the quality of meat has lagged behind that on pork and beef. Data on candidate genes that affect the performance and quality of meat have largely been lacking, warranting detailed studies in this direction.

The peroxisomal fatty acid β-oxidation pathway, which is an essential metabolic pathway that operates in all eukaryotic cells (Kunau et al., 1995; Shi et al., 2016), is the main metabolic destination of very long-chain fatty acids (VLCFAs). This pathway plays a key role in the infants of mammals, wherein lipids in the breast milk become the major source of energy (Ehara et al., 2015). Importantly, this pathway is regulated directly by the peroxisome proliferator-activated receptor alfa (PPARα), which is a master transcriptional regulator of the fatty acid metabolism (Poirier et al., 2006).

Acyl-coenzyme A oxidase 1 (ACOX1) is the first and the rate-limiting enzyme of the peroxisomal β-oxidation pathway. It is mainly activated in peroxisome proliferators (a

kind of organelle in the liver) and oxidizes long and medium straight-chain fatty acid substrates (Poirier et al., 2006; Wang et al., 2014). A deficiency of this enzyme leads to severe microvesicular steatohepatitis in mice (Fan et al., 1998; Yu et al., 2003). The lack of both *ACOX1* and *PPARα* causes the absence of spontaneous hepatic peroxisome proliferation in aged mice (Hashimoto et al., 1999; Tanaka et al., 2017), highlighting the close connection between *ACOX1* and *PPARα*. In humans, the absence of ACOX1 is associated with pseudoneonatal adrenoleukodystrophy (P-NALD), which is a disorder of the peroxisomes (El Hajj et al., 2012). The *ACOX1* gene holds great promise as a potential target for improving meat performance in pig and cattle. For instance, an A/C polymorphism in intron 9 was found to be significantly associated with fat deposition in pig (Zuo et al., 2007), and the other one (A1865C) in exon 13 was significantly related to back fat thickness and marbling score in cattle, as evidenced from ultrasound imaging (Jiao et al., 2011). These observations point to the critical role of ACOX1 in the fatty acid metabolism and suggest its potential functions in affecting the development and deposition of fat.

Alternative splicing (AS), which exists in almost all kinds of mammals (Kelemen et al., 2013), is a fundamental molecular event that results in the production of different mature transcripts from the same primary RNA sequence (Sammeth et al., 2008). These transcripts contribute to diverse biological processes, such as cell differentiation (Kianianmomeni et al., 2014; Cotter et al., 2015), disease development (Sohail et al., 2014) and the adipocyte metabolism (Fiszbein et al., 2017). Previous studies reported that the splicing variants of PPARG (PPARG1 and PPARG2) could affect the lipogenesis of mammary tissue via the upregulated expressions of key lipogenic gene networks (Shi et al., 2014). The different transcripts of the *SFRS18* gene (SFRS18_V1 and SFRS18_V2) were revealed to correlate with intramuscular fat content in pigs (Wang et al., 2009). Thus, AS may affect meat quality by regulating the expressions of key genes within the myocyte and adipocyte metabolism, especially the intramuscular lipocyte generation. Splicing variants of the *ACOX1* gene were detected in Nile tilapia (He et al., 2014), mice (Vluggens et al., 2010) and humans (Oaxaca-Castillo et al., 2007); however, they have not been reported in goats. These studies all lend credence to the notion that the *ACOX1* gene plays fundamental roles in meat quality. Therefore, this study aims to explore potential splicing transcripts of the goat *ACOX1* gene and the tissue expression pattern in kid and adult goats, which would be of benefit for further study on the function of the *ACOX1* gene and breeding programs in goats.

2 Materials and methods

Experimental animals and procedures used in this study were approved by the International Animal Care and Use Committee of the Fujian Academy of Agricultural Sciences (FAAS), Fujian province, China. The care and use of experimental animals fully complied with local animal welfare laws, guidelines and policies.

2.1 Animals and tissue collection

Seven Fuqing goats (four adult wethers and three female kids (lactation period)) were fed and slaughtered in the goat breeding farm of FAAS. Because the development of gonads in goats does not begin during the lactation period, the experiments would not be influenced by gender. Nine types of tissue were collected for the study: heart, liver, spleen, lung, kidney, leg muscle, the longissimus muscle, perirenal fat and brain. All the samples were snap-frozen in liquid nitrogen and subsequently stored at −80 °C.

2.2 Total RNA isolation, first-strand cDNA synthesis and cDNA pool construction

Total RNA was isolated from the collected tissue samples using an RNA extraction kit and RNase-free DNase I (TaKaRa, Dalian, China). The genomic DNA was removed by treatment with RNase-free DNase I, and the quality of RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and 1 % agarose gel electrophoresis (Li et al., 2013). Reverse-transcription polymerase chain reaction (RT-PCR) was performed to synthesize complementary DNA (cDNA) using a PrimeScript™ RT reagent Kit (TaKaRa), according to the manufacturer's recommended procedure (S. H. Zhang et al., 2016).

2.3 Identification of ACOX1 alternative splice variants

The primers were designed, based on the predicted goat ACOX1 mRNA sequence (XM_013972412.1), using Primer Premier 5 software (Premier BioSoft, Palo Alto, CA, USA); the sequences of the primers are shown in Table 1. A pair of primers (P1) was designed for the identification of different splice transcripts in the goat *ACOX1* gene (Table 1). The polymerase chain reactions (PCRs) were performed as described in our previous study (X. Y. Zhang et al., 2015).

2.4 Clone sequencing and validation of ACOX1 transcript variants

The PCR products were separated by electrophoresis on agarose gel and were cloned into a pGEM-T easy vector (Promega, Shanghai, China) after purification. The recombinant vector thus obtained was then transferred to *Escherichia coli* DH5α competent cells (TaKaRa) and sequenced (X. Y. Zhang et al., 2016).

Table 1. Sequences of PCR primers used for amplification of the goat *ACOX1* gene.

Loci	Primer sequences (5′→3′) (nucleotide position)	Tm (°C)	Sizes (bp)	Amplified region
P1	F: CGGCCGTCACCATGAATCA R: CGCAGTTTGTGTGACAGGTG	60	2065	Entire CDS region and AS variants for RT-PCR
P2	F: CGTAGCCAGCGTTATGAGGT R: TCCAGGCAGGCATGAAGAAG	60	220	EX1, EX2 for RT-qPCR (ACOX1)
P3	F: TTTGGCATCGCAGATCCTGA R: GTGCCCTGATTCAGCAAGGT	60	119	EX1, INS2 for RT-qPCR (ACOX1-SV1)
P4	F: TGTTTGTGATGGGCGTGAACCA R: ATGGCGTGGACAGTGGTCATAA	50	142	EX5, EX6, EX7 for RT-qPCR (GAPDH)

Note: NCBI reference sequence NC_022311.1.

2.5 Bioinformatics analysis

DNA and amino acid sequences were aligned using MEGA 5.1 (http://www.megasoftware.net/) and BioXM 2.6 (Nanjing Agricultural University). Phylogenetic trees were constructed using the neighbor-joining method implemented in MEGA (Lee et al., 2014) and by using NCBI pairwise alignments (http://www.ncbi.nlm.nih.gov/blast).

2.6 Measurement of ACOX1 and ACOX1-SV1 mRNA levels using quantitative real-time PCR

The specific primers used for reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) were shown in Table 1. RT-qPCR was performed with Eastep® qPCR Master Mix Kit (Promega) in an Eppendorf Mastercycler ep Realplex 4 Real-Time PCR system. The RT-qPCR reaction system was implemented as described in our previous study (X. Y. Zhang et al., 2015). The steps in RT-qPCR were as follows: denaturation at 95 °C for 30 s, followed by 39 cycles of 15 s at 95 °C, and 50 s at 63 °C. The individual samples were amplified in triplicates, and a no-template control (NTC) was included (which contained ddH_2O instead of cDNA template).

2.7 Statistical analysis

The popular $2^{-\Delta\Delta C_q}$ method was used to calculate the variations in the expression levels; *GAPDH* was used as the reference gene (Livak et al., 2001). The reactions in which water was used instead of the template were regarded as NTCs. The calculation in the $2^{-\Delta\Delta C_q}$ method was performed using the following formula: ratio $= 2^{-\Delta\Delta C_q}$, where $^{-\Delta\Delta C_q} = \text{GEOMEAN}\,(C_q \cdot \text{ACOX1}/\text{ACOX1-SV1}) - C_q \cdot GAPDH$ (Bustin et al., 2009). Nine tissue samples were used for the two variants. Three individuals were used for each tissue and different isoforms were detected from the same individual for each tissue. Statistical differences between the expression levels of the different ACOX1 isoforms were analyzed by Student's t test using the SPSS 18.0 software. The values with uppercase letters indicate statistical significance at $P < 0.01$ and those with lowercase letters indicate statistical significance at $P < 0.05$.

3 Results

3.1 Identification of a novel transcript of the *ACOX1* gene

The cDNAs prepared from the samples obtained from the kids and adults were pooled and the *ACOX1* CDS was amplified using Primer P1 (Fig. 1a). The amplified products were cloned and sequenced. We detected the presence of the full-length CDS of two ACOX1 isoforms: the known ACOX1 transcript (XM_013972412.1) and a novel variant, named ACOX1-SV1 (GenBank Accession number: KX828848). The coding sequences of both ACOX1 and ACOX1-SV1 were 1983 bp in length (Fig. 2a), comprising 13 exons. However, ACOX1-SV1 was different from the *ACOX1* transcripts, which had a novel exon 2 variation owing to an alternative 5′ splice site selection of exon 2 and partial intron 1 (NC_022311.1 nt4020–4120) (Fig. 1b).

3.2 Bioinformatics analysis of ACOX1 and ACOX1-SV1

The alignment of the ACOX1 and ACOX1-SV1 sequences (having the same number of amino acids) revealed a 40-amino acid (aa) difference (Fig. 2b). The amino acid sequence of goat ACOX1-SV1 was observed to share 95, 94, 92, 91, 91, 88, 88, 86, 85 and 82 % similarity with the corresponding sequences from *Bos taurus* (NP_001030366.1), *Ovis aries* (XP_014954428.1), *Bubalus bubalis* (XP_006045337.1), *Bos mutus* (XP_005908024.1), *Equus caballus* (XP_003362535.1), *Pan troglodytes* (XP_511690.2), *Homo sapiens* (NP_004026.2), *Sus scrofa* (NP_001094498.1), *Rattus norvegicus* (NP_059036.1) and *Mus musculus* (NP_056544.2). In the Basic Local

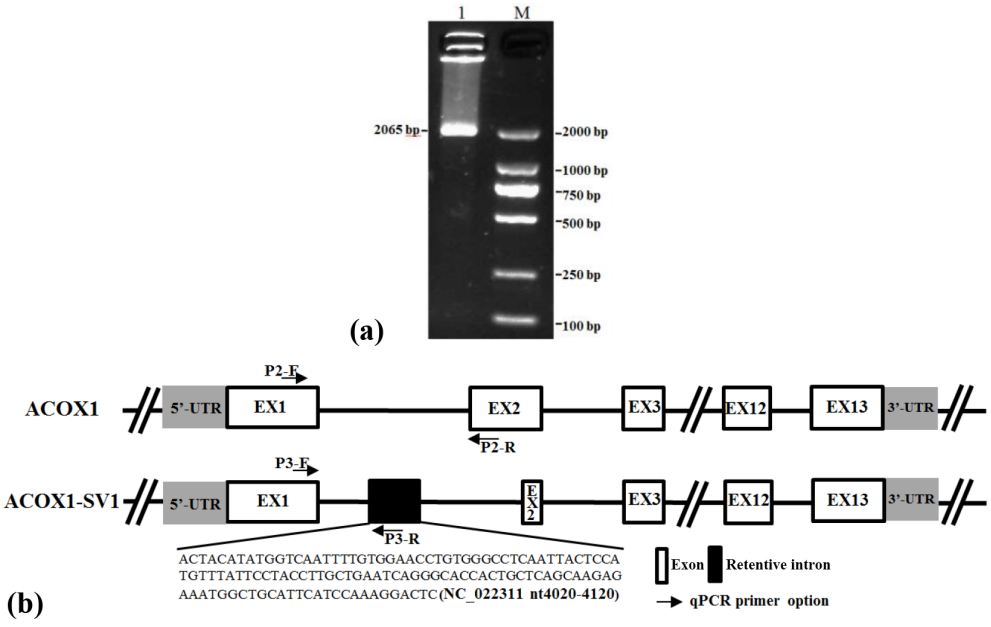

Figure 1. Detection of different isoforms (ACOX1, ACOX1-SV1) of ACOX1 in goats and schematic of genomic structure. **(a)** Electrophoresis of the products obtained by RT-PCR of the mixed cDNA pool; **(b)** goat ACOX1 (XM_013972412) was used as the reference sequence in the diagram. Note: basic exons are shown in white boxes and alternatively spliced intron regions are shown in black box. Lines represent the basic introns. Positions of the primers P2 and P3 for RT-qPCR of the ACOX1 and the ACOX1-SV1 transcript variants.

Alignment Search Tool (BLAST) search using the CDS of goat isoforms as queries, we detected a high degree of similarity of the goat ACOX1 (XM_013972412) with the functional human counterpart, ACOX1b (NM_004035.6), and that of ACOX1-SV1 (KX828848) with human ACOX1a (NM_007292.5) (Fig. 3a and b) (Vluggens et al., 2010). Moreover, the splice site of goat 121-nucleotide sequence in ACOX1-SV1 was highly similar (99.2 %) to that of human ACOX1a isoform.

A phylogenetic tree was constructed based on the ACOX1-SV1 sequence for studying evolutionary relationships. It was observed that the goat ACOX1-SV1 was conserved in different species; it was closest to *B. taurus* ACOX1 and most distant from the clade comprising sequences from *R. norvegicus* and *M. musculus* (Fig. 4).

3.3 Relative and differential expression of ACOX1 and ACOX1-SV1 in different tissue types from different goat groups

The expression profiles of ACOX1 and ACOX1-SV1 were determined using semiquantitative RT-PCR in both the kids and adults. ACOX1 was observed to be expressed only in six tissue types, namely heart, liver, spleen, the longissimus muscle, fat and brain (Fig. 5a). However, the ACOX1-SV1 isoform was detected in all of the collected tissue (heart, liver, spleen, lung, kidney, leg muscle, longissimus muscle, fat and brain) (Fig. 5b).

RT-qPCR was performed to determine the mRNA levels of the two isoforms in the experimental tissue samples (ACOX1: six tissue samples; ACOX1-SV1: nine tissue samples) collected from the two groups (Fig. 6). For kid goats, the expression of ACOX1 in the longissimus muscle was the lowest ($P < 0.05$) (Fig. 7a). The mRNA level of ACOX-SV1 was significantly higher in liver, spleen, kidney and lung samples than in the other tissue types ($P < 0.05$), followed by adipose tissue, the brain and the heart. Moreover, its expression in the leg muscle and longissimus muscle was low (Fig. 7c). In the adults, the ACOX1 expression level was the highest in adipose tissue; it was much higher in the spleen and liver compared to the levels in the heart, brain and longissimus muscle ($P < 0.01$) (Fig. 7b). The expression of the ACOX1-SV1 isoform was highest in the liver, followed by that in the spleen, lung and kidney. Moreover, the expression of ACOX1-SV1 in the brain, adipose tissue, the heart, the leg and the longissimus muscle was also low (Fig. 7d). Overall, in kids, the two isoforms had a relatively higher expression in the liver, the spleen, the brain and adipose tissue and displayed low levels of expression in the heart and longissimus muscle. In adult individuals, the expression of the two isoforms was totally different in liver and adipose tissue; the mRNA level of ACOX1 was much higher than that of ACOX1-SV1 ($P = 0.001$), whereas in the liver the expression of ACOX1-SV1 was higher than that of ACOX1 ($P = 0.02$). Both the transcripts are expressed moderately in the spleen and weakly in the heart, brain and longissimus muscle.

Figure 2. Alignment of nucleotide **(a)** and protein sequences **(b)** of ACOX1 and ACOX1-SV1 variants.

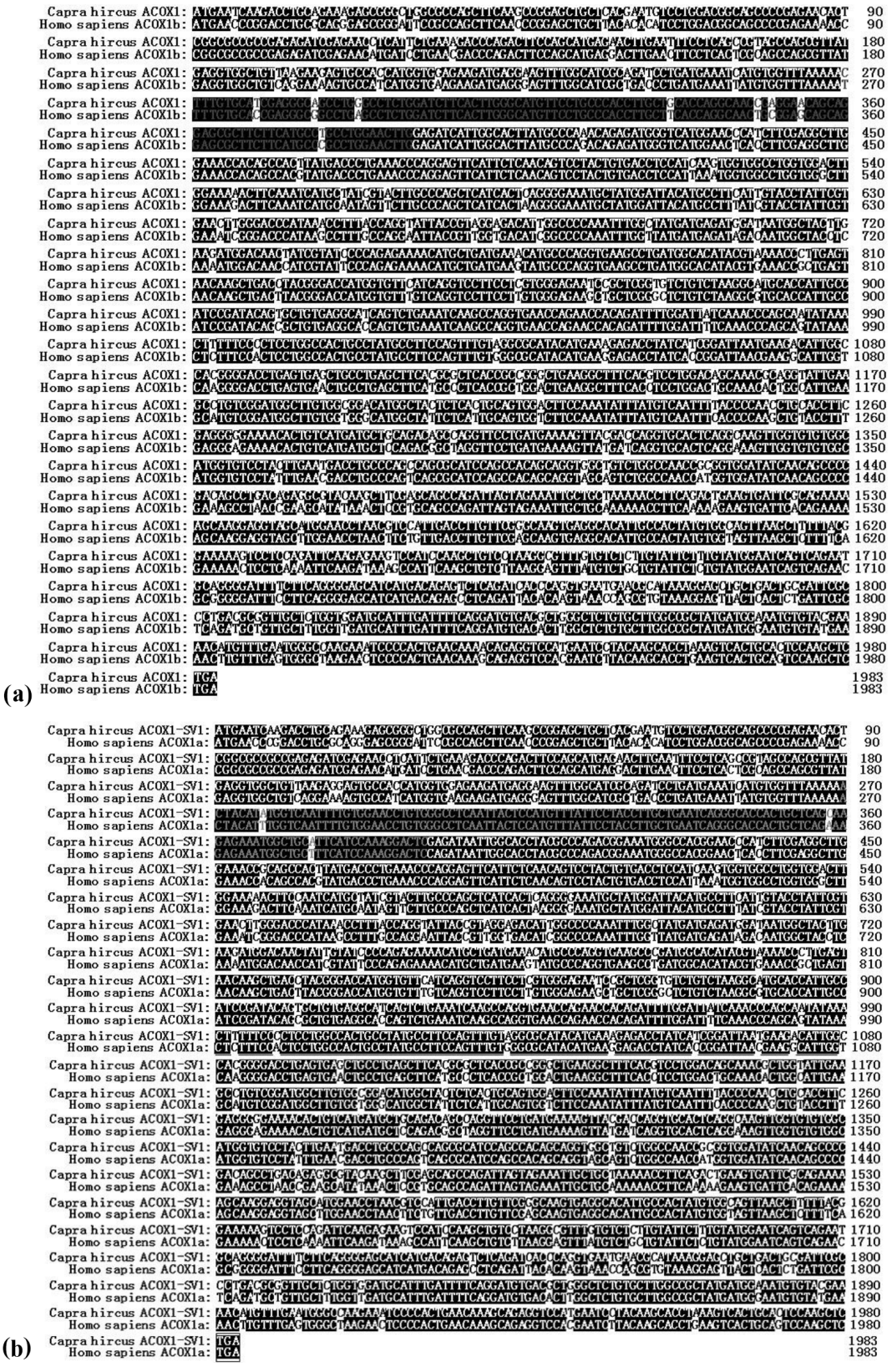

Figure 3. Sequence alignment of ACOX1 transcript variants from goat and human. **(a)** ACOX1 XM_013972412 is aligned with human ACOX1b NM_004035.6; **(b)** ACOX1-SV1 KX828848 is aligned with human ACOX1a NM_007292.5). Note: the gray uppercase letters represent the sequence of the novel exon 2 variation event in ACOX1-SV1. Boxes represent the initiation and termination codons.

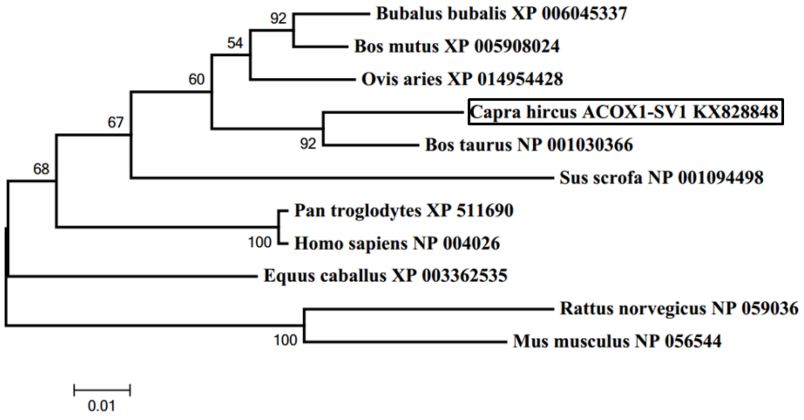

Figure 4. Phylogenetic tree of ACOX1-SV1 made by MEGA 5.1. Note: the tree was constructed based on amino acid similarities of the full mRNA sequences by MEGA 5.1. The accession numbers and description of the sequences used for the construction of the tree were shown in Table 2. The numbers on the joints were bootstrap test values, and the branch length represents the evolutionary time.

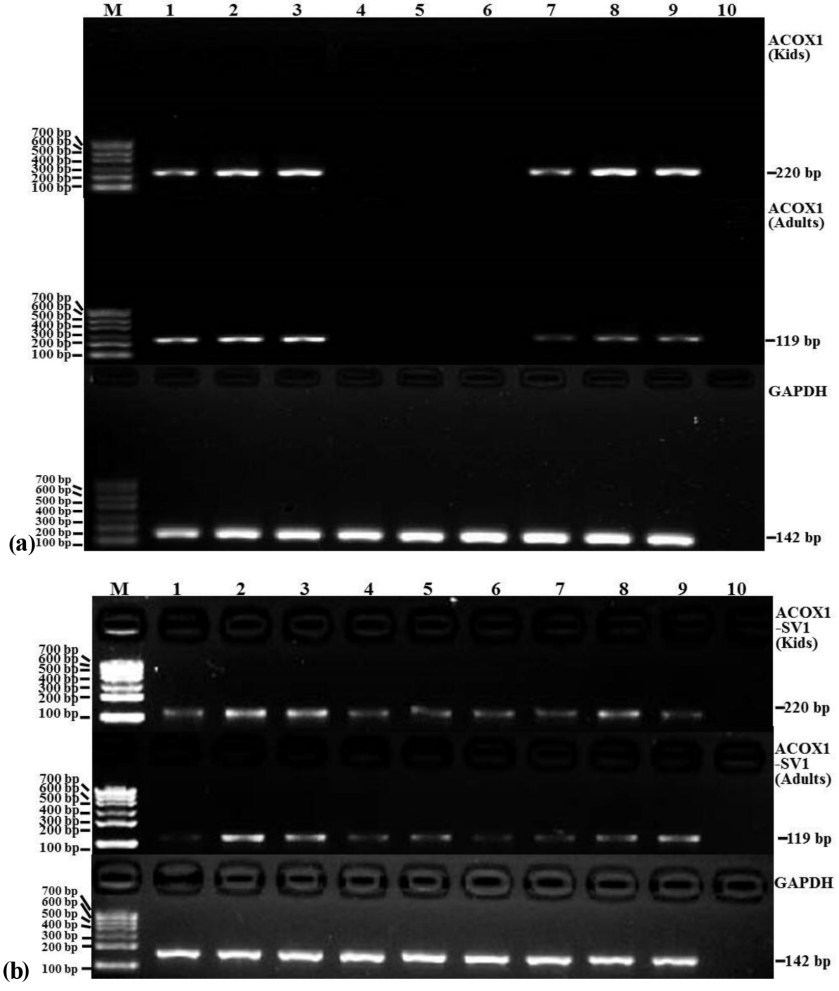

Figure 5. The mRNA expression patterns of ACOX1 **(a)**, ACOX1-SV1 **(b)** and GAPDH from various tissue types in the groups of kids and adults. Note: lanes (Ln) 1 – heart; Ln 2 – liver; Ln 3 – spleen; Ln 4 – lung; Ln 5 – kidney; Ln 6 – leg muscle; Ln 7 – longissimus muscle; Ln 8 – adipose; Ln 9 – brain; Ln 10 – NTC. Marker: marker I.

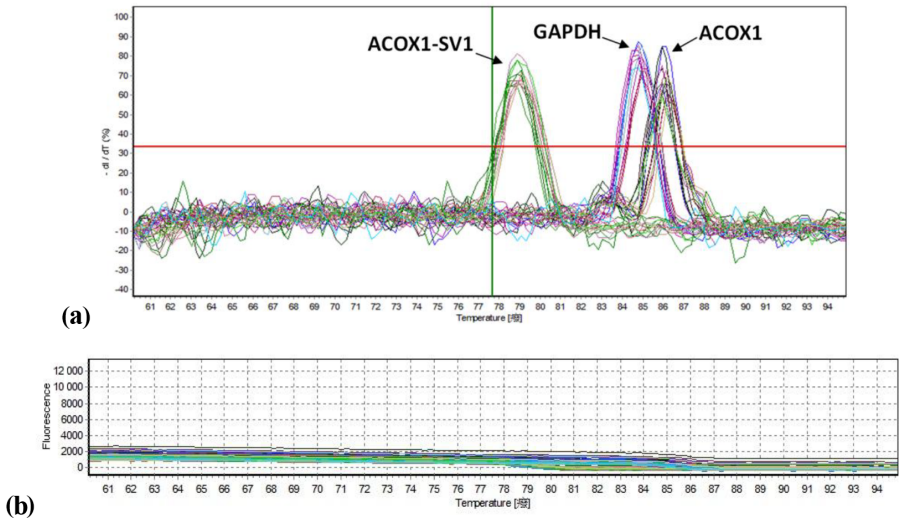

Figure 6. Melt peak **(a)** and melt curve **(b)** chart of ACOX1, ACOX1-SV1 and the reference gene *GAPDH* for RT-qPCR.

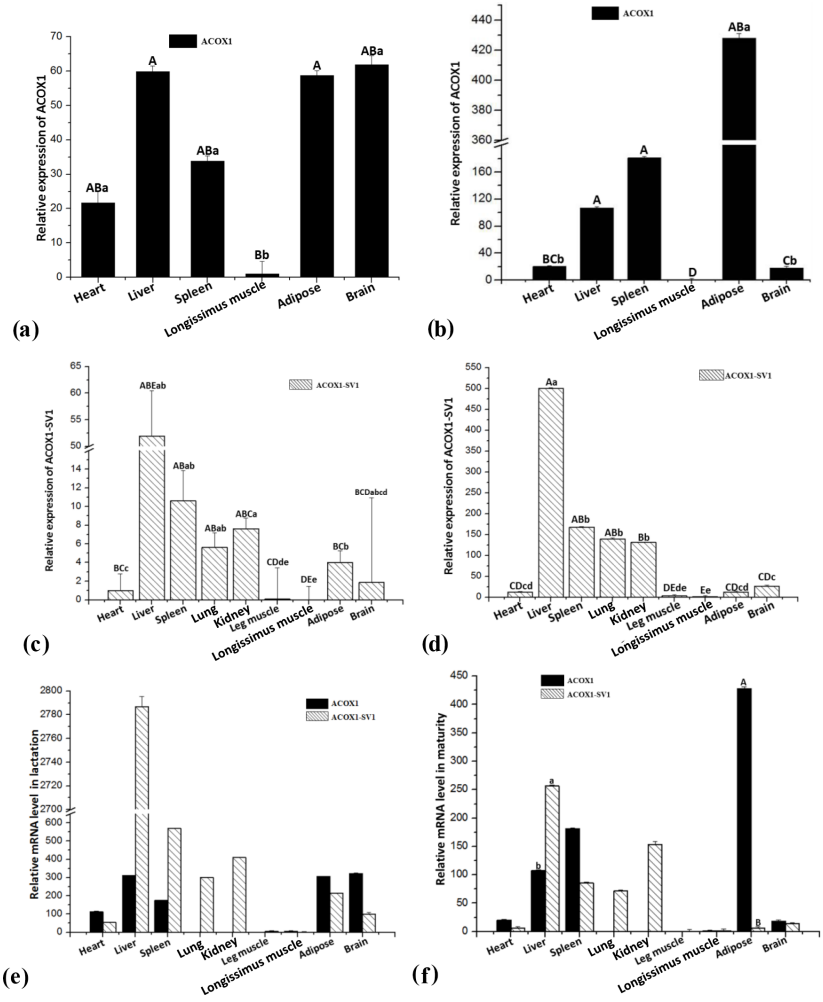

Figure 7. Relative ACOX1 and ACOX1-SV1 mRNA levels in the kids **(a, c)** and adults **(b, d)**, and differential expression levels of two different transcripts in various tissue types of kids **(e)** and adults **(f)**. Note: gene expression was normalized using *GAPDH* as a reference gene. Uppercase letters represent statistical significance at $P < 0.01$, and lowercase letters represent the statistical significance at $P < 0.05$.

Table 2. Accession numbers and description of the amino acid sequences used in tree building.

Accession number	Description	Sizes
KX828848	*Capra hircus* ACOX1-SV1	660
XP_014954428.1	*Ovis aries* ACOX1 isoform X1	660
NP_001030366.1	*Bos taurus* ACOX1	660
XP_006045337.1	*Bubalus bubalis* ACOX1 isoform X1	660
XP_005908024.1	*Bos mutus* ACOX1 isoform X1	661
XP_003362535.1	*Equus caballus* ACOX1 isoform X1	660
NP_059036.1	*Rattus norvegicus* ACOX1 isoform X1	660
NP_056544.2	*Mus musculus* ACOX1 isoform X1	660
NP_001094498.1	*Sus scrofa* ACOX1	660
XP_511690.2	*Pan troglodytes* ACOX1 isoform X1	660
NP_004026.2	*Homo sapiens* ACOX1 isoform a	660

4 Discussion

As a major mechanism for the expansion of transcriptome and proteome diversity (Bush et al., 2017), AS is a principal contributor to the evolution of phenotypic complexity in mammals. This type of evolution was also demonstrated for the *ACOX1* gene, a rate-limiting and relatively conserved enzyme in the peroxisomal fatty β-oxidation pathway. Several studies revealed similar sequences in other vertebrates, such as rodents, Nile tilapia, zebra fish and humans (Iyazawa et al., 1987; Morais et al., 2007; He et al., 2014). The ACOX1 transcripts were abundant in liver, adipose and kidney tissue. In this study, a novel splice variant, ACOX1-SV1 (KX828848), was first identified in goats; this variant was remarkably conserved in different species. Sequence alignment illustrated that the sequence of ACOX1-SV1 was highly similar to that of its human functional isoform, ACOX1a (Fig. 3b) (Vluggens et al., 2010), and ACOX1 was homologous to human ACOX1b (Fig. 3a). Splice variants of human ACOX1 (ACOX1a and 1b) were reported to be critical regulators in the fatty acid metabolism (Varanasi et al., 1994; Baes and Van Veldhoven, 2012), indicating that these variants were essential for the lipid metabolism in goats.

Interestingly, both the ACOX1 and ACOX1-SV1 variants were expressed spaciously and differently in the collected tissue samples. The mRNA levels of ACOX1 and ACOX1-SV1 were extremely different in the liver and fat, respectively. In liver tissue, ACOX1 had a moderate expression level, and the expression of ACOX1-SV1 was excellent in kid and adult goats, being 1.5 times higher than that of ACOX1 (Fig. 7e and f). Based on the results in humans, ACOX1 and ACOX1-SV1 might work as a dimer. Furthermore, the proteins encoded by these two isoforms should have critical functions in reducing PPARα activation and should subsequently affect the fatty acid metabolism in goats. The human ACOX1b isoform (compared to the goat ACOX1) reversed the ACOX1 null mice fatty liver phenotype (Oaxaca-Castillo et al., 2007). We, therefore, speculated that goat ACOX1 was the primary transcript used in the hepatic lipid metabolism of VLCFAs. Accordingly, ACOX1 and ACOX1-SV1 isoforms have a high probability of being the key regulators of the metabolism of VLCFAs in goats.

The ACOX1 isoform had high expression levels in fat when goats became mature. In contrast, the ACOX1-SV1 mRNA was rarely expressed in both the kid and adult individuals, suggesting that the ACOX1 isoform not only played a key role in the hepatic fatty acid metabolism but might also greatly affect the adipocyte differentiation and deposition of fat in goats. From the published reports, ACOX1 proteins are known to regulate adipose differentiation and are implicated in obesity (Baranova et al., 2013). They are used as markers of lipogenesis for RT-qPCR (Palou et al., 2009; Flachs et al., 2005). Furthermore, the expression level of ACOX in adults was considerably higher than that in kids, which could be explained by adipose deposition leading to a peak in expression after the individuals mature. In contrast, ACOX1-SV1 is likely to be involved in the hepatic fatty acid metabolism. The differential expression of ACOX1 and ACOX1-SV1 shows the existence of tissue-dependent splice regulation, which allowed for evolutionary selection (Kelemen et al., 2013).

The mRNA levels were found to change in the brain. In kid goat, ACOX1 and ACOX1-SV1 were abundantly expressed in the brain, whereas their expression was decreased in adults. Beyond their role in the lipid metabolism, ACOX1 and ACOX1-SV1 are involved in the synthesis of docosahexaenoic acid (DHA), which is best represented polyunsaturated fatty acid (PUFA) in brain and nervous tissue and is critical for the extension of neuronal axons and in neuronal processes (Trompier et al., 2014). When *ACOX1* is mutated, the plasma level of DHA is reduced (Baes and Van Veldhoven, 2012) and the neurological development of the infant dramatically deteriorates (Masson et al., 2016). The suggested transcripts of the *ACOX1* gene were thus crucial in the development of the brain in kid goats.

In conclusion, two alternative splice variants of ACOX1 (ACOX1 and ACOX1-SV1) were identified in kid and adult goats, and they were found to express at high levels in the liver, spleen, brain and fat tissue of kids. In adult individuals, the two isoforms were expressed at high levels in fat, the liver and the spleen. In both the cases lipid conversion and deposition were observed to be important factors involved in meat performance. These findings would provide a foundation for further investigation of the functions of ACOX1 and its variants in goat breeding, especially in the improvement of meat quality.

Data availability. The splice variant (ACOX1-SV1) that we have found has been uploaded to the National Center for Biotechnology Information (NCBI) Nucleotide Database (GenBank, accession number: KX828848), available at: https://www.ncbi.nlm.nih.gov/nuccore/KX828848 (Wu, 2017). The sequencing data of ACOX1-SV1 are provided in the Supplement.

Appendix A: Abbreviations

ACOX	acyl-coenzyme A oxidase
ACOX1	acyl-coenzyme A oxidase 1
ACOX1-SV1	acyl-coenzyme A oxidase 1 splice variant 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
PPARα	peroxisome proliferator-activated receptor alfa
P-NALD	pseudoneonatal adrenoleukodystrophy
VLCFAs	very long-chain fatty acids
AS	alternative splicing
mRNA	messenger RNA
CDS	coding sequence
cDNA	complementary DNA
PCR	polymerase chain reaction
RT-PCR	reverse-transcription polymerase chain reaction
RT-qPCR	reverse-transcription quantitative real-time polymerase chain reaction
aa	amino acid
bp	base pair
C_q	quantification cycle
SD	standard deviation
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
DHA	docosahexaenoic acid
PUFA	polyunsaturated fatty acid

Supplement. The supplement related to this article is available online at: https://doi.org/10.5194/aab-61-59-2018-supplement.

Competing interests. The authors declare that they have no conflict of interest.

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