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RESEARCH ARTICLE

Full-length sequencing and identification of novel polymorphisms in the *ACACA* gene of Valle del Belice sheep breed

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Abstract. The essential role of the acetyl-CoA carboxylase (ACACA) enzyme in milk fatty acid (FA) synthesis suggests that it may be responsible for the phenotypic variability observed in milk. Before attempting association analyses between this gene and/or enzyme and phenotypic traits, a study on the genetic variability within this locus is required. The aim of this work was to sequence the entire coding region of *ACACA* gene in Valle del Belice sheep breed to identify polymorphic sites. A total of 51 coding exons of *ACACA* gene were sequenced in 32 individuals of Valle del Belice sheep breed. Sequencing analysis and alignment of obtained sequences showed the presence of 23 polymorphic sites. The most polymorphic was exon 53 which showed presence of 12 single-nucleotide polymorphisms (SNPs), of which eight were missense mutations, caused amino acid changes and therefore may affect protein function or stability causing variation in phenotype. The identified polymorphisms showed high variability of the *ACACA* gene. Sequences analysis allowed to find six new SNPs in exon 53 (6832C>T; 6835C>A; 6840G>A; 6847G>T; 6852C>T and 6860G>C). A total of 31 haplotypes were inferred. Although this study could not provide association study with production traits, it shows finding of novel SNPs that might be important in future studies and laid the basis for further association analyses needed to evaluate the potential use of these SNPs as genetic markers for fat content and FAs composition in milk of Valle del Belice sheep breed.

Keywords. *ACACA* gene; single-nucleotide polymorphisms; sheep; Valle del Belice breed.

Introduction

Acetyl-coA carboxylase (ACACA) is the flux-determining enzyme in the regulation of fatty acid (FA) synthesis in animal tissues. The eukaryotic ACACA enzymes are multidomain and contain the biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT) domains (Wakil *et al.* 1983; Abu-Elheiga *et al.* 2001; Cronan and Waldrop 2002). ACACA is a complex multifunctional enzyme system that resides in the cytosol, which catalyses through a pathway of carboxylation ATP-dependent of acetyl-CoA to form malonyl-CoA, which is the substrate for the synthesis of palmitic acid and long-chain FAs (acyl-CoA>C22:0) (Smith *et al.* 2003) by the FA synthase (FAS) enzyme.

Based on *Ovis aries* v3.1 genome release, the ovine *ACACA* gene reported in Ensembl database (www.ensembl.org, ENSOARG00000000829) is located on chromosome 11 (OAR11) and consists of 53 exons of which 51 encode a protein of 2257 amino acids (ENSOARG00000000829, UniProtKB-W5NRT6).

Sequence and organization of *ACACA* gene is similar in ruminant species. In bovine species, *ACACA* gene is located on chromosome 19 (NM_174224.2/ENSBTAT00000023364) and is organized in 56 exons of which only 54 (7381 bp) encoded for 2346 amino acids. In caprine species, this gene is located on the same orthologous chromosome and its mRNA is 8498-bp long of which 7041 bp translated in 2346 amino acids.

This enzyme is ubiquitously expressed but the highest levels of ACACA are found in lipogenic tissues such as the liver, adipose tissue and the mammary gland during lactation. The expression of the mammary gland isoform is regulated by the acetyl-CoA carboxylase-α (*ACACA*) gene (Barber and Travers 1995). During lactation, ACACA facilitates the recruitment of FA precursors to the mammary gland that uses to synthesize fat in milk. This occurs due to the repression and induction of ACACA activity

in adipose tissue and the mammary gland, respectively (Barber and Travers 1998).

From a genetic point of view, the essential role of the ACACA enzyme in milk FA synthesis suggests that it may be responsible for the phenotypic variability observed in milk fat content and milk FA composition. In fact, different expression of this gene in the lactating mammary gland suggested a direct involvement in the FA synthesis during lactation (Moioli *et al.* 2007). Several studies have demonstrated the genetic influence of *ACACA* gene on FA composition in sheep milk (Carta *et al.* 2008; Sanchez *et al.* 2010). However, before attempting association analyses between this gene and/or enzyme and phenotypic traits of interest, a study on the genetic variability within this locus is required (García-Fernández *et al.* 2010). The aim of this work was to sequence the entire coding region of *ACACA* gene in Valle del Belice dairy ewes to identify polymorphic sites. In fact, the genetic diversity in the gene responsible of the FA synthesis could elucidate some peculiar characteristics of the local breeds that might affect fat yield and quality of their products.

Materials and methods

Sampling and DNA extraction

Sample collection, animal management and care followed the recommendation of EU Directive 2010/63/EU. A total of 32 individuals of Valle del Belice sheep breed were randomly collected from 10 farms. About 10 mL of blood were collected using vacutainer tubes containing EDTA as anticoagulant. Genomic DNA was extracted using salting-out method (Miller *et al.* 1988). Subsequently, NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to control the quantity and quality of the DNA, samples were diluted to a final concentration of 50 ng/μL with ultrapure water and stored at 4°C until use.

Amplification and purification protocols

A total of 51 coding exons (6774 bp, ENSOARG00000000829) of *ACACA* gene were amplified by polymerase chain reaction (PCR). The primer pairs and different protocols used for PCR amplifications are provided in table 1. The PCR reactions were carried out in 25 μL of final volume containing 0.5 μM of each primer, 0.6 mM of dNTP Mix, 1 U of *Taq* DNA polymerase (Fermentas, Hanover, USA), 1× PCR buffer with KCl, from 2 to 3.5 mM of $MgCl_2$, and ~100 ng of genomic DNA. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 50–70°C for 1 min and extension at 72°C for 1 min 30 s. A final extension step at 72°C for 5 min was performed (the sizes of the obtained fragments are shown in table 1.

The PCR products were checked by electrophoresis on 1.5% agarose gel stained with SYBR Safe (Invitrogen, Carlsbad, USA). The amplified fragments were purified using 10 U of *Exo*I and 1 U of Shrimp Alkaline Phosphatase (Fermentas) following manufacturer's protocol.

Sequencing protocol

Sequencing reactions were carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) with 5 μM of each PCR primer and internal primers for fragments greater than 530 bp (table 2). Cycle sequencing reaction was performed according to manufacturer's instruction following ethanol / EDTA / sodium acetate precipitation. Sequencing analyses were performed in an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Sequences data analysis

Obtained nucleotide sequences were checked using Sequencing Analysis v5.3.1 software (Applied Biosystems) and subsequently analysed with SeqScape v2.5 software (Applied Biosystems). Polymorphic sites were confirmed by visual examination of the electropherograms. Multiple alignments of the sequences were performed with Clustal W software (Thompson *et al.* 1994) using the mRNA ovine reference sequence (ENSOART00000000909).

Statistical analysis

The allele and genotypic frequencies, and deviations from Hardy–Weinberg equilibrium were estimated with the GenePop software v4.2 (Rousset 2008). Nucleotide diversity was estimated using DnaSP software v5.0 (Librado and Rozas 2009). Arlequin v3.5.1.2 (Excoffier and Lischer 2010) was used to infer haplotypes and Haploview v4.2 software (Barrett *et al.* 2005) was used to estimate linkage disequilibrium (r^2) among SNPs. ExPASy-Traslate tool was used to calculate the isoelectric point (pI) and molecular weight (MW) of the protein wild type and protein with the new SNPs found.

Results and discussion

This is the first report of sequence of the entire coding region of *ACACA* gene in Valle del Belice dairy sheep. Sequencing analysis and alignment of the obtained sequences showed the presence of 23 polymorphic sites (table 3). The average distance between polymorphic sites was ~296 bp. All point mutations were SNPs except an insertion of 17 bp in exon 53. Out of 23 polymorphic sites, five mutations were monomorphic in our breed and they were not considered in further analyses. The exact

Table 1. List of primer pairs used for amplification of *ACACA* gene in Valle del Belice sheep breed.

Exon	Forward	Reverse	*T*°C	Fragment size (bp)
Ex 3	CCTGTGGCTTTCCTCAGATGT	TGCTCTTCCTAAAACAAGATCAGT	60	402
Ex 4	GGCGGTGAAAAGCAGGTCTC	AAGCCACATGCCAGTTTCAAG	68	313
Ex 5	TTGCCTGCCTTTTCCACTTT	AGGTTGCGCCATTCAATGTTTT	68	253
Ex 6	CCCACGAATGTAGTTTGTCCC	GGATACCTGATTCACTTCGCTG	68	360
Ex 7	TGCTATGGCGCTATTGTACCT	ATGGACCAAAATGACAGAGAGA	66	260
Ex 8	ATGCAATGTGGGGGATACAAGA	CCCAGCCACAGTCCTAAATACC	66	290
Ex 9	AACTCCAAGGATTGATCAGGGTT	CCAGGCTCCAAATCCCCATTA	58	366
Ex 10	TGCCAGTTTTCTCCTTCTCTAC	GGTAGAGGCTACAGGGAATGA	64	318
Ex 11	ACATGGTCCCTGACACTTCC	ACTCCACTATTCTCAGTTGGGC	68	410
Ex 12	AGAAGCTTCAGACGAGCAGT	CACGAGACTTGGATAGGCGG	68	367
Ex 13–14	AGGTGGTGGCTACTGAAGTG	ACAAGACTCCCTTTCCCATGC	66	884
Ex 15–16	GGCACTGTCTGTTTCTTTCCC	GGTCTCTGAGCGTCAGTCCT	56	1206
Ex 17–18	AGCCAATCACCTTAGAGAGTCC	AGACCTTGAAAACCCCAAGAGT	66	1033
Ex 19	TGGCATTGTAAACCAAGATGC	CCTAGTCCATCCCAGCCAAC	64	380
Ex 20–21	CCAACCATGGCAAAAGTGGATT	AAACAGCTCTATCTCCCTCCCT	68	1111
Ex 22–23	GCAGTGCATAGTAAAGCGTG	GTGACCTGAAGGCAAACCTCT	70	1503
Ex 24	CATGGTTGGCTATTTAACGGTGT	TGTTCGTGTCAAGTGGTGGT	70	280
Ex 25	AACCACACTGCCAATTCTGTAG	TCACACAAGGCAGGAGCTTT	68	369
Ex 26	TCGGCCACACATCTACAAGC	GGAAGAGCTCATGGAACGGA	68	321
Ex 27–28	CCCTCAGGAACTTAACGCCTC	CGGCCAGAAGCTGAGAAAAAG	68	1327
Ex 29	TTCTGTCAGCAGAGACCACC	CAGATAGAGCTCTGGTTGGC	62	297
Ex 30	TTGTGGCTTTCCCCTAACCTC	CAGGACCTCCGAGCAGAAA	66	197
Ex 31–32	AGTGGGTCAGAAACCAAGCC	CCCACGTGCCACAACTAAGA	70	1250
Ex 33	ACTTTTCTCTGCCCCCGTCT	AAGGATGGATACGTCACACAGG	70	241
Ex 34–35	GGGCCTTCTGAGGACTGAAC	AGGGACCAAATATCAGACCAGC	70	525
Ex 36–37	CCCCAAAGAGTCGGTCGTAT	ACATACTGCAAGCCGAGTGG	62	1441
Ex 38	GGGTTGGCCAAAATCCAAACA	TCCAGGTCCAAGTGCAAACA	64	317
Ex 39	CGTTCCTCCCTTCCCAAAGA	GCTGAGCGAGGATAATTCCCA	54	413
Ex 40	GTCCCTCTAGGCAACTCATGT	AAGCTTTTCTCTCGGGCACA	68	289
Ex 41	GTCCATGTTGAAATGCTGATTGT	AGTGGGCACAGAGATAGTGG	62	245
Ex 42	TCAAGGAGCCTGGAACAAAA	AAACCTCTACTTCTCTCCCACA	60	437
Ex 43	TTGCTGGGTCTTCTGGTCAT	TGCGACAAGCAGTCTTCATT	66	280
Ex 44–45	TGTTGTTACTGTGTTTAACCTGTCT	TTCCCCCACCAATCAAGACC	66	1265
Ex 46–47	GCAGTTACGAGGCAGACCTT	TGCTATCGGGCAGGAATTGG	66	1020
Ex 48	CAGGTCCAACCTTCCACTCG	GCTCCTCGGTCCTTCACTAC	66	296
Ex 49–50	AAGAGTCCGATTTCCCCCTG	CCCTCTGCTACAGGGTTCAG	68	1351
Ex 51	TCTGTGTACCGTGATGGGAAC	CTTGGTTAGACAGGCTCTCCC	68	335
Ex 52	TAACCGGCCTCTCTCTGTTGT	CTGAACGGCTCACTGACTTG	68	236
Ex 53	CCAGTTATCAGCAGAGGCGG	GTGGGACTCAGTTTCCCGTC	68	528

T°C, annealing temperature.

positions of the SNPs were assigned according to the sequence ENSOART00000000909 (http://www.ensembl.org) and the exons in which they were found and their accession numbers (LT627649–LT627657) were reported in table 3. Among the 53 sequenced exons, only 11 showed polymorphism in Valle del Belice sheep breed. In particular, the polymorphic sites were found in exons 9, 11, 13, 17, 19, 37, 42, 51, 53. The most polymorphic was exon 53 which showed the presence of 12 SNPs. The number of polymorphisms identified in Valle del Belice breed showed high variability of the *ACACA* gene. In a previous study, García-Fernández *et al.* (2010) sequenced ~6.6 kb of the *ACACA* gene in sheep and identified a total of 22 synonymous SNPs, some of which overlapped with those found in our breed. *ACACA* gene has been reported to be less variable in other livestock species. Badaoui *et al.* (2007) in a study on goat breeds, showed only one silent SNP in exon 45, whereas Matsumoto *et al.* (2012) in cattle, reported five synonymous SNPs in exons 7, 22, 30, 39, and 48, respectively. Therefore, the *ACACA* gene in sheep presents a great variability in contrast to other species. Currently, most studies on *ACACA* gene have focussed their attention on different promoter regions. Two promoter regions (PI and PII) were identified in mammals (Lopez-Casillas *et al.* 1991) and a third promoter region (PIII) that initiates transcription of the gene in sheep (Barber and Travers 1998), cattle (Mao *et al.* 2001) and goat (Signorelli *et al.* 2009). The genomic regions encoding the three promoters of the *ACACA* gene in sheep were directly sequenced with the identification of 10 SNPs, and the association analysis with milk traits was performed for one SNP of PIII (GenBank AJ292286, g.1330G>T) showed a significant

Table 2. List of internal primer pairs used for sequencing fragments >530 bp in *ACACA* gene of Valle del Belice sheep breed.

Exon	Forward	Reverse	Fragment size (bp)
Ex 13	AGGTGGTGGCTACTGAAGTG	CCAGGGGAGGTCCCAAGATA	377
Ex 14	GGCCCGCAAACCTTAATGAC	ACAAGACTCCCTTTCCCATGC	391
Ex 15	GGCACTGTCTGTTTCTTTCCC	GGCACAAGAACGCTTACTTACA	448
Ex 16	ACTCTCCGATTGGCTCAGTG	GGTCTCTGAGCGTCAGTCCT	407
Ex 17	AGCCAATCACCTTAGAGAGTCC	GCTCTGATGGTTCTCTGTCTCT	406
Ex 18	GATCAGGGATTGAGCCTGGG	AGACCTTGAAAACCCCAAGAGT	413
Ex 20	CCAACCATGGCAAAAGTGGATT	TGCATGATACCAAAGGCACA	409
Ex 21	CGCCTGGATGGCTGATTCTT	AAACAGCTCTATCTCCCTCCCT	415
Ex 22	GCAGTGCATAGTAAAGCGTG	CAGACAGTGAGGAGCATCCA	403
Ex 23	TCCAATTAAAACAACAACAGCAGGA	GTGACCTGAAGGCAAACCTCT	305
Ex 27	CCCTCAGGAACTTAACGCCTC	GGACACATCCATCAAGGCCA	385
Ex 28	AAGCCAAGGCAGAGAGAAGG	CGGCCAGAAGCTGAGAAAAAG	303
Ex 31	AGTGGGTCAGAAACCAAGCC	GTACCAGCGAGGGCTACATC	368
Ex 32	CACGTCCTGTCTAGCCACTG	CCCACGTGCCACAACTAAGA	384
Ex 36	CCCCAAAGAGTCGGTCGTAT	AGCAGTCCTTTCTTAGTACATCAT	382
Ex 37	GGCACAACATGGCTTATGTTTCT	ACATACTGCAAGCCGAGTGG	394
Ex 44	TGTTGTTACTGTGTTTAACCTGTCT	AGAATCCTGCACCGCAATCA	387
Ex 45	TGGCTTTAACCAGGAAATTGTGT	TTCCCCCACCAATCAAGACC	376
Ex 46	GCAGTTACGAGGCAGACCTT	GTCTCACATGCTGAGGCAGT	300
Ex 47	TCCAGCTGATGGATGGGACT	TGCTATCGGGCAGGAATTGG	339
Ex 49	AAGAGTCCGATTTCCCCCTG	TCAAGGCTGCTGACTGTCTC	358
Ex 50	GGGTGGTGGAATCTAGGCTG	CCCTCTGCTACAGGGTTCAG	467

Table 3. List of the SNPs identified in *ACACA* gene of Valle del Belice sheep breed.

Exon	Fragment size (bp)	Positions (bp) Ensemble	Identified SNPs (position)	Accession number	Codon variant	Mutation type	Amino acid change
Exon 9	107	11:13,203,582	SNP1_1040G>T	LT627650	CTG/CTT	Silent	Leu/Leu
Exon 11	210	11:13,192,321	SNP1_1289C>T	LT627649	GTC/GTT	Silent	Val/Val
		11:13,192,243	SNP2_1367T>C	LT627649	TTT/TTC	Silent	Phe/Phe
Exon 13	162	11:13,184,797	SNP1_1709T>G	LT627651	GCT/GCG	Silent	Ala/Ala
		11:13,184,746	SNP2_1760C>T	LT627651	GTC/GTT	Silent	Val/Val
Exon 17	82	11:13,178,385	SNP1_2273C>T	LT627652	TAC/TAT	Silent	Tyr/Tyr
Exon 19	151	11:13,176,713	SNP1_2501A>G	LT627653	TCA/TCG	Silent	Ser/Ser
Exon 37	216	11:13,120,399	SNP1_4460C>T	LT627654	CTC/CTT	Silent	Leu/Leu
		11:13,120,354	SNP2_4505G>A	LT627654	AGG/AGA	Silent	Arg/Arg
Exon 42	270	11:13,092,108	SNP1_5309C>T	LT627655	ATC/ATT	Silent	Ile/Ile
Exon 51	178	11:13,044,974	SNP1_6572G>A	LT627656	GCG/GCA	Silent	Ala/Ala
Exon 53	320	11:13,029,628	SNP1_6832C>T	LT627657	ACG/ATG	Missense	Thr/Met
		11:13,029,625	SNP2_6835C>A	LT627657	CCG/CAG	Missense	Pro/Gln
		11:13,029,620	SNP3_6840G>A	LT627657	GAG/AAG	Missense	Glu/Lys
		11:13,029,613	SNP4_6847G>T	LT627657	GGC/GTC	Missense	Gly/Val
		11:13,029,608	INS_AYGTGAGTATG CGGCCC	LT627657	ACG/ATG	Missense	Thr/Met
		11:13,029,600	SNP6_6860G>C	LT627657	CTG/CTC	Silent	Leu/Leu
		11:13,029,566	SNP7_6894T>C	LT627657	TGT/CGT	Missense	Cys/Arg
		11:13,029,562	SNP8_6898C>T	LT627657	CCG/CTG	Missense	Pro/Leu
		11:13,029,552	SNP9_6908G>A	LT627657	GCG/GCA	Silent	Ala/Ala
		11:13,029,526	SNP10_6934G>A	LT627657	GGG/GAC	Missense	Gly/Glu
		11:13,029,483	SNP11_6977C>G	LT627657	CCC/CCG	Silent	Pro/Pro
		11:13,029,471	SNP12_6989T>C	LT627657	CCT/CCC	Silent	Pro/Pro

effect of allelic substitution (Moioli *et al.* 2013). Other studies on the promoter regions were also conducted on cattle and goats. In particular, Matsumoto *et al.* (2012) in cattle have identified 28 SNPs in PI and three SNPs in PIII, whereas Zhang *et al.* (2009) identified eight SNPs in the PI; in goat, Signorelli *et al.* (2009) reported three SNPs in PIII. In our study, all SNPs reported in exons 9, 11, 13, 17, 19, 37, 42, 51 and four SNPs of exon 53 (SNP6, SNP9,

Table 4. Allele and genotype frequencies of polymorphic SNPs ($n = 18$) identified in *ACACA* gene of Valle del Belice sheep breed.

SNP	Allele frequency		Genotype frequency			HWE
EX9_SNP1	G	T	GG	GT	TT	*
	0.4828	0.5172	0.34	0.28	0.38	
EX11_SNP1	C	T	CC	CT	TT	ND
	0.9828	0.0172	0.97	0.03	0	
EX11_SNP2	T	C	TT	TC	CC	NS
	0.1034	0.8966	0.03	0.14	0.83	
EX13_SNP1	T	G	TT	TG	GG	*
	0.6207	0.3793	0.59	0.07	0.34	
EX13_SNP2	C	T	CC	CT	TT	*
	0.3793	0.6207	0.27	0.21	0.52	
EX17_SNP1	C	T	CC	CT	TT	*
	0.4310	0.5690	0.31	0.24	0.45	
EX19_SNP1	A	G	AA	AG	GG	NS
	0.7759	0.2241	0.62	0.31	0.07	
EX37_SNP1	C	T	CC	CT	TT	NS
	0.5862	0.4138	0.41	0.35	0.24	
EX37_SNP2	G	A	GG	GA	AA	NS
	0.8966	0.1034	0.83	0.14	0.03	
EX42_SNP1	C	T	CC	CT	TT	NS
	0.8276	0.1724	0.69	0.28	0.03	
EX51_SNP1	G	A	GG	GA	AA	NS
	0.9310	0.0690	0.86	0.14	0	
EX53_SNP5	T	C	TT	TC	CC	NS
	0.4828	0.5172	0.30	0.35	0.35	
EX53_SNP6	G	C	GG	GC	CC	NS
	0.8276	0.1724	0.72	0.21	0.07	
EX53_SNP7	T	C	TT	TC	CC	*
	0.5172	0.4828	0.38	0.28	0.34	
EX53_SNP8	C	T	CC	CT	TT	NS
	0.4828	0.5172	0.31	0.35	0.34	
EX53_SNP9	G	A	GG	GA	AA	NS
	0.7586	0.2414	0.62	0.28	0.10	
EX53_SNP11	C	G	CC	CG	GG	NS
	0.4828	0.5172	0.31	0.35	0.34	
EX53_SNP12	T	C	TT	TC	CC	NS
	0.4828	0.5172	0.31	0.35	0.34	

ND, not detected; NS, not significant; *$P < 0.05$. A significant P value indicates deviation from HWE.

SNP11, and SNP12) were silent mutations, while the others eight SNPs of exon 53 were missense mutations and caused amino acid changes (table 3). This is notable as they are nonsynonymous changes and therefore may affect protein function or stability causing variation in phenotype.

The silent mutations in biotin carboxylase domains were found in exons 9, 11 and 13. In acetyl-CoA carboxylase central domain, the silent mutations were found in exons 17 and 37. In acetyl-CoA carboxyl transferase domain, it was found in exon 42, whereas the missense mutations were found in the domain acetyl-CoA carboxyl transferase C-terminal in exon 53.

The sequences analysis allowed to find six new SNPs in exon 53 of Valle del Belice sheep breed (6832C>T; 6835C>A; 6840G>A; 6847G>T; 6852C>T and 6860G >C), which are not reported in the reference sequence available in Ensembl database. Further, at position 6852C >T, an insertion of 17 bp has been highlighted, inside which a SNP is present. This insertion leads to a frameshift and the presence of SNP determines a premature stop codon. This mutation caused the formation of a new protein that, in respect to the wild type which consists of 2257 amino acids, lacks the final 46 amino acids and presents 2211 amino acids. Results of ExPASy-Translate tool showed differences among the isoelectric points (pI) of the wild-type protein and other proteins considering the insertion of 17 bp and SNPs. The three pI were 6.14, 5.94 and 6.09, respectively. Moreover, the MWs were different among proteins and precisely 254325.25 Da for the wild-type protein and 249189.00 and 254303.15 Da for protein with amino acids substitutions and with the premature stop codon.

Table 5. List of best inferred haplotypes and their frequencies identified in *ACACA* gene of Valle del Belice sheep breed.

	Haplotype	Frequency
H1	TCCTTTATGCGCGCTGGC	0.259
H2	GCTTCCACATGCGCTGGC	0.052
H3	GCCTTTATGCGCGCTGGC	0.034
H4	GCCGTTGCGCGTGTCACT	0.017
H5	TCCTTTACGCGTGTCACT	0.034
H6	TCCGCTACGCGTGTCACT	0.069
H7	TCCGCTACGTGTGTCACT	0.017
H8	GCCTCCACATGCGCTGGC	0.017
H9	GCCGCTACGCGTGTCACT	0.034
H10	GCTTCCACGCGTGTCACT	0.017
H11	GCCTCCGCGTGTGTCGCT	0.017
H12	GCCGCCGCGCGTCTCGCT	0.017
H13	GCCGTCGCGTGTGTCGCT	0.017
H14	TCCGCTGTGCGCGCTGGC	0.034
H15	GCTTCCACGCGTCTCGCT	0.017
H16	GCCTCCGCGCGTCTCGCT	0.017
H17	GCCTCCACATATCTCGCT	0.017
H18	TTCGCCACGTATGTCACT	0.017
H19	GCCGCCGTGCATGTCGCT	0.017
H20	GCCGTCGCGCGTGTCACT	0.017
H21	GCCGTCACGCGTCTCGCT	0.034
H22	GCCGTCGCGCGCGCTGGC	0.017
H23	GCCGTCATGCGCGCTGGC	0.034
H24	GCTTCCACACGCGCTGGC	0.017
H25	TCCTTCATGCGCGCTGGC	0.017
H26	GCCTTCATGCGCGTTGGC	0.017
H27	GCCTTCGCGCGTGTCGCT	0.017
H28	GCCGTCGCGCGTCTCGCT	0.017
H29	GCCGTCACGTATGTCACT	0.017
H30	TCCTTTACGCGTCTCGCT	0.052
H31	TCCTTTATGCGCGTTGGC	0.017

The six novel substitutions reported in this study, together with the substitutions previously observed (García-Fernández *et al.* 2010) suggest ovine *ACACA* gene is highly variable among breeds and that further potential exists for variation of this gene. Allele and genotypic frequencies and HWE for all SNPs are reported in table 4. Of 18 SNPs, only five were not in HWE ($P < 0.05$). The SNP1 on exon 51 was the SNP with the lowest MAF (0.0690) while the SNP2 on exon 11 was the one with the highest MAF (0.8966).

The nucleotide diversity (π) was used to measure the degree of polymorphism within the breed. This parameter indicates the average number of nucleotide differences per site between sequences calculated from pairwise comparisons. We obtained a value of 0.00097 that shows low nucleotide diversity.

A total of 31 haplotypes were inferred considering the 18 heterozygous polymorphic sites (table 5). Haplotype H1 was the most frequent (0.259), whereas the other haplotypes showed similar frequencies ranged from 0.017 to 0.069. Haplotype-based analysis can provide higher power, precision and quality to assess the relationship between genetic variation and phenotypes compared to single SNP analysis (Tan *et al.* 2005; Tolone *et al.* 2016). The linkage disequilibrium patterns (r^2) between SNP pairs within the ovine *ACACA* gene were estimated. The mean value of r^2 between pairwise combinations of SNPs was 0.17.

In conclusion, in this study, we demonstrate high genetic variability in the *ACACA* gene in Valle del Belice sheep breed. Although this study could not provide any association study with production traits, it shows finding of novel SNPs that might be important in future studies. In fact, the results suggest that ovine *ACACA* gene presents high variability and requires further characterization among different breeds. The characterization of *ACACA* gene reported here laid the basis for further association analyses needed to evaluate the potential use of these SNPs as genetic markers for fat content and FAs composition in milk of Valle del Belice sheep breed.

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