Characterization of the *SREBP-1* Gene Polymorphisms and Milk Traits in Dairy Sheep

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Summary

The *SREBP* genes (Sterol Regulatory Element-Binding Proteins) are involved in the milk fat synthesis. In dairy cows some polymorphisms at the *SREBP-1* gene sequence have been related with milk fat content. The aim of this study was to characterize the entire coding regions of the SREBP-1 gene in Sarda sheep breed, in order to highlight any polymorphisms and their association with milk traits. Four-hundred adult and lactating Sarda ewes were selected. Individual milk yield was recorded monthly from Day 30 to Day 150 of lactation, and fat and protein concentration were analysed. A blood sample from each ewe was taken for DNA extraction; thus, all the 19 coding exons of the *SREBP-1* gene were amplified by polymerase chain reaction (PCR). Single-strand conformation polymorphism analysis (SSCP) and sequencing were used to scan mutations. Results provide, for the first time, the entire coding DNA sequence (CDS) of the *SREBP-1* gene in sheep, and by sequences analysis 8 polymorphisms have been detected. The statistical analysis exhibited no relationship between polymorphisms and milk traits. The low *SREBP-1* gene diversity that emerged from the present study, may be linked to the important role of this gene in the mechanism of milk fat synthesis or to the severe genetic selection performed in the Sarda sheep. However, it would be necessary to extend the study, including other breeds and other genes, in order to expanding the knowledge about the process of milk fat synthesis in dairy sheep.

Key words

single nucleotide polymorphism; *SREBP-1* gene; milk fat synthesis; dairy sheep

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Introduction

Lipids are important components of milk for their biological value, and their nutritional and organoleptic properties (Lock and Bauman, 2004; Harvatine et al., 2009). The concentration of fatty acid in milk is influenced by nutrition and by genetic traits (Bauman et al., 2011). Among the genes involved in the regulation of the fatty acid secretion, the *SREBP* genes have recently received increased interest due to their role in the synthesis of mammary lipids (Nafikov et al., 2013). Mammalian genomes have two different *SREBP* genes, named *SREBP-1* and *SREBP-2*. In dairy cows single nucleotide polymorphisms (SNPs) in the intron 5 and exon 14 of the *SREBP-1* gene were found to be related with different milk fat content (Cecchinato et al., 2011; Rincon et al., 2012; Hoaschi et al., 2007). Contrarily, in Sarda sheep no variation was found in the intron 5 and exon 14 of the *SREBP-1* gene (Cosso et al., 2012). However, in dairy sheep different expression levels of the *SREBP-1* gene are found to be associated with milk fat yield (Carcangiu et al., 2013). Thus the aim of the present study was to clarify the entire coding region of the *SREBP-1* gene and to evidence possible polymorphisms and their association with milk traits in Sarda breed sheep.

Material and methods

The research was conducted on 400 adult lactating Sarda breed ewes from two farms, with the same management and feeding conditions, located in North Sardinia. Feeding consisted of natural extensive pasture supplemented by 300g per head daily of commercial pellets (crude protein 20.4% and 12.5 MJ ME/kg DM). Hay (crude protein 11.1% and 7.2 MJ ME/kg DM) and water were *ad libitum*. A jugular blood sample was collected from each ewe using vacuum tubes with EDTA as an anticoagulant (BD Vacutainer Systems, Belliver Industrial Estate, Belliver Way, Roborough, Plymouth, PL6 7BP, U.K.). Monthly individual milk yield was recorded from Day 30 to Day 150 of lactation. For each sample fat and protein were analysed (CombiFoss 6000, Foss Electric, Slangerupgade 69, DK 3400 Hillered, Denmark). DNA was extracted from whole blood using a commercial DNA extraction kit (NucleoSpin®Blood, Macherey–Nagel Postfach 101352 D-52313, Düren Neumann Neander Str. 6-8 D-52355 Düren, Germany). The primer set (reported in Table 1) were designed on the *SREBP*-1 gene bovine sequence using Primer 3 plus software. Each exon was amplified by polymerase chain reaction (PCR) in a reaction mix containing 2.5 µl of template DNA (100 ng), 2.5 µl of 10X PCR Buffer (minus $MgCl_2$) (20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2 mM Sodium Phosphate, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50% (v/v) glycerol), 1.5 mM $MgCl_2$, 4 µl of 1.25 mM of each dNTPs, 10 pM of each primer and 0.2 µl of 5U *Taq* DNA polymerase (Platinum *Taq* DNA Polymerase Invitrogen, Carlsbad, CA, USA), in a final volume of 25 µl. The PCR reaction was started by a 2.30 min template denaturation at 95°C, followed by a 35-cycle program with 20 s denaturation at 95°C, 30 s annealing at different temperatures among 54.7°C and 67°C for the different segment, elongation 72°C; finally a 10 min final extension at 72°C for all the fragments on a Mastercycler® 5333 (Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany). Amplified fragments were named F1-F19. The F19 was very long and no restriction enzyme has been found to cut its sequence, so it was the only fragment to be sequenced directly after amplification. All the other fragments (F1-18) were subjected to single-strand conformation polymorphism analysis (SSCP), to scan mutations. Aliquots of 2.5 µl of PCR products were denatured at 95°C for 10 min in a 7.5 µl of denaturing solution containing 1 mg/ml of xylene-cyanol (Sigma–Aldrich Corporation, St. Louis, MO 63103, Missouri, USA), 1 mg/ml of bromophenol blue (Amersham Pharmacia Biotech, AB Vinstandelsstift, Blasieholmstrong 12, 111 48 Stockholm, Sweden), 10 mM of EDTA (pH 8), 8 ml of deionised formammide (Sigma–Aldrich Corporation, St. Louis, Missouri, USA) and then chilled on ice before loading. SSCP analysis, has been conducted using the vertical electrophoresis DCode TM Universal mutations detection system for SSCP (Bio-Rad Laboratories, Via Cellini 18/A, 20090 Segrate, MI, Italy). Denatured amplicons were subject to electrophoresis using different combinations of Watt and V depending on the amplicons length. After SSCP analysis 5 samples for each migration pattern were sequenced using a commercial service (Bio-Fab Research srl, Roma, Italy).

Table 1. *SREBP-1* gene primer sequence, fragments length and annealing temperature

Fragments name	Primer sequence (5' to 3')†	Length (bp)	T annealing (°C)
F1	F: CCCAGTTTCCGAGGAACTTTTC R: GGCCCTGACGCACCTTCTAT	221	60.5
F2	F: ACGGCTGCTCACGGCTTT R: AGCCTGCAAACTCCTCTACCA	523	64.3
F3	F: AGCCCCAGCCTTCATCTCT R: TCCCTGATGCCAGCCAGAC	238	62.0
F4	F: CCTCCCAGATACAGCAGGTC R: GGCAGAGTTAGCAGGTGGAC	333	64.0
F5	F: CCTGACGACCATGAAAACAG R: TATTAGGGCCTCAGCCCACA	436	61.0
F6	F: CTCTGCCCTCTTGCTTCAGT R: AACTTCCAGGGACACCAG	228	54.7
F7	F: CCTGGTGTCCCTGGAAGTT R: CCCTCAGCCTTGTCTTTCTTC	495	59.0
F8	F: GCTGAAGGGTTCCCACAGTA R: CACAGGACGGGATCCACATA	365	63.1
F9	F: GATCTTGTCCTGTGGGCTTG R: AGCACCTTCCCAGGCACT	327	67.0
F10	F: CCCAAGATGGAGGAGTAGCA R: TGGAAGATAAGAGGGCGTGA	398	62.0
F11	F: ATGGGTATGCGGGTGAGG R: GCTGTTGAGGAGGGAATGG	387	61.0
F12	F: GTGAGGGCTGCACAGAAAG R: AGGCAAGGGACAAGACACTG	388	65.0
F13	F: GGTGCGTGTGCAAAGGAG R: CCCAGAGAGGAACCGAAATG	284	56.0
F14	F: AGCCATGTTGACCGCCTGT R: GCAGAAACTCAGCCACACTG	222	61.0
F15	F: GCTGAGTTTCTGCCTCCTGT R: CTCTGCCCTGGTTCTGGAT	276	60.1
F16	F: ATCCAGAACCAGGGCAGAG R: CATCCAGGGAGTGGAAAGG	287	64.0
F17	F: TTGTGAGGCAGGTGCAGTG R: AGTCGGGCAGTGGCTTCAT	455	64.0
F18	F: GGGACAGGCATGAGGTGT R: CATCTTACGGTCTCCCTCTG	246	62.1
F19	F: CTTCTGGACCGTAGCCTGAG R: AGCTGGAGGTCACAGTGGTC	603	57.0

† F is forward primer and R is reverse primer

Nucleotide sequence alignments were performed using the Bioedit software (www.mbio.ncsu.edu/bioedit/bioedit.html). The genotypic, allelic frequencies and Hardy-Weinberg equilibrium were calculated using GENEPOP population genetics software. (4.1- http://kimura.univ-ontp2.fr/~rousset/Genepop.htm). Statistical analysis was performed using R-Project software (R Development Core Team (2011) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). Data, normally distributed ($P > 0.05$, Anderson-Darling Test) were expressed as mean ± standard deviation (SD). General Linear Model (GLM) was applied to analyse association between *SREBP-1* gene polymorphisms and milk yield, fat and protein contents.

Results

The length in bp of the fragments obtained by PCR analysis, and confirmed by sequencing, are shown in Table 1. Results from the sequencing and SSCP analysis exhibited 7 nucleotide substitutions within the coding regions of the different exons, and one at intronic level (Table 2). Among these mutations, G981T and G5688A also exhibited an amino acid change: Gly/Val and Gly/Ser, respectively (Table 2). The population resulted in Hardy-Weinberg equilibrium for all the found polymorphisms. The SNPs position, allelic and genotypic frequencies are shown in Table 3.

Milk yield resulted higher at the 3rd sample (Day 90) while fat and protein percentage have been decreased at the higher production but did not differ significantly between the different samples. Statistical analysis revealed no significant association between SNPs and the milk traits (yield, fat and protein contents) (Table 3). No significant associations between haplotypes and milk traits were found.

Discussion

The sequence identified in the present study resulted similar to that from bovine, deposited in database (98% homology). The detected ovine SREPB-1 gene sequence, exhibited 8 different mutations. Also in cattle the polymorphisms in this gene associated with production traits are few, and this may indicate that this gene is highly conserved in both species. In addition, in the last 100 years the Sarda breed sheep has been subjected to intense genetic selection (Luridiana et al., 2014) with the subsequent possible reduction of variations, and this may explain the low number of SNPs detected in this breed, in the present

Table 2. Polymorphisms and their location within the *SREBP-1* gene in Sarda breed sheep

SNP Position	980	981	1686	4294	5448	5657	5688	6872
SNP Location†	Ex3	Ex3	Ex5	In11	Ex15	Ex16	Ex16	Ex19
Chrom. position	34.185.884	34.185.885	34.186.568.	34.188.499	34.190.588	34.190.797	34.190.828	34.191.385
SNP Alleles	GT	GT	CT	CT	CT	GA	GA	CT
Amino Acid Change	–	Gly/Val	–	–	–	–	Gly/Ser	–

† Ex is for Exons and In is for Introns; SNPs underlined are present in dbSNP

Table 3. *SREBP-1* gene SNPs position, allelic and genotypic frequency and associations with milk traits in Sarda breed sheep

SNP position	Allelic frequency	Genotypic frequency	Yield (g/die)	P-value	Fat (%)	P-value	Protein (%)	P-value
G980T	G 0.90	GG 0.84	974.9±268.1	0.148	6.3±0.7	0.383	5.8±0.5	0.060
	T 0.10	GT 0.15	875.3±99.5		6.5±0.7		6.2±0.5	
		TT 0.01	–		–		–	
G981T	G 0.90	GG 0.84	974.9±268.1	0.148	6.3±0.7	0.383	5.8±0.5	0.060
	T 0.10	GT 0.15	875.3±99.5		6.5±0.7		6.2±0.5	
		TT 0.01	–		–		–	
C1686T	C 0.70	CC 0.49	993.9±276.7	0.262	6.3±0.8	0.936	5.8±0.4	0.542
	T 0.30	CT 0.46	918.6±222.5		6.3±0.7		5.9±0.5	
		TT 0.05	1091±265.3		6.3±0.5		5.9±0.6	
C4294T	C 0.60	CC 0.35	924.7±236.9	0.593	6.2±0.6	0.314	5.9±0.5	0.893
	T 0.40	CT 0.52	986.1±269.8		6.3±0.8		5.8±0.5	
		TT 0.13	987.3±243.4		6.6±0.8		5.9±0.5	
C5448T	C 0.98	CC 0.95	936.1±254.4	0.839	6.3±0.7	0.714	5.9±0.5	0.477
	T 0.02	CT 0.05	989.7±280.9		6.4±0.7		5.7±0.4	
		TT 0.00	–		–		–	
G5657A	G 0.80	GG 0.68	880.6±188.6	0.054	6.1±0.5	0.238	6.0±0.5	0.214
	A 0.20	GA 0.32	1003.3±271.8		6.3±0.8		5.8±0.5	
		AA 0.00						
G5688A	G 0.85	GG 0.75	1060.7±355.0	0.284	5.8±0.4	0.129	5.7±0.6	0.102
	A 0.15	GA 0.21	882.2±268.5		6.0±0.8		5.6±0.5	
		AA 0.04	983.3±244.2		6.4±0.7		5.9±0.5	

study (Mura et al., 2012). In Sarda breed sheep these SNPs did not show to influence the milk composition. This result was unexpected because the *SREBP-1* gene expression is found to be correlated significantly with the daily production of individual fatty acids in ovine milk (Carcangiu et al., 2013). In the present study, as opposed to what has been found in cattle, no deletion at intron 5 or SNP at exon 14 were found, and all the analysed ewes carried C/C genotype at position 66 of the exon 14, that in cattle occurs in animals exhibiting a reduced milk fat synthesis (Rincon *et al.* 2012). Conversely, Sarda sheep produces high milk fat levels, thus it might be that in sheep this site could be not crucial for the milk fat synthesis or that in cattle this mutation is linked to other variation in other part of the sequence which perform the milk fat-decreasing effect. The present study evidenced no association between polymorphisms and the milk production traits and this is probably the consequence of a strong genetic selection for improvement in the milk production traits. Thus, it is reasonable to think that in the course of the years the animals carrying genotypes influencing negatively the organoleptic and technological properties of the milk, were removed.

Conclusion

In conclusion, data from the current research provided, for the first time, the entire coding sequence and part of the intronic regions of the *SREBP-1* gene in the ovine specie. This result is of considerable importance because identification of the entire *SREBP-1* gene sequence provides the basis for future investigations on genetic control of milk fat synthesis.

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