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Full Length Research Paper

New allelic variant of the ovine calpastatin gene

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Calpastatin, a specific inhibitor of the calpains, participates in muscle growth and tenderization. Amplified fragment of 12 intron of *CAST* was used; the gene encoding calpastatin, from 164 lambs from synthetic lines for restriction fragment length polymorphism analysis. Although, *CAST* has previously been investigated in sheep, the observed polymorphism incidence in this population was higher. Furthermore, three previously identified alleles as well as one new allelic variant (allele "e") have been detected. Eight genotypes were observed in this population. The results of this study may be useful in further research supporting associations between polymorphism in the ovine *CAST* gene on both the function and expression gene.

Key words: Polymorphism, sheep, calpastatin, RFLP, meat quality.

INTRODUCTION

Calpastatin, an endogenous and specific calpain inhibitor, inhibits calpain activity, thus is involved in the degradation of myofibrillar proteins both in living muscle and in post-mortem tissue. Thus, calpastatin also regulates protein turnover, muscle growth, and the rate and extent of post-mortem tenderization (Koohmaraie et al., 2002). A number of studies have reported associations between variations in the *CAST* gene and carcass and meat quality traits in cattle (Casas et al., 2006) and pigs (Koćwin-Podsiadła et al., 2003). The single nucleotide polymorphisms (SNPs) identified in the 3' untranslated region and in intron 5 of bovine *CAST* are currently used in the IGENITY Tender Gene marker and in the GeneSTAR Tenderness tests for marker-assisted selection (Schenkel et al., 2006). Although sheep research has not advanced extensively as research in other animals, consumers desire high lamb quality, especially with regards to tenderness (Savell and Shackelford, 1992). However, tenderness is highly variable between carcasses, suggesting that marker-assisted selection may increase the intensity and effectiveness of the selection for lamb quality.

This study was undertaken to identify the polymorphisms in the ovine *CAST* gene.

MATERIALS AND METHODS

The investigation was carried out in the Research Experimental Station of Small Ruminants, Bezek, Poland, a part of the University of Life Sciences in Lublin. The group of animals comprised of 164 ram lambs of the two synthetic meat-prolific lines: BCP- 62 animals (37.5% Polish Lowland Sheep, 12.5% Finnsheep or Romanov, 25% Berichon du Cher and 25% Charolaise) and SCP- 102 animals (37.5% Polish Lowland Sheep, 12.5% Finnsheep or Romanov, 25% Suffolk and 25% Charolaise). These animals are described as having high reproductive levels and high meat utility (Gruszecki and Lipecka, 2002).

In total, blood was drawn from 164 animals of the two synthetic meat-prolific lines for DNA isolation. Approximately, 5 ml of blood was gathered from the main zygomatic vein in tubes with ethylenediaminetetraacetic acid and were transferred at -20°C. Genomic DNA was purified from blood leukocytes with a commercial kit according to the manufacturer's instructions (Qiagen™). The concentration of DNA extracted from each sample was determined by spectrophotometry at 260 nm. The region of the ovine *CAST* gene encoding a part of the first repetitive domain was amplified using PCR with the forward primer 5'TGGGGCCCAATGACGCCATCGATG3' and the reverse primer 5'GGTGGAGCAGCACTTCTGATCACC3', which captured a fragment spanning intron 12 and exon 13 (622 bp) (Palmer et al., 2000). Primers were synthesized by Operon Biotechnologies. Amplification was performed in a reaction containing 100 ng genomic DNA, primers (10 pmol/µl), PCR reaction mix (REDTaq® ReadyMixTM, Sigma), and water in a total volume of 20 µl. The PCR cycling parameters were 2 min at 95°C, 35 cycles of 60 s at 95°C, 60 s at 62°C, and 2 min at 72°C, followed by a final extension for 10 min at 72°C.

Genotypes were determined by RFLP analysis (Palmer et al., 2000) with the restriction enzymes *MspI*, *NcoI* and *Hin6I* (Fermentas™). The digested PCR products were separated by electrophoresis in a 2% agarose gel (PRONA Agarose) with ethidium bromide for 3 h in the SubCell GT System (BioRad). The

MspI

Ovine *CAST*- a 241 gcttctcttg ttgcagagcc **g**g**g**gctctgg gtgcacaggc ttcagttgtt gtg**g**cttgag

Ovine *CAST*- b 241 gcttctcttg ttgcagagcc **a**g**g**gctctgg gtgcacaggc ttcagttgtt gtg**g**cttgag

Ovine *CAST*- c 241 gcttctcttg ttgcagagcc **g**g**g**gctctgg gtgcacaggc ttcagttgtt gtg**a**cttgag

Ovine *CAST*- e 241 gcttctcttg ttgcagagcc **a**g**a**gctctgg gtgcacaggc ttcagttgtt gtg**g**cttgag

NcoI *Hin6I*

Ovine *CAST*- a 301 agctctagag cacaggctca gtggtc**g**tgg c**g**cacaggct tactcca**c**gg catgtgggat

Ovine *CAST*- b 301 agctctagag cacaggctca gtggtc**a**tgg c**g**cacaggct tactcca**t**gg catgtgggat

Ovine *CAST*- C 301 agctctagag cacaggctca gtggtc**g**tgg c**t**cacaggct tactcca**c**gg catgtgggat

Ovine *CAST*- e 301 agctctagag cacaggctca gtggtc**g**tgg c**g**cacaggct tactcca**c**gg catgtgggat

Figure 1. Nucleotide sequences of fragment of the ovine intron-12 *CAST* alleles: “a”, “b”, “c” and “e”. Mutations sites are presented in bold. Restrictions sites of *MspI*, *NcoI* and *Hin6I* are marked. The ovine sequences were sourced from GenBank (ID: AF016006.1, AF016007.1, AF016008.1 and EU486168.1).

gel was examined under ultraviolet light and documented in Vilber Lurmat ECX. The ovine DNA samples that produced different expected RFLP patterns were selected for sequencing in a GS FLX Titanium (454) sequencer (Roche) and were sequenced in both directions with forward and reverse primers after purification (Clean-up Kit, A&A Biotechnology, Blood Mini^{TM}). Sequence data were checked by FASTA program (http://www.ebi.ac.uk/Tools/sss/fasta/) and sequence alignments were carried out using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The frequency of alleles and genotypes observed and expected heterozygosity and F_{IS} were calculated using Popgene v.1.32., while the Hardy-Weinberg equilibrium test was estimated based on the program Cevus v.3.03.

RESULTS AND DISCUSSION

We genotyped 164 rams belonging to the BCP and SCP synthetic lines and detected three previously identified SNPs (Nassiry et al., 2006, Palmer et al., 1997) in the interrogated region of *CAST*: a G/A transition was recognized by *MspI*, a T/C transition was detected by *NcoI* and a G/T transversion was the cutting site for *Hin6I*. Thus far, the polymorphisms in this region have been revealed with single-strand conformational polymorphism and RFLP in Dorset Down, Corriedale, Coopworth, Ruakura, Dorset Down x Coopworth, Arabic and Kurdi sheep (Mohammadi et al., 2008; Nassiry et al., 2006; Palmer et al., 1997; Roberts et al., 1996). In addition, a new restriction pattern was observed in more than one animal, suggesting that it did not result from PCR or RFLP errors. Sequencing of PCR amplicons which are representatives of the various RFLP patterns revealed a new allelic variant (allele “e”) that has not been reported in other breeds, confirming the assumption that the existence of new alleles in other breeds is very possible (Palmer et al., 2000). The sequences were deposited in NCBI GenBank with accession number ID: EU486168.1. The sequences of the “a” and “e” alleles differed by eight SNPs in this region; allele “e” had 11 SNPs that differed from allele “b,” and 10 SNPs that differed from “c” (Figure 1).

All eight genotypes of the four-allele system were detected in our sheep population (Figure 2). Allele “a” was predominant in this flock (0.7) (Table 1). A similar frequency for this allele was previously detected in Dorset Down, Coopworth and Kurdi sheep (Nassiry et al., 2006; Palmer et al., 1997). Allele “e,” the novel allele, was the second most frequent allele (0.16) (Table 1). Our BCP and SCP sheep exhibited a “c” allele frequency similar to

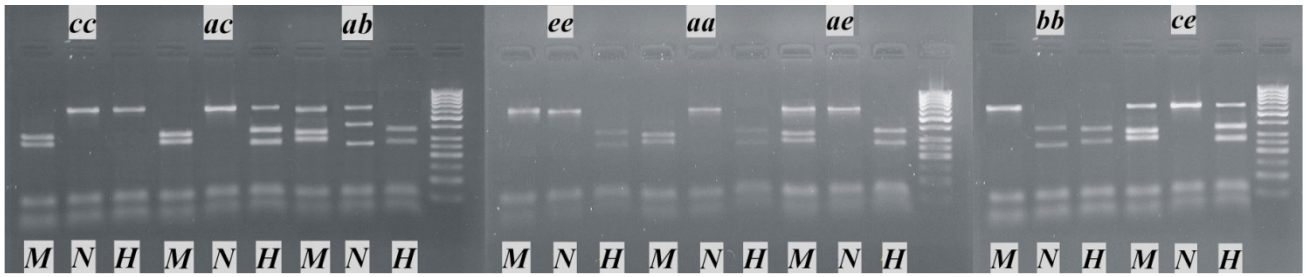

Figure 2. Restriction fragment length polymorphism genotyping of the ovine *CAST* gene. Agarose (2.5%) gel showing eight *CAST* genotypes after digestion of the 622-bp amplification product with *MspI* (M), *NcoI* (N) or *Hin6I* (H). Genotypes: ee, aa, ae, cc, ac, ab, bb and ce indicate the genotypes of individual sheep.

Table 1. Allele and genotype frequencies of ovine *CAST*, intron 12.

Parameter		Breed					
		BCP		SCP		Total (entire population)	
		n	f	n	f	n	f
Allele frequency	a	86	0.694	145	0.711	231	0.704
	b	2	0.016	8	0.039	10	0.030
	c	3	0.024	30	0.147	33	0.101
	e	33	0.266	21	0.103	54	0.165
Genotype frequency	aa	27	0.435	47	0.461	74	0.451
	ab	-	-	8	0.078	8	0.049
	ac	3	0.048	23	0.225	26	0.159
	ae	29	0.468	20	0.196	49	0.299
	bb	1	0.016	-	-	1	0.006
	cc	-	-	3	0.029	3	0.018
	ce	-	-	1	0.010	1	0.006
	ee	2	0.032	-	-	2	0.012

F, Frequency; n, number of animals.

that reported in Dorset Down and Kurdi sheep (Nassiry et al., 2006; Palmer et al., 1997). However, we detected a lower frequency of allele “b” in our flock than that reported in Correidale, Dorset Down and Kurdi sheep (Nassiry et al., 2006; Palmer et al., 1997).

Only four genotypes were reported in previously investigated flocks: “aa”, “ab”, “ac” and “bb” (Nassiry et al., 2006; Palmer et al., 1999). In comparison with the dominant frequencies of the “aa” genotype in SCP population and in Kurdi sheep (0.46 and 0.55, respectively; Table 1), our BCP sheep had a slightly higher “ae” frequency (0.468) (Table 1) than the “aa” frequency (0.43) (Table 1). In addition, the observed “ac” frequency in this study (0.048; Table 1) was nearly double the previously reported frequency (Nassiry et al., 2006). In comparison with SCP sheep, in BCP sheep, the homozygous “ee” was observed, but the frequency was very low (0.012; Table 1).

The results of the observed and expected heterozygosity F_{IS} parameter and the results of the test for deviation from Hardy-Weinberg equilibrium are presented in Table 2. There were no large discrepancies between the values of H_O and H_E. In each analyzed lines of the animal, the value of the observed heterozygosity was higher than the expected heterozygosity. There were no significant deviations from Hardy-Weinberg equilibrium. Another estimators connected with genetic differentiation is population inbreeding estimate F_{IS}, which indicates heterozygote deficiency. A negative value was observed for both populations of sheep, suggesting an excess heterozygote and that there was no inbreeding in BCP and SCP lines. The analysis of genetic variability reveals that these populations do not exhibit a decrease in genetic variation.

The variation of the CAST gene in the studied region was also the subject of interest in cattle. Chung et al.

Table 2. The coefficient of observed (H_O) and expected (H_E) heterozygosity, a test for deviation from Hardy-Weinberg equilibrium and inbreeding estimate (F_{IS}) in the population of lambs of synthetic line BCP and SCP calculated for *CAST* locus.

Description		H_O	H_E	Hardy- Weinberg test	Inbreeding estimate (F_{IS})
Line	BCP	0.516	0.451	ns	-0.15
	SCP	0.510	0.463	ns	-0.11

(1999) observed two alleles (A and B) in 12 intron of the *CAST* gene of Angus cattle. Allele frequencies for A and B were calculated as 0.29 and 0.71, respectively. In the analysed genomic DNA of the bovine CAST different cattle breeds, four new SNPs were found within intron 12 and almost equal frequencies of haplotypes and alleles suggested that CAST SNPs were linked with different causative alleles of an unknown locus affecting carcass and meat quality (Juszczuk-Kubiak et al., 2008).

A number of studies have suggested that calpastatin performs its regulatory function by binding calpain through its I domain. Since we investigated SNPs in intron 12 of *CAST*, it seems unlikely that these genotypes directly exert their effects (for example on carcass and meat quality) at the protein level. These SNPs may be linked to another mutation elsewhere in the coding or regulatory regions of the gene, and this other mutation may directly impact gene/protein expression and/or function.

We identified a larger number of allelic variants in our flocks than has been previously reported for sheep (Nassiry et al., 2006; Palmer et al., 1997); as more animals of various breeds are analyzed, it is possible that even more alleles will be found. These genotyping data will provide a background for more extensive characterization of the ovine *CAST* gene, its diversity in various breeds, and for potential commercial relevance of these *CAST* alleles, including the novel allele uncovered by this study in genetic markers for carcass and meat-quality traits.

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