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Comparison of goat, sheep, cattle and water buffalo leptin (*LEP*) genes and effects of the Intron 1 microsatellite polymorphism in goats

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Abstract. We report and compare the partial genomic sequence (from part of Intron 1 to part of Exon 3) of goat, sheep, cattle and water buffalo leptin (*LEP*) genes. Genomic DNA was obtained from leukocytes of 117 goats belonging to six breeds (Angora, Alpine, Garganica, Girgentana, Maltese and Red Syrian); 30 sheep belonging to five breeds (Altamura, Sarda, Apulian Merino, Leccese, Apennine) 50 water buffaloes and 43 Italian Friesian cattle. All the four species had a microsatellite region in Intron 1. According to the results of a population analysis, we observed 10, 5, and 2 alleles, in cattle, water buffalo and goats, respectively, in this region. No nucleotide variation was observed in sheep. The results of this study show that in Red Syrian goats the two alleles are associated with significantly different effects on β-hydroxybutyric acid ($P = 0.04$) and free thyroxine ($P = 0.018$) levels, and milk somatic cell counts ($P = 0.034$). The same microsatellite region was tendentially associated with variation in insulin-like growth factor-1 ($P = 0.082$) and triglycerides ($P = 0.072$) levels. The results of this study are further evidence for the role of leptin as an indicator of metabolism and mammary gland health in dairy ruminants.

Additional keywords: beta-hydroxybutyric acid, free thyroxine, milk somatic cell counts, ruminants.

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Introduction

The leptin (*LEP*) gene encodes for a 167 amino acid protein known as leptin. The first 21 amino acids at the N-terminal end constitute the signal peptide and the remaining 146 amino acids constitute the excreted protein. Leptin is synthesised mainly by the adipose tissue and, to a lesser extent, by muscle cells, stomach epithelium, placenta, fetal tissues, and mammary glands (Smith-Kirwin *et al*. 1998; Ahima and Flier 2000). Leptin is involved in the regulation of several behavioural, metabolic and hormonal pathways affecting, for example, food intake, body energy homeostasis, reproduction, nutrient partioning between tissues and body composition, hormone secretion by several endocrine glands, immune and renal function, haematopoiesis (Gautron and Elmquist 2011).

In cattle, polymorphisms at the *LEP* gene have been associated with effects on traits of economic interest such as: dry matter (DM) intake, feed intake, milk energy output, energy storage, calf prenatal mortality, calving interval, carcass traits, lean meat yield, growth rate and meat fatty acid composition (Giblin *et al*. 2010; Glantz *et al*. 2011; Pinto *et al*. 2011; Orrù *et al*. 2012). In sheep, polymorphisms at the *LEP* gene (Barzehkar *et al*. 2009; Zhou *et al*. 2009) have been associated with effects on some traits such as muscle growth, fat tail percent and body and carcass weight (Boucher *et al*. 2006; Barzehkar *et al*. 2009; Hajihosseinlo *et al*. 2012). In dairy goats, changes in maternal nutrition does not affect plasma and milk leptin concentration and the latter is negatively correlated with kids' liveweights and average daily growth rate (Celi *et al*. 2008). By comparison with cattle, a relatively limited number of studies have been conducted on leptin in other ruminants and in dairy goats in particular.

The *LEP* gene maps to chromosome 4q32 in cattle (Pomp *et al*. 1997; Taniguchi *et al*. 2002), sheep and goat (Perucatti *et al*. 2006), and to chromosome 8q32 in water buffalo (Vallinoto *et al*. 2004). In this study, we report and compare the partial genomic sequence (from part of Intron 1 to part of Exon 3) of goat, sheep, cattle and water buffalo leptin (*LEP*) genes and evaluate the effects of the genetic variation at this locus on some metabolic, hormonal, and dairy traits in goats.

Materials and methods

Animals

Genomic DNA was obtained from leukocytes of 117 goats belonging to six breeds (Angora, Alpine, Garganica, Girgentana,

Maltese and Red Syrian); 30 sheep belonging to five breeds (Altamura, Sarda, Apulian Merino, Leccese, Apennine) 50 water buffaloes and 43 Italian Friesian cattle.

Sequence and PCR analyses

Genomic DNA sequences were performed on overlapping PCR products obtained with primers designed on bovine *LEP* gene sequence (NCBI accession number NC_007302) using an ABI PRISM 377 automated DNA sequencer (ABI PRISM, Foster City, CA, USA). Polymerase chain reactions for analysis of microsatellite polymorphisms were performed in 20 μL volume consisting of the following reagents: 50–100 ng DNA, 1 × PCR buffer (Promega, Madison, WI, USA); 2.5 mM $MgCl_2$; 200 μM each dNTPs; 0.2 μM each primer (forward 5′-CCC AGCTCAGGCGACAC-3′; reverse 5′-CCAGGATGCCACAG TGAACA-3′); 1 U *Taq* DNA polymerase.

Phenotypic data collection

The Red Syrian goats (22 females in their second lactation) were homogeneous for days of lactation (50 ± 5 days), milk production (1.3 ± 0.3 kg/day), bodyweight (42.1 ± 1.2 kg), and feed intake (1.45 ± 0.06 kg/DM.day). The goats received pelleted feed including 65% of alfalfa hay (5.8 MJ NEl/kg DM; 15.2% crude protein; 44% neutral detergent fibre on DM) according to their energy and protein requirements. Milk samples, consisting of proportional volumes of morning and evening milk, were analysed for fat, protein, lactose and somatic cell count (SCC) by an infrared method (Combi-foss 6000, Foss Electric, Hillerød, Denmark; Ferdowsi and Rezakhani 2013). Milk casein was calculated from total nitrogen fraction and non-casein nitrogen fraction by Fédération Internationale du Lait - International Dairy Federation (FIL-IDF) standard procedures (International Dairy Federation 1964). Milk urea content was determined using a differential pH meter (CL10, Eurochem, Savona, Italy). Blood samples (8 mL) were taken from all goats on the same day and before morning feeding, by using vacutainer tubes containing lithium heparin (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and immediately placed on ice. Within 1 h from collection, blood samples were centrifuged at 1400*g* at 4°C for 20 min, plasma harvested and stored at −20°C pending analysis. A TARGA model 2000 (Technology Advanced Random Generation Analyser, Biotecnica Instruments, Roma, Italy) automated analyser was used to determine glucose, cholesterol, triglycerides, urea, total protein and albumin. Non-esterified fatty acids and β-hydroxybutyric acid (BHBA) were analysed by using respectively FA 115 and Ranbut commercial kits (Randox Laboratories, Crumlin, Antrim, UK). Commercially available ELISA kits were used to determine insulin (Mercodia, Uppsala, Sweden), insulin-like growth factor-1 (IGF-1, DRG Instruments GmbH, Marburg, Germany), free triiodothyronine (fT3; DiaMetra Srl, Milano, Italy) and free thyroxine (fT_4; DiaMetra). Before statistical analysis, triglycerides, fT_4 and SCC data were transformed into log(y + 10), log(y + 10) and ln values, respectively.

Statistical analyses

The association between the observed leptin microsatellite genotypes and metabolic, hormonal and dairy data was evaluated with the ANOVA procedure (SYSTAT 2009) using the following statistical model:

$$Y_{ij} = \mu + A_i + e_{ik},$$

where Y_{ij} is the observation of the metabolic, hormonal and dairy trait, μ is the means for each trait, A_i is the effect of the genotype, and e_{ik} is the random residual effect. Differences with $P < 0.05$ were considered significant and those with $0.05 < P < 0.1$ were considered a trend.

Results and discussion

We sequenced the goat (4955 nucleotides, GenBank Accession number AM114 397), sheep (4686 nucleotides, GenBank Accession number HE605 296), cattle (4385 nucleotides, GenBank Accession number HE605 298), and water buffalo (4929 nucleotides, GenBank Accession number HE605 297) *LEP* genes. All the deposited sequences span from part of Intron 1 to part of Exon 3 and include the whole coding sequence for the 167aa pre-mature leptin.

Similarity analysis of the four orthologous *LEP* genes by using CLUSTAL4 algorithm (Higgins and Sharp 1989) showed that *Capra hircus* and *Ovis aries LEP* genes are more similar than *Bos taurus* and *Bubalus bubalis LEP* genes (Fig. 1). The same results were obtained comparing the four coding sequences since only two silent mutations between *Capra hircus* and *Ovis aries* and 12 mutations (9 silent and 3 missense) between *Bos taurus* and *Bubalus bubalis* were observed. These results are in agreement with those obtained by Denver *et al*. (2011) by comparing leptin amino acid sequence.

In addition, the alignment of the four DNA sequences indicates that goat and sheep Introns 2 are 80 nt longer than cattle and water buffalo counterparts because of two insertions: a 30-nt insertion starting at 519 nt downstream from the end of Exon 2 and a 50-nt insertion starting 547 nt upstream from the beginning of Exon 3. This result indicates that both insertions were present in the ancestor common to goat and sheep. Similarity analyses using sequences deposited in the EBI database showed that the 30-nt insertion is specific to both the goat and sheep leptin gene, whereas the 50-nt insertion is highly similar (92%) to part of a bovine SINE A repeat (Band and Ron 1996) and, therefore, shows homology with thousands of deposited bovine sequences.

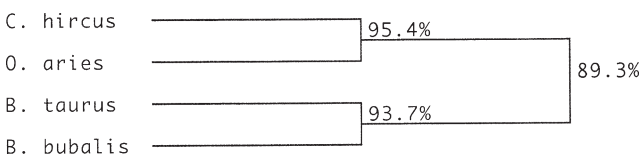

Fig. 1. Phylogenetic analysis of goat (GenBank Accession number AM114 397), sheep (GenBank Accession number HE605 296), cattle (GenBank Accession number HE605 298) and water buffalo (GenBank Accession number HE605 297) *LEP* gene. Compared sequences correspond to nucleotides residues from 1005 upstream the second exon to 1085 downstream the STOP codon. The dendrogram was produced by applying the unpaired geometric mean analysis method using the Higgins–Sharp algorithm (CLUSTAL4) supplied in the MacDNASIS software package (Hitachi Software). Calculated matching percentages are indicated at each branch point of the dendrogram.

The four species showed a microsatellite region [A(n)TA(n) in cattle, water buffalo and goat and A(5)CATA(2)TA(2) in sheep] ~800 nt upstream of Exon 2. Population analyses of this region showed 10 alleles in 43 samples of the Italian Friesian bovine breed. Only 6 (281 bp, 284 bp, 285 bp, 286 bp, 291 bp and 293 bp) of these alleles should correspond to those (173 bp, 176 bp, 177 bp, 178 bp, 183 bp and 185 bp, respectively) already observed by Wilkins and Davey (1997). The other four alleles were observed for the first time. In 50 water buffaloes, five (279 bp, 284 bp, 285 bp, 287 bp and 291 bp) alleles were observed and should correspond to five (171 bp, 176 bp, 177 bp, 179 bp and 183 bp, respectively), of the seven alleles already identified by Vallinoto *et al*. (2004) (Table 1). In goat, two alleles (266 bp and 264 bp) were observed in 117 samples belonging to six different breeds (Table 2). Sequence analysis of *LEP* gene coding regions of 11 goats, belonging to six breeds and with different genotypes at this microsatellite region, did not show any difference among individuals. In sheep, analysis of 30 DNA samples belonging to five different breeds (Altamura, Sarda, Apulian Merino, Leccese, and Apennine) did not show any polymorphism at this microsatellite region.

Some metabolic, hormonal, and milk traits data were only available for the 22 Red Syrian goats indicated in Table 2. These goats, 14 homozygous 266 bp/266 bp (L genotype) and 8 heterozygous 266 bp/264 bp (H genotype), were homogeneous for days of lactation, milk production, bodyweight, and food intake. The least-squares means and standard errors of the considered metabolic, hormonal and dairy traits in goats with the two genotypes are shown in Table 3. Goats with the L genotype showed significant higher BHBA (P = 0.04) and SCC (P = 0.034) values than the H genotype. The effect of leptin gene polymorphisms on BHBA was also detected in Holstein cows (Oikonomou *et al*. 2009). Ketone bodies, especially BHBA, are routinely used as an indicator of energy status in dairy animals and the observed increased in BHBA concentration in goats with the L genotypes indicates incomplete oxidation of non-esterified fatty acids in the tricarboxylic acid cycle. The concomitant higher concentration of triglycerides observed in the L genotype goats suggests that this group of animals were utilising adipose reserves rather than storing fat. Furthermore, L genotype goats showed a significant lower plasma level of fT4 (P = 0.018) than H genotype ones. Thyroid hormones are indicators of the nutritional and metabolic status

Table 1. Allele frequencies at the *LEP* gene Intron 1 microsatellite region in 43 Italian Friesian cattle and 50 water buffalo

Allele (bp)	Frequency
Cattle	
281	0.08
284	0.41
285	0.03
286	0.05
291	0.01
293	0.06
295	0.27
305	0.02
311	0.06
314	0.01
Water buffalo	
279	0.66
284	0.15
285	0.07
287	0.05
291	0.07

Table 2. Frequencies of the two alleles observed at the *LEP* gene Intron 1 microsatellite region in six goat breeds

Breed	Alleles		*n*
	266 bp	264 bp	
Malta	0.81	0.19	26
Alpine	0.54	0.46	14
Girgentana	0.87	0.13	8
Angora	1	–	18
Garganica	0.90	0.10	29
Red Syrian	0.82	0.18	22

Table 3. Least-squares means and standard errors for metabolic, hormonal and milk traits of the two leptin genotypes observed in Red Syrian goats

H, heterozygous genotype; L, homozygous genotype. NEFA, non-esterified fatty acids; BHBA, β-hydroxybutyric acid; IGF-1, insulin-like growth factor-1; fT3, free triiodothyronine; fT4, free thyroxine; SCC, somatic cell count. Means within a row followed by different lowercase letters are significantly (P < 0.05) different from each other, and means within a row followed by different uppercase letters tend to be significantly ($P \leq 0.082$) different from each other

Trait	Leptin genotype		*P*-value
	H (mean ± s.e.)	L (mean ± s.e.)	
	Metabolic profile		
Glucose (mg/100 mL)	39 0.0 ± 2.70	39.35 ± 2.03	0.917
NEFA (mmol/L)	0.231 ± 0.094	0.275 ± 0.070	0.712
BHBA (mmol/L)	0.379b ± 0.044	0.558a ± 0.033	0.040
Cholesterol (mg/100 mL)	52.18 ± 3.4	48.95 ± 2.6	0.455
Triglycerides (mg/100 mL)	2.46F ± 0.45	4.55E ± 0.33	0.072
Urea (mg/100 mL)	35.68 ± 1.42	33.65 ± 1.07	0.185
Total protein (g/L)	66.59 ± 1.32	65.75 ± 0.99	0.425
Albumin (g/L)	46.24 ± 0.46	45.53 ± 0.34	0.159
Globulin (g/L)	20.36 ± 1.48	20.21 ± 1.11	0.939
Calcium (mg/100 mL)	5.39 ± 0.098	5.20 ± 0.074	0.134
Inorganic phosphorus (mg/100 mL)	4.42 ± 0.23	4.56 ± 0.17	0.646
Magnesium (mg/100 mL)	3.03 ± 0.15	3.06 ± 0.11	0.898
	Hormonal profile		
Insulin (μg/L)	0.169 ± 0.039	0.132 ± 0.029	0.449
IGF-1 (ng/mL)	295.07E ± 28.80	229.51F ± 21.60	0.082
fT3 (pg/mL)	3.613 ± 0.196	3.445 ± 0.147	0.501
fT4 (ng/dL)	0.73a ± 0.03	0.57b ± 0.02	0.018
	Milk traits		
Fat (%)	4.05 ± 0.39	3.76 ± 0.29	0.552
Protein (%)	3.63 ± 0.14	3.55 ± 0.10	0.616
Lactose (%)	4.79 ± 0.08	4.73 ± 0.06	0.547
Casein (%)	2.95 ± 0.12	2.87 ± 0.09	0.595
Urea (mg/L)	382.4 ± 18.5	372.3 ± 13.9	0.670
SCC [ln(ncell/mL)]	13.623b ± 0.292	14.461a ± 0.227	0.034

of animals and in ruminant species are positively correlated with feed intake and energy and nitrogen balance; in sheep, energy deficiency reduces concentrations of fT3 and fT4, whereas subsequent overfeeding increases them (Todini 2007). Finally, goats with the H genotype showed, as a tendency, higher IGF-1 ($P = 0.082$) and lower triglycerides ($P = 0.072$) levels compared with L genotype ones. The values of metabolic and hormonal profile and milk traits were within the physiological range of Red Syrian goats (Celi *et al*. 2008). As the levels of all metabolites and hormones were within physiological range and most of them did not differ significantly between the two genotypes it could be argued that that the observed differences in lipid metabolism (higher BHBA and triglycerides) and hormone concentrations (lower fT4 and IGF-1) in the goats with the L genotype could be due to an increased demand for energy. The observed link with leptin polymorphisms could be attributable to the well known role that leptin plays in the regulation of metabolism and energy expenditure. The results of this study bring further evidence for the role of leptin as an indicator of metabolism and mammary gland health in dairy goats. The observed associations between *LEP* and metabolites and hormones should be confirmed by other investigators before the polymorphisms can be used in gene-assisted selection programs.

Even though this polymorphism is determined by the variation of a microsatellite region and, therefore, it is not likely causative of the observed differences, and it needs to be validated in a larger population of animals, data on SCC (Table 3) are in agreement with results obtained by Kulig *et al*. (2010) in Jersey cows, where two different polymorphisms at the leptin locus significantly affect SCC. Monitoring and reducing SCC is the primary objective for dairy producers since this parameter is considered as an indicator trait for mastitis and, according to Interbull (2008), is used as an indirect selection criterion for improving mastitis resistance. Of course, attention should be paid in order to avoid excessive reduction of SCC that could affect immune response (Suriyasathaporn *et al*. 2000*a*).

Furthermore, in the analysed goats the 264-bp microsatellite allele is associated with a lower serum BHBA level. Abnormally high levels of BHBA in blood and other body fluids affect immune functions in cattle and sheep (Lacetera *et al*. 2001; Grinberg *et al*. 2008). In dairy cows it has been demonstrated that high serum, urine, and milk levels of BHBA are associated with higher risk for mastitis (Gröhn *et al*. 1989; Suriyasathaporn *et al*. 2000*b*). In small ruminants clinical mastitis has a frequency of ~5% and subclinical mastitis a frequency of ~5–30% (Contreras *et al*. 2007). Finally, the 264-bp microsatellite allele is also associated with a significant higher plasma level of fT4. In ruminants the thyroid hormones are involved in the metabolic response of animals to certain nutritional, environmental and/or disease-related challenges (Todini 2007), as well as in regulation of fibre shedding (Celi *et al*. 2003). For example, induction of hypothyroidism was successful in increasing liveweight and body condition score gains, and in suppressing milk production during the treatment period in Brahman cows (Thrift *et al*. 1999). Furthermore, a slightly negative correlation was established between blood concentrations of fT3 and fT4 and milk production (Nixon *et al*. 1988).

Conclusions

Until now only a very limited number of studies have been conducted on effects of *LEP* gene differences in goats. In this study we observed a low level of variability of *LEP* genes in goat and sheep compared with cattle and water buffalo orthologs. However, in goat, Intron 1 microsatellite polymorphism is associated with effects on BHBA, fT4, IGF-1 and triglycerides levels, and milk SCC. Further analyses, based on a large population of animals and in different breeds, are needed to increase the body of knowledge on the role of leptin and its association with health and production traits in goats. Further studies should investigate the association of the *LEP* gene polymorphisms with traits related to energy balance and efficiency during different physiological stages, namely, growth, pregnancy and lactation. These studies are required to confirm that genotyping at the Intron 1 microsatellite region of the goat *LEP* gene can be useful for selection of traits of economic interest.

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