Journal of Molecular Biology Research; Vol. 5, No. 1; 2015
ISSN 1925-430X E-ISSN 1925-4318
Published by Canadian Center of Science and Education

αS1-Casein Lineage Assessed by RFLP in the Endangered Goat Breed "Retinta Extremeña"

José L. Fernández-García[1] & María P. Vivas Cedillo[1]

[1] Genetics and Animal Breeding, Veterinary Faculty, Universidad de Extremadura, 10071 Cáceres, Spain

Correspondence: José L. Fernández-García, Genetics and Animal Breeding, Veterinary Faculty, Universidad de Extremadura, 10071 Cáceres, Spain. Tel: 34-927-257-105. E-mail: pepelufe@unex.es

Received: December 7, 2014 Accepted: December 29, 2014 Online Published: March 3, 2015

doi:10.5539/jmbr.v5n1p32 URL: http://dx.doi.org/10.5539/jmbr.v5n1p32

Abstract

The Retinta Extremeña goat is a well-adapted breed to "Dehesa" environment. Traditionally their raw milk is used to make artisan cheese. However, crosses with specialized breeds are occurring since the eighties, this goat breed has been declared of special protection by the Spanish Agriculture Ministry (R.D. 1682/1997 and R.D. 229/2008). Genetic studies about casein variants have been mainly performed on Spanish goats of high milk yields because the caseins are a relevant fraction of milk. But recent studies claimed to study the caseins in all breeds, including threatened goat breeds to decide about its conservation value. This study was focused on the αS1-casein in the endangered "Retinta Extremeña" goat for the first time to enhance its conservation interest. Genomic DNA of seventy five pureblood goats was studied. A PCR-RFLP assay was designed to find a B*my*I target that distinguishing A versus B2 lineages (including recombinant variant M and B1, respectively) of the αS1-casein locus. The allelic frequency of variants related to A lineage (CAG triplet) was 14.0% similar to other southwestern Spanish breeds. It is suggested that individuals or families carrying A lineage should be more studied to detect less allergen null alleles while the opposite allele pools of the B2 lineages should be tested for alleles associated to unsaturated fatty acid content. Therefore, the priorities for conservation plans of animal genetic resources as threatened goat breeds; more investigation is claimed in the aim to study for proved useful alleles of certain genes, as casein variants.

Keywords: αS1-lineage, casein variant, endangered goat breed, genetic resources

1. Introduction

The Retinta (due to show uniform red coat color) Extremeña goat breed is geographically located almost exclusively in Extremadura Autonomy at the Southwestern Spanish territory. This goat is well adapted to "Dehesa" environment and often related to the most depressed socioeconomic uses within their distribution area. Moreover, it has been historically exploited as dual purpose (meat/milk) in a context of familiar subsistence and exclusively under extensive production systems based on browsing and grazing. More recently, the high quality cheese "Queso Ibores" (a Protected Designation of Origin artisan product) used raw milk of this breed and other autochthonous breeds. Despite, its substitution and crossing with more specialized milk yield breeds has occurred since the eighties. This fact led a census reduction of pureblood below 2,000 animals, but in geographically fragmented flocks. From here, the Retinta goat was considered to be on genetic threatening situation reason why the Spanish Agriculture Ministry declared of special protection this breed (R.D. 1682/1997 and R.D. 229/2008). Breeders Association and herd book have been established (Decreto 296/2011) for which the breed was first described with morphological, reproductive and productive data available. However, there is a shallow genetic characterization of the breed in general, much less on casein variants in particular. This is important because this goat is being used to product artisan cheese.

Goat milk synthesized in the mammary gland has six different types of milk proteins (the four caseins -αS1, αS2, β and κ - being 80% of milk protein), among which αS1-casein (αS1-cn: localized to the fourth goat chromosome; Hayes, Petit, Bouniol & Popescu, 1993) has been largely studied due to its impacts and positive correlation with goat milk composition (the amount of total protein, total solids, milk fat concentration and fatty acid composition; see Valenti, Pagano & Avondo, 2012; and references there in) and cheese-making properties (Remeuf, 1993; Pirisi, Colin, Laurent, Scher, & Parmentier, 1994; Clark & Sherbon, 2000; Chilliard et al., 2006). The 18 allelic variants of αS1-cn have been subdivided into four categories according to its quantity in goat milk (Moioli,

D'Andrea, & Pilla, 2007) as follow: (1) high expressing or strong alleles (A, B1, B2, B3, B4, C, H, L and M), (2) intermediate alleles (E and I) and (3) weak alleles (D, F and G) and null alleles (01, 02 and N) with levels of 3.6 g/L, 1.1 g/L and 0.45 g/L (cero for nulls) per allele, respectively (Valenti, Pagano, Pennisi, Lanza & Avondo, 2010). So, strong variants triple the performance levels associated to the αS1-cn and to which should be added the corresponding by the correlation with other milk components already mentioned. Also it has been reported the positive relationship between variants and the physicochemical properties of milk that affects the technological characteristics related to the clotting time of curd, firmness and rennet yield (Grosclaude et al., 1987).

Therefore, genetic studies about casein in threatened goat breed may be reasoned not only to analyze for high yield genes but also to decide about its conservation value based on traits of especial interest for its sustainable use in rural areas. This last statement includes one important of several priorities for conservation of specific animal genetic resources (Boettcher et al., 2010).

The aims of this study was to show for the first time in pureblood "Retinta Extremeña" goat breed valuables casein αS1-lineages to enhance the interest for its conservation.

2. Materials and Methods

Seventy five complete blood samples were collected of the pureblood "Retinta Extremeña" goats from the Selection Centre of Animal Selection and Reproduction (CENSYRA) of the Extremadura Government (4 males and 71 females, around the 45% of the total herd at CENSYRA). Although limited pedigree information was available, only three partial generations was able to assess Mendelian inheritance of the locus. Since all sampled animals belonged to the CENSYRA herd, where this and other endangered breeds are maintained institutionally as pureblood nucleus, the data presented here can be considered to represent this pureblood breed.

Genomic DNA was isolated by non-commercial procedure as in Fernández-García et al. (2012). The identification of natural variants for αS1-cn precursor was obtained in the SWISS-PROT database (CAS1-CAPH1 locus, acc. nº P18626). At 77 (amino acid position) all phylogenetically related casein variants to the most ancestral A variant shows a CAG triplet (glutamine: Q) but other variants wear the triplet GAG (glutamic: E) (Devilacqua et al., 2001). This polymorphism can be identified at 1045-1047 triplet in a segment of the casein coding sequence expands from exon 9 to exon 11 (Leroux, Mazure & Martin,1992) (accession number: GenBank X56462) (Figure 1). Two restriction targets for the endonuclease B*my*I (GDGCH/C) was predicted using software PROPHET (The Prophet Group at BBN Systems and Technologies; http://www-prophet.bbn.com). A primer pair was designed with PRIMER3 software (Koressaar and Remm 2007) that extend partly the intron 9, the exon 10, the intron 10, the exon 11 and partly the intron 11, but flanking both B*my*I target (C-A1 Forward 5´AAGCTATGATGTGTCTGGTT and C-A1Reverse 5´AACATTCTTGCTCATTCCCT). The PCR reaction is performed in 50 µL of a mixture containing template DNA (20-50 ng), primers [10pmol] and other general PCR reagents. Thermal cycling profile was as follow: [97° 5'] / [94ºC - 1'; 59° - 1'; 72° - 1'] * 33 cycles / 72º - 5' final extension; 4º C-∞. 10 µL PCR products were run in agarose gel to assess amplification. After, 10 µL of purified PCR products were digested overnight with 4 U of B*my*I according to the manufacturer **(**Boehringer Mannheim, Germany**).** For detection of genotypes the PCR-RFLP fragments were separated in 2.0 % agarose gel. CERVUS ver 3.0.3 (Marshall, Slate, Kruuk, & Pemberton, 1998) was used to verify HWE for the B*my*I polymorphic site of this locus.

3. Result.

One fragment of 318 bp was always present as expected after PCR assays based on Leroux et al. (1992) sequence (GenBank X56462). Based on knowledge obtained from databases (Figure 1), sequences from the F variant have a 3 bp insertion at intron nine between the selected primers but these should not be resolvable by agarose gels two percent (Figure 1). Digestion with B*my*I revealed the two target sites in the PCR product but only one of them was polymorphic (Figure 2). Accordingly, profiles for three possible genotypes were observed after digestion.

The homozygous goats for the GAG triplet showed profiles consisting of two fragments of 84 and 234 bp (n = 55 genotype GAG/GAG). Therefore, the 234 bp band was present in goats carrying the B2 lineage (see Bevilacqua, Ferrant, Garro, Veltri & Lagonigro, 2002) (Figure 2). In heterozygous goats (n = 19 genotypes GAG/CAG) were seen four bands with 234 bp, 122 bp, 112 bp, but these two last bands almost co-migrant and worst resolved in 2 % agarose gels but signaling the A lineage gene, and 84 bp (Figure 2). The homozygous goats for the CAG triplet have had profiles with 122, 112 and 84 bp bands (n = 1 genotype). Furthermore, the presence of another target for B*my*I was conserved in all samples and therefore it could be used as an internal control of the digestion or enzymatic activity.

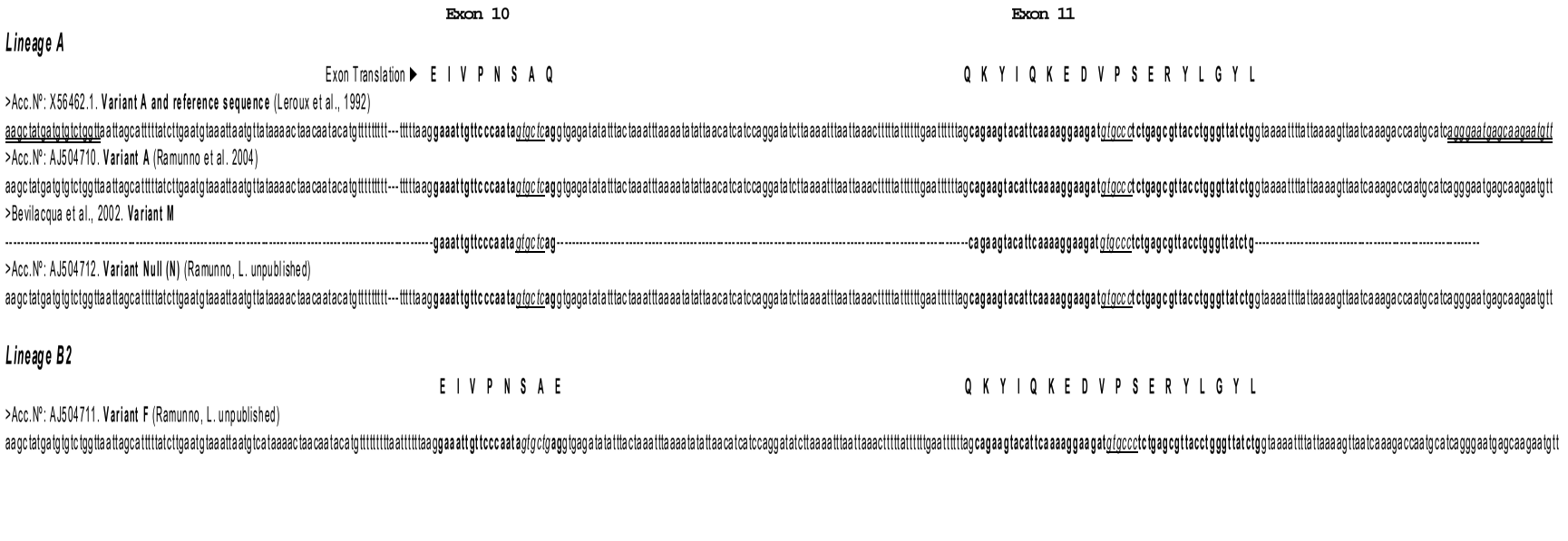

Figure 1. Sequence alignment of different alleles of the αS1-casein.The sequence of the variant A with Accession Number X56462.1 in GenBank (Leroux et al., 1992) was used to design primers and size predictions. Partial sequence of the allele M was transcribed from Bevilacqua et al. (2002). Exon translation: IUPAC code for amino acids of the exon 10 and 11. Double underline for primers sequences, bold type for the nucleotide sequence of each exon, underline-italics for B*my*I target

Co-dominant segregation of B*my*I target was observed in only three parental- descendant available families (data not showed). No discrepant segregation was observed for mendelian transmission of the polymorphic site. The allelic frequency of the CAG triplet was 14.0 % in the total sample, but 37.5 % in the four breeding males. By χ^2 test it was verified HWE equilibrium of the locus using only unrelated animals ($p = 0.801$). So the higher percentage of the GAG triplet in breeding males should be attributed to stochasticity.

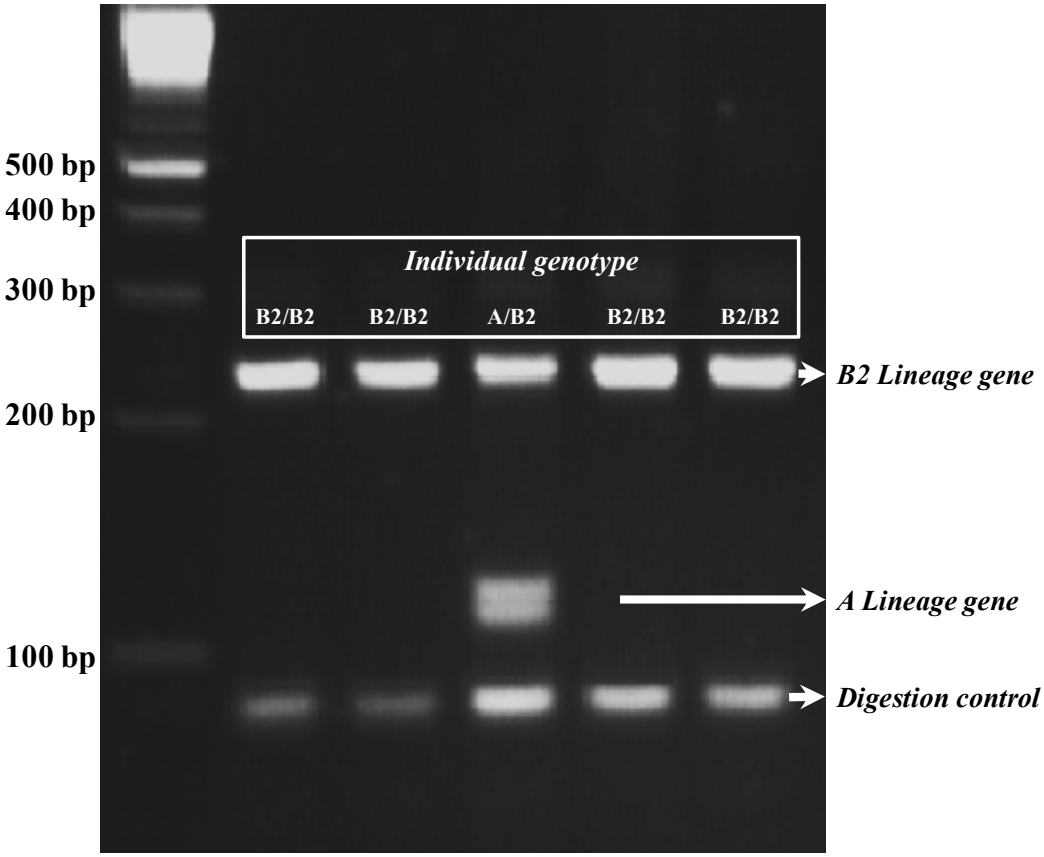

Figure 2. RFLP profile with B*my*I endonuclease from five individuals of the Retinta Extremeña goat. (Right) Restriction fragment corresponding to the gene of each lineage (B2 or A) and the 84 bp fragment digestion Control at the bottom and (left) fragment size of the molecular weight marker

4. Discussion

Structural analysis of goat casein performed both at the protein and the genomic level (Leroux, Martin, Mahè, Leveziel, & Mercier, 1990, Grosclaude & Martin 1997, Sacchi et al., 2005) have showed high variability and complex relation among polymorphic variants for the casein of the goat milk (Mahè & Grosclaude, 1989). This is especially certain for the locus αs1 and κ -casein which showed the highest number of variants (number of variant ≥ 16; Moioli et al., 2007). This high polymorphism feature provides further evidence that the allelic diversity come from multiple pathways, including recombination events between both ancestral lineages (A vs B2) as it has been hypothesized for the αs1- casein locus (Bevilacqua et al., 2002 and references there in)(Figure 3). In this study it was showed that the BmyI-RFLP can be useful to discriminate between lineages A and B2 (including recombinant variant M and B1, respectively) and thus for screening the "allele pools" associated with each one of both in the individual, familiar or population scale (Figure 3). This is important because null alleles (O1, O2 and N) have been exclusively associated to the A lineage (Grosclaude & Martin 1997; Bevilacqua et al., 2002).

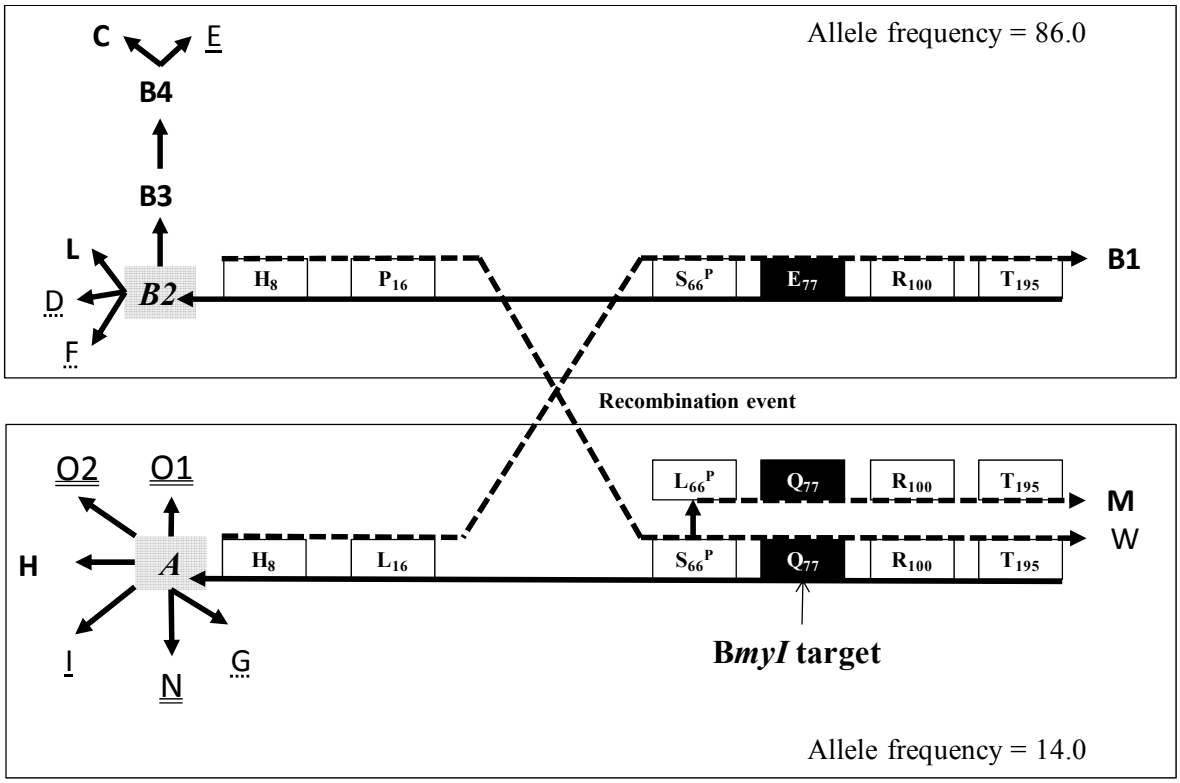

Figure 3. Schematic view of lineages of the αS1-casein locus using a simplified formula for haplotypes. The small boxes mimic exons but in some of them were annotated the polymorphic amino acid residues that occurred between lineages and also it is indicated the amino-acid residue targeted by B*my*I. Both large boxes regroup variants of casein: top for lineage B2 and bottom for lineage A. The numbers at right (down and top) are the frequency of each lineage in "Retinta Extremaña" goat. The continuous lines under the small boxes regroup the amino acid residues belonging to the respective ancestral lineages A or B2 and derived variants but dashed lines are signalling sites of inter-allelic recombination event between both as causes of the B1, W and M variants. Dark arrows indicate the possible evolutionary pathway scenario. Underline of variants: not underlined, simple underline dotted underline and double underlined for those of high yield, average yield, low yield and null (adapted from Bevilacqua et al., 2002)

Several studies revealed that the genotypes with strong or medium alleles of the B2 lineage of the αs1-casein predominated among goat breeds from the Mediterranean range (Sarda, Maltese, Moroccan, Tunisian; Moioli et al., 2007 and references there in) and, within the Iberian Peninsula, in southern breeds as the Murciano-granadina, Malageña or Payoya (Jordana et al., 1996; Caravaca et al., 2009) but allele E being at high frequency (Jordana et al., 1996). Regarding αs1-casein lineages, the genotypic frequencies observed in the Retinta Extremeña breed was similar to other Mediterranean breeds. For example, by pooling alleles belonging to the lineages B2, which carried the GAG triple that coded for the E (glutamic acid) (Bevilacqua et al., 2002), for the murciano-granadina breed (Caravaca et al., 2009) the estimated frequency was around the 88% (the complementary 12% for lineage A). As a whole, the native goat breeds from the Iberian Peninsula showed frequencies of the lineage A within a range between 10% and 20% (Jordana et al., 1996), what includes the results of the Retinta Extremeña reported here. Then, alleles of low frequency but high technological interest might be easily screened for a first glance by the B*my*I-RFLP and thus uncovering hidden αs1-casein lineages on endangered and/or bottlenecked breeds.

Contradictory results about the effect on total protein, fat and casein contents reported for this casein locus (Mahé, Manfredi, Ricordeau, Piacere, & Grosclaude, 1993; Manfredi, Ricordeau, Barbieri, Amigues, & Bibe, 1995; Grosclaude & Martin 1997; Caravaca et al., 2009; Valenti et al., 2010) suggested to be cautious in making decisions about this particular locus regarding the Retinta breed. Both, complicated interactions among genotypes, other *loci* and environmental factors as certain dependence on the particular polymorphism present in populations under consideration should be deemed (Caravaca et al., 2009, Valenti et al., 2010). Recent studies suggested that although some interaction genotype x diet should be expected; comparisons between AA vs FF genotypes affected to nine different fatty acids. But it should stress that monounsaturated fatty acids (especially C16:1 and cis-9 C18:1) were

lower in milk from AA (lineage A) than FF genotypes (lineage B2) (Valenti et al., 2010). Other studies suggested that milk defective in αs1-cn (null alleles) could be less allergenic as reported by Bevilacqua et al. (2001). The goats with these particular genotypes may to be of interest because these have been proposed as substitute of cow milk for people having allergies to cow proteins (Moioli et al., 2007). Regarding to the conservation of goat breeds, all these studies are suggestive because somehow give support to research on the different casein variants within endangered breeds, such as the retina breed. In fact, actions aimed to genotype for proved useful genes should be among the priorities for conservation of specific animal genetic resources (Boettcher et al., 2010), especially for breeds well adapted to difficult territories as the Retinta Extremeña breed. As claimed Caravaca et al. (2009), more investigations in different breeds regarding the effects of casein polymorphism on goat milk yields and the features of their products should be addressed, because it may have an especial value in future comprehensive conservation plans.

Acknowledgements

We thank to CENSYRA (Badajoz, Junta de Extremadura) for providing all samples of the endangered Retinta Extremeña breed and to support this study. We also would like to thank anonymous reviewers for their valuables advices.

References

Bevilacqua, C., Ferrant, P., Garro, G., Veltri C., & Lagonigro, R. (2002). Interallelic recombination is probably responsible for the occurrence of a new αs1-casein variant found in the goat specie. *European Journal* of *Biochemistry, 269*, 1293-1303. http://dx.doi.org/10.1046/j.1432-1033.2002.02777.x

Bevilacqua, C., Martin, P., Candalh, C., Fauquant, J., Piot, M., Roucayrol, A. M., ... Heyman, M. (2001). Goats' milk of defective alpha(s1)-casein genotype decreases intestinal and systemic sensitization to beta-lactoglobulin in guinea pigs. *Journal of Dairy Research, 68*, 217-227. http://dx.doi.org/10.1017/S0022029901004861

Boettcher, P. J., Tixier-Boichard, M., Toro, M., Simianer, H., Eding, H., Gandini, G., … the GLOBALDIV Consortium. (2010). Objectives, criteria and methods for using molecular genetic data in priority setting for conservation of animal genetic resources. *Animal Genetics, 41*(Suppl. 1), 1-14. http://dx.doi.org/10.1111/j.1365-2052.2010.02050.x

Caravaca, F., Carrizosa, J., Urrutia, B., Baena, F., Jordana, J., Amills, M., Badaoui, B., Sanchez, A., Angiolillo, A., & Serradilla, J. M. (2009). Effect of αS1-casein (*CSN1S1*) and κ-casein (*CSN3*) genotypes on milk composition in Murciano-Granadina goats. *Journal of Dairy Science*, 92, 2960-2964. http://dx.doi.org/10.3168/jds.2008-1510

Chilliard, Y., Rouel, J., & Leroux, C. (2006). Goat's alpha-s1 casein genotype influences its milk fatty acid composition and delta-9 desaturation ratios. *Animal Feed Science and Technology, 131*, 474-487. http://dx.doi.org/10.1016/j.anifeedsci.2006.05.025

Clark, S., & Sherbon, J. W. (2000). Alpha S1- casein, milk composition and coagulation properties of goat milk. *Small Ruminant Research, 38*, 123-134. http://dx.doi.org/10.1016/S0921-4488(00)00154-1

Fernández-García, J. L. (2012). The endangered *Dama dama mesopotamica*: genetic variability, allelic loss and hybridization signals. *Contributions to Zoology, 81*(4), 223-233.

Grosclaude, F., & Martin, P. (1997). Casein polymorphisms in the goat. IDF Seminar`Milk Protein Polymorphism II' (pp. 241-253). International Dairy Federation, Palmerston North, New Zealand.

Grosclaude, F., Mahé, M. F., Brigonon, G., Di Stasio, L., & Jeunet, R. (1987). A. Mendelian polymorphism underlying quantitative variation of goat αs1-CN. *Genetics Selection Evolution, 19*, 399-412. http://dx.doi.org/10.1186/1297-9686-19-4-399

Hayes, H., Petit, E., Bouniol, C., & Popescu, P. (1993). Localization of the alpha S2-casein gene (CASAS2) to the homologous cattle, sheep and goat chromosomes 4 by *in situ* hybridization. *Cytogenetics and Cell Genetics, 64*, 281-285. http://dx.doi.org/10.1159/000133593

Jordana, J., Amills, M., Díaz, E., Angulo, C., Serradilla, J. M., & Sanchez, A. (1996). Gene frequencies of caprine αs1-casein polymorphism in Spanish goat breeds. *Small Ruminant Research, 20*, 215-221. http://dx.doi.org/10.1016/0921-4488(95)00813-6

Koressaar, T., & Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics, 23*, 1289-1291. http://dx.doi.org/10.1093/bioinformatics/btm091

Leroux, C., Martin, P., Mahè, M. F., Leveziel, H., & Mercier, J. C. (1990) Restriction fragment length polymorphism identification of the goat as1-casein alleles. A potential tool in selection of individuals

carrying alleles associated with a high level protein synthesis. *Animal Genetics, 21*, 341-351. http://dx.doi.org/10.1111/j.1365-2052.1990.tb01979.x

Leroux, C., Mazure, N., & Martin, P. (1992). Mutations away from splice site recognition sequences might cis-modulate alternative splicing of goat αs1-casein transcripts. Structural organization of the relevant gene. *Journal of Biological Chemistry, 267*, 6147-6157.

Mahè, M. F., & Grosclaude, F. (1989). aS1-CnD, another allele associated with a decreased synthesis rate at the caprine αS1-casein locus. *Genetics Selection Evolution, 21*, 127-129. http://dx.doi.org/10.1186/1297-9686-21-2-127

Mahè, M. F., Manfredi, E., Ricordeau, G., Piacere, A., & Grosclaude, F. (1993). Effets du polymorphisme de la caseine αs1 caprine sur les performances laitieres: Analyse intradescendance de boucs de race Alpine. *Genetics Selection Evolution, 26*, 151-157. http://dx.doi.org/10.1186/1297-9686-26-2-151

Manfredi, E., Ricordeau, G., Barbieri, M. E., Amigues, Y., & Bibe, B., (1995). Genotype caseine αs1 et selection des boucs sur descendance dans les races Alpine et Saanen. *Genetics Selection Evolution, 27*, 451-458. http://dx.doi.org/10.1186/1297-9686-27-5-451

Marshall, T. C., Slate, J., Kruuk, L. E. B., & Pemberton, J. M. (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology, 7*, 639-655. http://dx.doi.org/10.1046/j.1365-294x.1998.00374.x

Moioli, B., D'Andrea, M., & Pilla, F. (2007). Candidate genes affecting sheep and goat milk quality. *Small Ruminant Research, 68*, 179-192. http://dx.doi.org/10.1016/j.smallrumres.2006.09.008

Pirisi, A., Colin, O., Laurent, F., Scher, J., & Parmentier, M. (1994). Comparison of milk composition, cheesmaking properties and textural characteristics of the cheese from two groups of goats with high or low rate of alfa-s1-casein synthesis. *The International Dairy Journal, 4*, 329-345. http://dx.doi.org/10.1016/0958-6946(94)90030-2

Ramunno, L., Cosenza, G., Rando, A., Illario, R., Gallo, D., Di Berardino, D. & Masina, P. (2004). The goat alphas1-casein gene: gene structure and promoter analysis. *Gene, 334*, 105-111. http://dx.doi.org/10.1016/j.gene.2004.03.006

Remeuf, F. (1993). Influence of genetic polymorphism of caprine αs1-casein on physicochemical and technological properties of goat's milk. *LAIT, 73*, 549-557. http://dx.doi.org/10.1051/lait:19935-652

Sacchi, P., Chessa, S., Budelli, E., Bolla, P., Ceriotti, G., Soglia, D., ... Caroli, A. (2005). Casein haplotype structure in five Italian goat breeds. *Journal of Dairy Science, 88*, 1561-1568. http://dx.doi.org/10.3168/jds.S0022-0302(05)72825-3

Valenti, B., Pagano R. I., & Avondo, M. (2012). Effect of diet at different energy levels on milk casein composition of Girgentana goats differing in CSN1S1 genotype. *Small Ruminant Research, 105*, 135-139. http://dx.doi.org/10.1016/j.smallrumres.2011.11.013

Valenti, B., Pagano, R. I., Pennisi, P., Lanza, M., & Avondo, M. (2010). Polymorphism at αs1-casein locus. Effect of genotype diet × interaction on milk fatty acid composition in Girgentana goat. *Small Ruminant Research, 94*, 210-213. http://dx.doi.org/10.1016/j.smallrumres.2010.07.023