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# A point mutation in the splice donor site of intron 7 in the as2-casein encoding gene of the Mediterranean River buffalo results in an allele-specific exon skipping

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*Source/description*: The CSN1S2 cDNA of 10 unrelated Mediterranean River Buffaloes reared in Southern Italy was amplified by RT-PCR, while the region from the 6th to the 8th exon of the CSN1S2 gene was amplified from genomic template. All amplicons were sequenced twice and in both directions. Fiftythree individuals randomly chosen from four breeding herds were genotyped for an AluI-restriction fragment length polymorphism (RFLP). Primer sequences and PCR conditions are given in Tables S1 & S2. Three individual milk samples from buffaloes with different genotypes at CSN1S2 were analysed by reverse-phase-high pressure liquid chromatography (RP-HPLC).

*Polymorphism detection*: cDNA sequence comparisons showed that five individuals had a normal transcript only (lodged on EMBL, accession FM865618, named CSN1S2A), one had a deleted transcript only (lodged on EMBL, accession FM865619, named CSN1S2B), because of the splicing out of the 27-bp of exon 7, and the remaining four had a heterozygous pattern. Analysis of the genomic sequences revealed a FM865620:g.773G>C transversion that caused inactivation of the

intron 7 splice donor site and, consequently, the allele-specific exon skipping characteristic of the CSN1S2B allele. The g.773G>C mutation creates a new AluI restriction site enabling a PCR–RFLP rapid genotyping assay (Fig. S1). PCR–RFLP genotypes for the AluI site were consistent with the cDNA sequence data for all 10 animals. The cDNA sequences showed three additional exonic mutations forming an extended haplotype with the g.773G>C polymorphism: FM865618: c.459C>T, c.484A>T and c.568A>G homozygous and heterozygous respectively in the CSN1S2BB and CSN1S2AB buffaloes. The first is silent, while the remaining two are non-conservative (p.Ile162Phe and p.Thp200Ala respectively). Chromatographic analysis of three individual samples with CSN1S2 AA, AB and BB genotypes showed the same retention time for the as2 casein fraction (Fig. S2), but the hydrophobic characteristics of each allele do not allow their chromatographic separation. Allelic frequencies: The genotype frequencies (37 CSN1S2A/A, 15 CSN1S2A/B and one CSN1S2B/B) are in agreement with Hardy–Weinberg equilibrium (v2 = 0.13, d.f. = 1), with the frequency of the deleted B allele being 0.16.

*Comments*: The results indicate that buffalo, similar to goats1,2 and cattle,3,4 have a CSN1S2 allele resulting from a non constitutive splicing event. The predicted bubaline as2B protein is 198 aa long instead of 207 aa and would also be characterized by the presence of Phe at position 147 and Ala at 185.

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*References*

1 Ramunno L. et al. (2001) Anim Genet 32, 264–8.

2 Martin P. et al. (1999) Int Dairy J 9, 163–71.

3 Bouniol C. et al. (1993) Gene 128, 289–93.

4 Mohr U. et al. (1994) Gene 143, 187–92.

*Supporting information*

Additional supporting information may be found in the online version of this article.

Table S1 Oligonucleotide primer sequences and positions.

Table S2 Thermal amplification programmes for (a) RT-PCR, (b) PCR and (c) AluI PCR-RFLP.

Figure S1 Observed genotypes after AluI digestion of fragments obtained by PCR of the DNA region spanning the 7th exon and flanking regions of the Mediterranean river buffalo CSN1S2 gene.

Figure S2 RP-HPLC chromatogram of individual buffalo milk samples.

**Table S1.** Primer sequences and amplification conditions for PCR.

| ***CSN2*-amplified fragment** | **Position[1]** | **Primer sequence (5'-3')** | **Use** | **Annealing temperature** |
|---|---|---|---|---|
| Promoter | 1292-1311 | F: TCCATTATAGCTTAAGCACT | AS-PCR | 58.5 °C |
| | | F: TCCATTATAGCTTAAGCACC | | |
| | Complementary to 1457-1475 | R: TGGGATGCACGGAAGTTTT | | |
| Exon 9 | 10323-10340 | F: GGGGGTGAGATGAAGAGT | Internal control | |
| | Complementary to 10663-10682 | R: AATGACTGGTTAGGAAATAG | | |

[1] Numbering of primers was according to the goat *CSN2* gene (AJ011018).

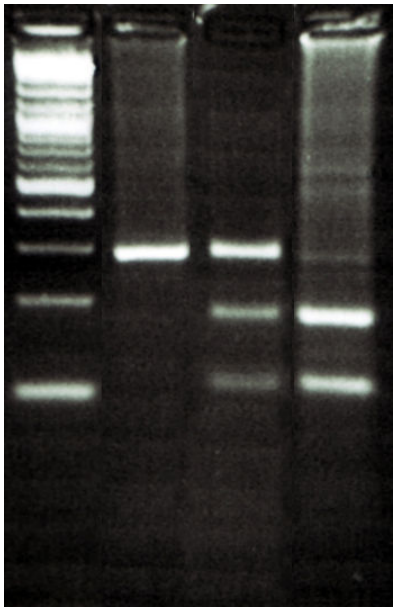
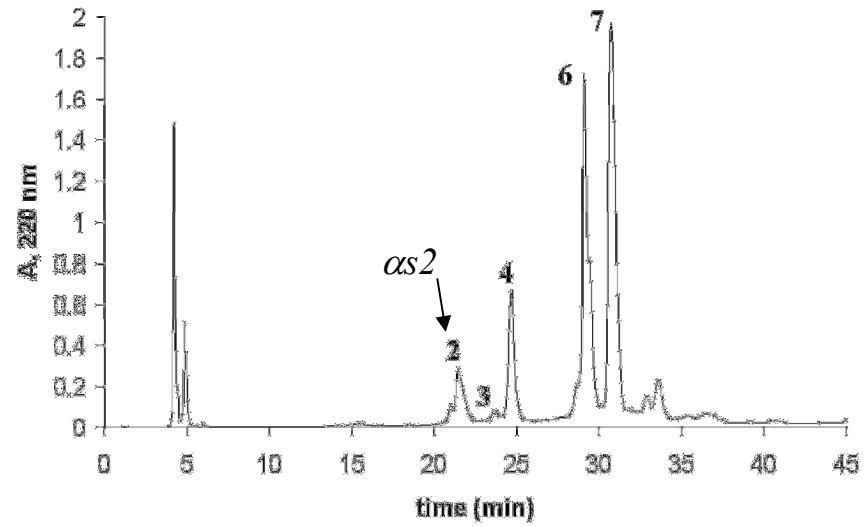
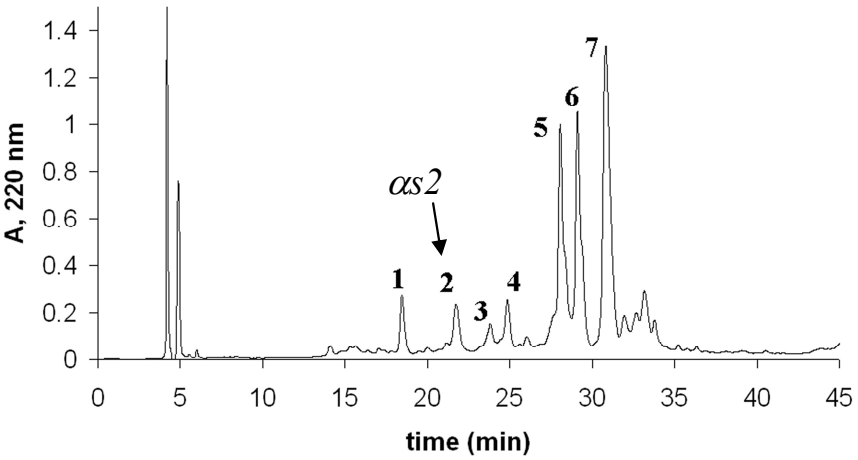


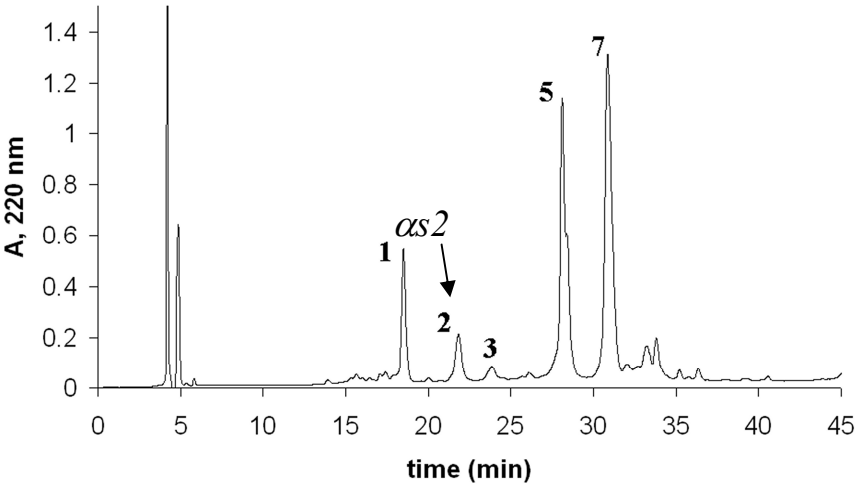

**Figure S1**. Observed genotypes after AluI digestion of fragments obtained by PCR of the DNA region spanning the 7$^{th}$ exon and flanking regions of the Mediterranean river buffalo *CSN1S2* gene. M=100 bp DNA ladder (Promega); lane 1: *CSN1S2* A/ A; lane 2: *CSN1S2* A/ B; lane 3 *CSN1S2* B/ B.




***CSN1S2*AA**




***CSN1S2*AB**




***CSN1S2*BB**

**Figure S2**. RP-HPLC chromatogram of individual buffalo milk samples. Subjects with different genotypes at the *CSN1S2 locus* (*CSN1S2*AA, AB and BB) showed the same retention time for the αs2 casein fraction. Peaks 1, 3 and 4: κ casein, peaks 5 and 6: αs1 casein, peak 7: β casein, peak 2: αs2 casein.

**Table S1** Oligonucleotide primer sequences and positions.

| | Position nt | Primers sequence (5'-3')* | | EMBL | Amplicon size |
|---|---|---|---|---|---|
| RT-PCR** | 1-18 | Forward | ATATTCCATTGCCTGGAC | DQ133467 | 796 bp<br>769 bp |
| | Complementary to: 775-796 | Reverse | ATGTGAAACTGTAGAAGATAGA | | |
| PCR ex 6-8 | 175-190 | Forward | GAGAACCTTTGCTCCA | FM865618 | ~1750 bp |
| | Complementary to: 235-251 | Reverse | CTAGATGAGCCGATAGA | | |
| AluI PCR-RFLP*** | 668-687 | Forward | TTCCTTTTATATTCAGGAGA | FM865620 | 288 bp |
| | Complementary to: 938-955 | Reverse | GATTTAGGTGGACATTAC | | |
| PCR Mix composition | The 100-µl PCR reaction mix comprised: 20 µl of RT reaction product or 100 ng of genomic DNA, 1x PCR Buffer, 10 pmol of each primer, dNTPs each at 0.2 mM, 5 U of Taq DNA Polymerase (Promega), 3 mM $MgCl_2$ | | | | |

* Primers were designed using OLIGO 5.0 software (National Biosciences Inc., Plymouth, MN)

**The RT was performed using Improm-IIk Reverse Transcriptase (Promega)

***Digestion with 10 U of AluI endonuclease of 17µl of PCR product was carried out for 5 h at 37 °C following the buffer manufacturer's instructions (Promega).

**Table S2** Thermal amplification programs for a) RT-PCR b) PCR and c) AluI PCR-RFLP

a)

| Cycle | Denaturation | Annealing | Extension |
|---|---|---|---|
| 1 | 97 ºC – 2 min | 57.5 ºC – 45 sec | 72 ºC – 1.5 sec |
| 29 | 94 ºC – 45 sec | 57.5 ºC – 45 sec | 72 ºC – 1.5 sec |
| 1 | 94 ºC – 45 sec | 57.5 ºC – 45 sec | 72 ºC – 10 min |

b)

| Cycle | Denaturation | Annealing | Extension |
|---|---|---|---|
| 1 | 97 ºC – 2 min | 54 ºC – 45 sec | 72 ºC – 2 min |
| 29 | 94 ºC – 45 sec | 54 ºC – 45 sec | 72 ºC – 2 min |
| 1 | 94 ºC – 45 sec | 54 ºC – 45 sec | 72 ºC – 10 min |

c)

| Cycle | Denaturation | Annealing | Extension |
|---|---|---|---|
| 1 | 97 ºC – 2 min | 54 ºC – 45 sec | 72 ºC – 1.5 sec |
| 29 | 94 ºC – 45 sec | 54 ºC – 45 sec | 72 ºC – 1.5 sec |
| 1 | 94 ºC – 45 sec | 54 ºC – 45 sec | 72 ºC – 10 min |