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Short communication:

A single nucleotide polymorphism in the bovine *beta-casein* promoter region across different bovine breeds.

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The nucleotide sequence data reported in this paper have been submitted to GenBank and has been assigned the accession number AJ973327.

Introduction:

The bovine *beta-casein* (*CSN2*) gene has been shown to span a region of 8.5kb, containing nine exons and eight intervening introns (Bonsing *et al.,* 1988; Martin *et al*., 2002). The exons range in size from 24 to 498bp, however the introns are much larger and account for 85% of the gene. Twelve genetic variants in the coding sequence of the *beta-casein* gene have been reported (Farrell *et al*., 2004). The A^2 allele of the *beta-casein* gene has been associated with a higher milk production (Lin *et al.,* 1986; Bech and Kristiansen, 1990) while the B variant has been associated with an increase in protein content and better cheese-making properties (Marziali and Ng-Hang-Kwai, 1986). The *beta-casein* gene codes for a protein of 209 amino acids with varying regions at codons 67, 106 and 122. The A^1 variant differs from A^2 at position 67, where a histidine replaces a proline (Lien *et al.,* 1992). The *beta-casein* A^2 variant has histidine and the A^3 variant has glycine at position 106 (Lien *et al.,* 1992); the *beta-casein* A^2 variant has serine at position 122 and the *beta-casein* B variant has arginine at this codon (Stewart *et al.,* 1987; Damiani *et al.,* 1992).

The *beta-casein* promoter has been characterised and contains a number of binding sites for transcription factors c/ebp, Stat5, Oct and GR (Doppler *et al.,* 1995; Lechner *et al.,* 1997; Raught *et al.,* 1995). A *beta-casein* enhancer element, sited in the distal bovine promoter between –1562 to –1613, contains binding sites for Stat5, c/ebp, YY1 and GR (Raught *et al.,* 1995). In addition, analysis of the murine *beta-casein* promoter has shown the functional significance of the Runx2 transcription factor in full transcriptional activation of the *beta-casein* gene (Inman *et al*., 2004). Polymorphisms have been investigated in the *beta-casein* gene promoter of different bovine breeds. The *beta-casein* promoters from Jersey, Brown Swiss and Holstein

bulls (one of each), were sequenced and the only difference found was a single base deletion at position –516 (Bleck *et al.,* 1996). Four additional sequence differences (single base deletions) were found when comparing sequences to the database sequence, however these are more likely to be sequencing errors in the original sequence (Bonsing *et al.,* 1988). Another investigation into the incidence of polymorphic sites in the *beta-casein* gene promoter identified seven polymorphic sites in the region (Schild and Geldermann, 1996). A study by Szymanowska *et al*. (2004) screened Polish Black-and-White (n = 81) and Polish Red (n = 195) cows for the incidence of the G to C change at –109 identified in the Schild and Geldermann study (1996) but no polymorphism was identified (Szymanowska *et al*., 2004). Promoter studies have not indicated that differences in casein gene expression are due to these variations but it has been suggested that gene expression changes may instead result from a combination of promoter variants, i.e. that certain haplotypes influence casein gene expression (Martin *et al.,* 2002).

In this study, polymorphism incidence in the *beta-casein* gene promoter in nine bovine breeds typical of the Irish herd were investigated. The bovine breeds chosen included dairy, dual-purpose and non-dairy (beef) breeds. Potential links between promoter polymorphisms and structural gene polymorphisms were also investigated.

Materials and Methods:

DNA isolation. Blood was obtained from the coccygeal vein of animals from nine bovine breeds, namely high genetic merit Holstein-Friesian (n=4), low genetic merit Holstein-Friesian (n=4), Irish-Friesian (n=4), Dutch-Friesian (n=4), Limousin (n=6), Montbeliarde (n=4), Charlois (n=2), Normande (n=4), Norwegian Red (n=2) and Kerry (n=8). DNA extractions were carried out using the Gentra Capture Column™ (Gentra, UK) system from approximately 200µl of whole blood per animal. Blood was stored at –80°C and DNA was stored at –20°C until further use.

Polymerase Chain Reaction. Primers (located at positions 97-120 and 1799-1824 in the NCBI database sequence X14711) were designed to amplify a 1728bp fragment of the *β-casein* gene promoter (MWG Biotech, UK). A second set of primers (located at positions 7574 – 7593 and 8287 – 8306 in NCBI database sequence M55158) were used to amplify a 732bp fragment encompassing the polymorphism that distinguishes the A^1 and A^2 coding sequence variants. PCR was carried out from a starting template of approximately 200ng of genomic DNA in a final volume of 50µl containing 1X *Taq* DNA polymerase buffer (Invitrogen, UK), 1.5mM $MgCl_2$, 200µM dNTPs (Promega, UK), 0.3µM each primer, and 1U *Taq* polymerase (Invitrogen, UK). Conditions were an initial incubation at 95°C for 2min, followed by 35 cycles of 95°C for 1min, 58°C for 1min and 72°C for 1min.

Restriction digestion. Digestion of PCR products was carried out in a final volume of 20µl containing 10µl of PCR product, 1X reaction buffer and 1U *Eco*RI restriction enzyme. Reactions were incubated at 37°C for 2h and resolved on a 2% agarose gel in 1X Tris Borate EDTA (TBE) buffer at 90V for 1h.

Sequencing and bioinformatics. Sequencing of PCR products was carried out by MWG Biotech (Germany). The resulting sequences were analysed using the Vector

NTI® Suite of software (Informax™, US). Alignment of sequences for all 42 animals was carried out, and potential polymorphic sites identified. Examination of chromatogram sequence files to detect homozygotic and heterozygotic animals was also performed.

Statistical analysis. Observed allele frequencies were analysed for equilibrium using the Hardy Weinberg equation. Results were analysed by chi-square test to determine whether observed allele frequencies and allele frequencies predicted by the Hardy Weinberg equations were significantly different. Results of promoter and coding sequence variant screens were analysed by chi-square test. Null hypothesis was that no association occurred between variants.

Results and Discussion:

Bovine breeds chosen. Blood samples were obtained from nine bovine breeds chosen to represent the animals typical of the Irish herd, but also to increase the likelihood of genetic variation. The breeds chosen were: dairy - high and low genetic merit Holstein Friesian (n=4 of each), Irish Friesian (n=4) and Dutch Friesian (n=4); dual-purpose - Norwegian Red (n=2), Normande (n=4), Montbeliarde (n=4) and Kerry (n=8); and beef - Limousin (n=6) and Charlois (n=2).

A single nucleotide polymorphism (SNP) from T to A was identified at position -851 from the transcriptional start site. In addition, it was noted that compared to the database sequence all animals had a T insertion at -848. These two variations introduced a recognition site for the *Eco*RI restriction enzyme that allowed development of a PCR-RFLP rapid screen to determine which variant of the *β-casein* promoter is present (either β-TT, β-TA or β-AA) (Figure 1). The TT allele was undigested and showed a band of 1728bp. The AA allele was digested through the introduction of an *Eco*RI site and showed two bands of 880bp and 848bp. The heterozygote TA allele showed bands at 1728bp, 880bp and 848bp. The incidence of the T/A SNP was TT-45%, TA-38% and AA-17%. The allele frequencies are in Hardy Weinberg equilibrium ($p = 0.97$). When breed differences were observed the incidence of the A allele differed between breeds. The dairy breeds had an incidence of 50%, compared with dual-purpose breeds with an incidence of 38% and the beef breeds with a 100% incidence (Table 1). No transcription factor has as yet been identified that binds at this location, however the study by Schild and Geldermann (1996) suggests that the progesterone receptor may bind at this location.

The *β-casein* exon VII was also analysed for the presence or absence of the base change at position 67 which encodes either the A^1 or A^2 variants. This base change is also present in the B variant, so for the purposes of this study that A^1 + B are designated A^1. The promoter and coding sequence genetic variants for the *β-casein* gene for all forty-two animals screened are listed in Table 2. These allele frequencies were also in Hardy Weinberg equilibrium ($p = 0.24$). The results of the promoter and coding sequence variant screen were analysed statistically and it was indicated that an association existed between the coding sequence variant A^2A^2 and the promoter β-AA variant pair and also between the coding sequence variant A^1A^1 and the promoter variant β-TT ($p = 0.00002154$). Analysis of a larger group of animals is required to confirm these findings.

The occurrence of polymorphism in the *β-casein* gene promoter may have an effect on the transcriptional activity of the gene and thus provide an opportunity to improve expression of this important milk protein gene. A previous study of polymorphisms in the promoter region examined thirteen animals and noted seven potential sites of variability (Schild and Geldermann, 1996). However, five of these polymorphisms were seen in only one of the fourteen animals analysed (a different animal with each polymorphism). A further study to determine the incidence of the C to G change at –109 identified in this original screen did not show the change in any of a large number of animals (n = 276) (Szymanowska *et al*., 2004). Although two of these seven polymorphisms would appear to be quite common, the other five may be rare and only found in specific breeds.

In this present study, ten animals were originally screened for polymorphism in the entire 1728bp region of the *β-casein* promoter. In all ten animals sequenced, a T insertion appeared at –848 which differs from the original database sequence (Bonsing *et al*., 1998). This insertion was also noted in other studies suggesting that the original sequence is incorrect at this position. (Schild and Geldermann, 1996; Bleck *et al*., 1996). The only other variable site noted was a T to A base change at –851. This change was noted in four of the ten animals sequenced and fortuitously introduced a recognition site for the *Eco*RI restriction enzyme. This change was also noted in the Schild and Geldermann (1996) study. Forty-two animals were screened by PCR-RFLP and although the number of animals screened per breed was small, the differences between animals bred for different production purposes was noteworthy. In dairy animals the A allele frequency was 50%, with a homozygous AA genotype frequency of 5.5%. In beef animals, however, the A allele frequency was 100%, with a homozygous AA genotype frequency of 25%. The animals bred for both beef and dairy (dual-purpose) were also higher than dairy animals with an A allele frequency of 38% and a homozygous AA frequency of 25%.

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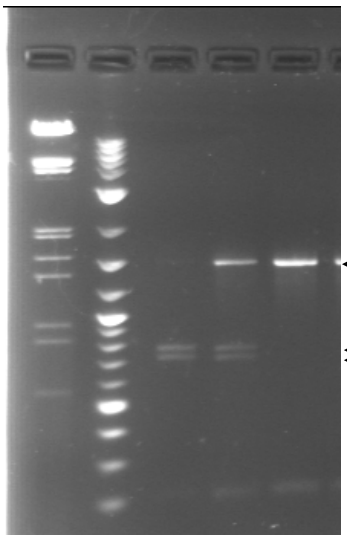
239 Association of nucleotide-sequence polymorphism in the 5'-flanking regions of
240 bovine casein genes with casein content in cow's milk. Lait. **84**: 579-590.
241

242 **Figure 1:**

243



β-TT 5'-GAAG**T**ATTCTC-3'

β-AA 5'-GAAG**A**ATTCTC-3'

*Eco*RI

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Table 1.

Breed	TT	TA	AA	n
Holstein Friesian	37.5	50	12.5	8
Irish Friesian	75	25	0	4
Dutch Friesian	50	50		4
Norwegian Red	50	50		2
Normande	75	25		4
Montbeliarde	50	25	25	4
Kerry	62.5		37.5	8
Limousin		100		6
Charlois		66	33	2

Table 2.

Animal	β-Casein CDS variant	β-Casein promoter variant
	Dairy Breeds	
	Holstein Friesian	
0011	A1A2	β-TA
0026	A1A2	β-TT
3048	A1A2	β-TA
9615	A2	β-TA
0050	A1A2	β-TT
0059		β-TA
0081	A2	β-AA
0876	A1	β-TT
	Irish Friesian	
0599	A1A2	β-TT
1668	A1A2	β-TT
1270	A2	β-TA
1257	A1A2	β-TT
	Norwegian Red	
0407	A1A2	β-TA
0287	A1	β-TT
	Dutch Friesian	
0188	A1	β-TT
0508	A1A2	β-TA
1535	A1	β-TT
0191	A2	β-TA
	Dual purpose Breeds	
	Normande	
0163	A1	β-TT
0166	A1	β-TT
1226	A1	β-TT
1267	A1A2	β-TA
	Montbeliarde	
1212	A1	β-TT
0130	A2	β-AA
1023	A1A2	β-TA
1545	A1	β-TT
	Kerry	
39	A2	β-AA
40	A2	β-AA
41	A1A2	β-TT
42	A1A2	β-TT
43	A2	β-TT
44	A2	β-AA
45	A1A2	β-TT
46	A2	β-TT
	Beef Breeds	
	Charlois	
0292	A2	β-TA
191C	A1A2	β-TA
	Limousin	
0183	A2	β-AA
0094	A2	β-TA
42L	A1A2	β-TA
215W	A1A2	β-TA
0069	A2	β-AA
0086	A1A2	β-TA

Figure legends.

Figure 1. RFLP analysis of the *β-casein* promoter.
Lane 1: λ*Hin*dIII/*Eco*RI marker. Lane 2: 100bp marker. Lane 3: β-AA 880bp and 848bp fragment. Lane 4: β-TA = 1728bp, 880bp + 848bp fragment. Lane 5. β-TT = 1728bp fragment.

Table 1. The percentage incidence of promoter variants in bovine breeds surveyed.

Table 2. The incidence of variants in the promoter and coding sequence of the β-casein gene.