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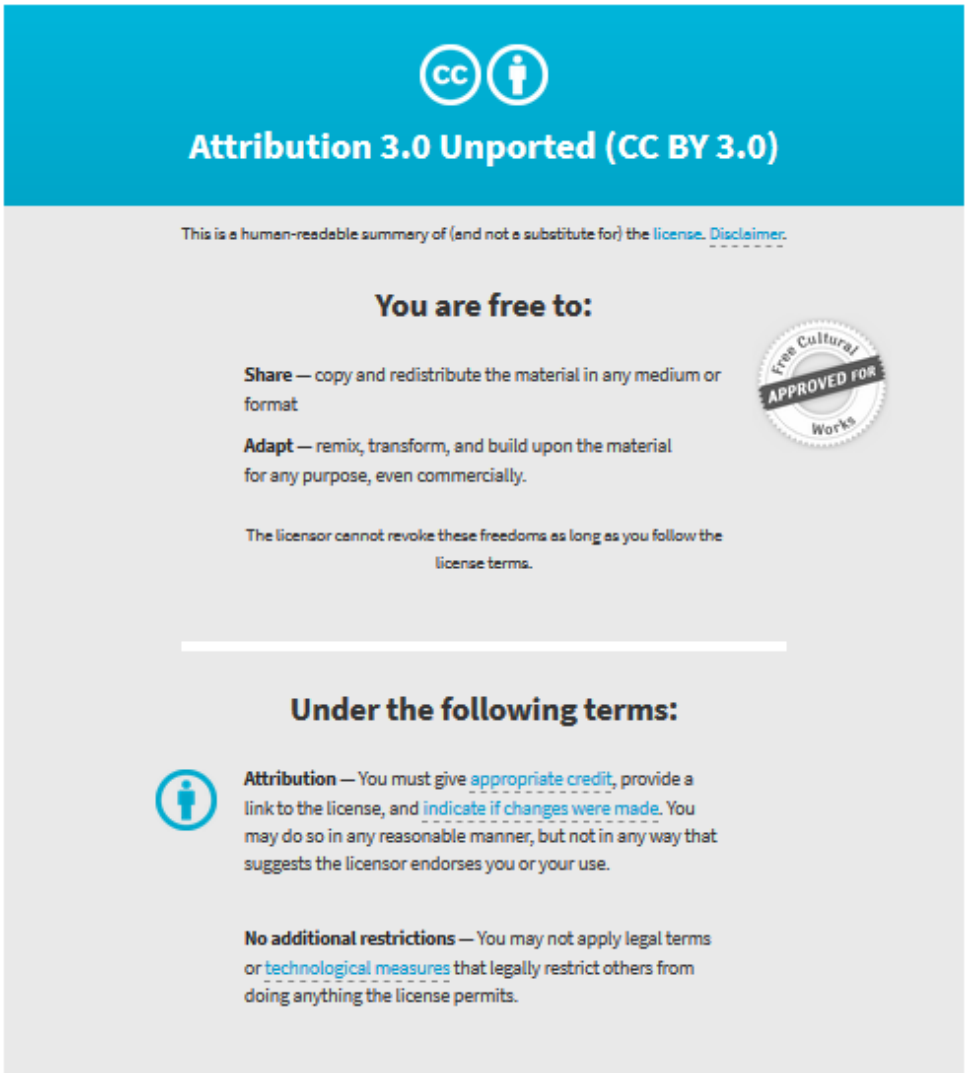

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Intragenic haplotypes at the bovine *CSN1S1* locus

Dedicated to Prof. Drs. h. c. Franz Pirchner PhD, on the occasion of his 75th birthday

Summary

A new alternative genotyping method based on PCR-SSCP was developed for direct differentiation of the *CSN1S1* alleles *B* and *C* in the coding region. In addition a PCR-RFLP test based on a *Mae*III restriction site in the promoter region of *CSN1S1*, reported in the literature as an alternative test for the differentiation of *CSN1S1*B* and *C* was used and the alleles named *b* and *c*. Genotyping of 649 animals belonging to 17 European and Turkish cattle breeds showed differences in occurrence and frequency of the alleles. *CSN1S1*B* occurred in all breeds with frequencies varying from 0.50 in Anatolian Blackup to 1.0 in e.g. Ayrshire. *CSN1S1*b* on the other hand varied from 0.63 in Jersey, 0.97 in Ayrshire to 1.0 in e.g. Angler.
Comparison of the results from both typing methods and positions in the gene showed that both mutations do not always occur together. From the resulting four intragenic haplotypes (*B-b, B-c, C-c* and *C-b) B-b* is predominant in all breeds with frequencies varying from 0.3450 in Anatolian Black to 1.0 in Angler and Scottish Highland. The number of haplotypes varied from only one in Angler and Scottish Highland, two in Ayrshire, three in Asturian Valley and Turkish Grey Steppe to all four in the other 12 breeds. Correlation between allele frequencies and the geographic origin of the breeds was significant for the *Mae*III promoter polymorphism.

Key Words: casein, haplotype, SSCP, cattle

Zusammenfassung

Titel der Arbeit: **Intragene Haplotypen im bovinen α_{s1}-Kasein Gen (*CSN1S1*)**
Es wurde ein neuer auf PCR-SSCP basierender DNA-Test zur direkten Unterscheidung von *CSN1S1*B* und *C* in der kodierenden Genregion entwickelt. Zusätzlich wurde ein PCR-RFLP Test auf der Basis einer *Mae*III Schnittstelle in der Promotor Region des *CSN1S1*, der in der Literatur als alternativer Test für die Differenzierung der *CSN1S1*B* und *C* Varianten genannt wird, zur Typisierung derselben Tiere verwendet und die Allele als *b* und *c* bezeichnet. Die Genotypisierung von 649 Tieren aus 17 europäischen und türkischen Rinderpopulationen ergab Unterschiede im Auftreten und der Frequenz der Allele. *CSN1S1*B* trat in allen untersuchten Rassen mit Frequenzen zwischen 0,50 in Anatolischem Schwarzvieh und 1,0 in Ayrshire auf. An der anderen polymorphen Position variierten die *CSN1S1*b* Frequenzen zwischen 0,63 bei Jersey über 0,97 bei Ayrshire und 1,0 bei Angler .
Der Vergleich der Ergebnisse beider Typisierungsmethoden an den beiden Positionen im Gen zeigte, dass beide Austausche nicht in allen Fällen übereinstimmen. Von den sich daraus ergebenden vier intragenen Haplotypen (*B-b, C-c, C-c* und *C-b*) war *B-b* in allen Rassen am häufigsten vertreten mit Frequenzen zwischen 0,3450 bei Anatolischem Schwarzvieh und 1,0 bei Angler und Schottischem Hochlandrind. Die Zahl nachweisbarer Haplotypen schwankte zwischen nur einem bei Angler und Schottischem Hochlandrind, zwei bei Ayrshire, drei bei Asturischem Niederungsvieh und Türkischem Grauen Steppenrind und bis zu allen vier möglichen bei den übrigen 12 untersuchten Rassen. Es wurden signifikante Korrelationen zwischen dem *Mae*III Promotorpolymorphismus und der geographischen Herkunft der Rassen gefunden.

Schlüsselwörter: Kasein, Haplotyp, SSCP, Rind

Introduction

Variants of different milk protein genes in cattle are discussed in the context of studies regarding quantitative and qualitative traits and are used within evolutionary and diversity studies. For αs_1-casein (*CSN1S1*) 9 alleles (*A, B, C, D, E_{Yak}, E_{Bali}, F, G, H*) have been described within different cattle breeds, with *CSN1S1*B* being the predominant allele in *Bos taurus* and *CSN1S1*C* in *Bos indicus* and *Bos grunniens* breeds (EIGEL et al., 1984; FORMAGGIONI et al., 1999). The other alleles are rare in other breeds that have been studied. It has been postulated that *CSN1S1*B* has positive effects on milk yield (LIN et al., 1986), whereas higher milk protein content is found in animal heterozygous for *CSN1S1*BC* compared to *BB* homozygous animals (NG-KWAI-HANG et al., 1990; ALEANDRI et al., 1990, BOVENHUIS et al., 1992). In Nordic cattle breeds LIEN et al. (1999) identified an allele frequency gradient with low frequency of *CSN1S1*B* in native breeds to high frequency of *CSN1S1*B* and loss of *CSN1S1*C* in high selected dairy cattle. This is in agreement with the reported very high frequencies of *CSN1S1*B* in breeds selected for milk production, up to fixation or nearly-fixation in Ayrshire, Angler, or Holstein Friesian (ERHARDT, 1993; IKONEN et al., 1996).

Development of several DNA tests for genotyping milk protein genes offered the possibility to type animals independent from age, sex, and lactation (LÉVEZIEL et al., 1988). SCHLEE & ROTTMANN (1992) and DAVID & DEUTCH (1992) developed allele specific PCR tests (ASPCR) for differentiation of *CSN1S1*B* and *C*, while LIEN et al. (1993) used an amplification created restriction site (ACRS) for *Hph*I to discriminate between *CSN1S1*B* and *CSN1S1*C.* The latter test detects the causal nucleotide substitution inside exon 17 of the gene. KOCZAN et al. (1993) described a polymorphism in the promoter of the *CSN1S1* gene affecting a *Mae*III restriction site. The resulting PCR-RFLP test was suggested as an alternative to the ASPCR test for the differentiation of *CSN1S1*B* and *C*. However, TURECKOVÁ et al. (2001) recently reported that in Czech and German Red Cattle the *Mae*III promoter polymorphism is not always linked to the *Hph*I ACRS in exon 17.

Single strand conformation polymorphism (SSCP) analysis is a rapid and sensitive screening technique, that allows mutation identification without restriction enzyme digests or special primers (ORITA et al., 1989). PCR-SSCP analysis for milk protein genotyping identified new alleles in both - endangered and production breeds – which were subsequently characterized by DNA sequencing (PRINZENBERG et al., 1999; CAROLI et al., 2001).

The aim of this study was to determine the occurrence of the two *CSN1S1* polymorphisms described in different cattle breeds and to define intragenic haplotypes as genetic markers using a PCR-SSCP based DNA test for typing the causal nucleotide substitution in exon 17.

Materials and Methods

Sample collection: Blood samples of 649 animals belonging to 17 cattle breeds (Anatolian Black n=13, Jersey n=43, Chianina n=32, Casta Navarra n=34, Turkish Grey Steppe n=17, Maremmana n=39, Aberdeen Angus n=36, Piemontese n=42, Fighting Bull n=36, Asturian Valley n=42, Hereford n=46, Pezzata Rossa Italiana n=47, Charolais n=51, British Friesian n=38, Ayrshire n=48, Angler n=46, Scottish Highland n=39) were collected and DNA was extracted by the method of MONTGOMERY and SISE (1990).

DNA test (PCR-RFLP) for CSN1S1 promoter polymorphism: A 310 bp fragment containing the first 274 bp of the promoter and parts of exon 1 was amplified, digested with *Mae*III and separated on an agarose gel as described by KOCZAN et al. (1993). Uncut fragments of the 310 bp fragment (indicating G in nucleotide position 1957) were named *c*, fragments cut by *Mae*III (indicating A in nucleotide position 1957) were named *b*.

*PCR-SSCP test for CSN1S1*B and C:* For differentiation of the *CSN1S1* alleles *B* and *C* a SSCP-based DNA test was developed using 58 DNA samples from animals with known *CSN1S1* genotypes as standard samples. Genomic DNA was amplified by PCR to give a 223 bp-fragment containing exon 17 of the *CSN1S1* gene (Position 17644-17867) of GenBank Acc. No. 59856). PCR was in a final volume of 25 µl, containing 100 ng genomic DNA, 15 pmol of each primer (*CSN1S1*-5: 5` CAC TGT TGC TTT TTC AAT GGT C 3` *CSN1S1*-3: 5` AAG GCA ACA ATA TGC AGT CAT TT 3`), 1 U *Taq*-polymerase (*Peqlab Biotechnologie GmbH, Erlangen*), 200 µM dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.8, 50 mM KCl with an initial denaturation step at 94°C for 5 min, followed by 30 cycles with a denaturation at 94°C for 1 min, annealing of 56°C for 1 min, elongation of 72°C for 1 min and a final elongation of 72°C for 5 min. Four microlitres of the PCR product were mixed with 6 µl formamide buffer (95% formamide, 0.025% bromphenolblue, 0.025% xylenecyanol FF, 20 mM EDTA), denatured at 91°C for 3 min and immediately chilled on ice. Samples were run 3 h at 200 V at 10°C on a 10% acrylamide:bisacrylamide gel (37:1) with 2% glycerol. DNA fragments were visualised by silver staining (BASSAM et al., 1991).

Haplotype definition: Haplotypes were determined based on the results of PCR-RFLP analyses determining nucleotide position (nt) 1957 (b=nt1957: A, c=nt1957: G) and exon 17 PCR-SSCP discriminating alleles B and C (Table 1).

Table 1
Nucleotide sequence of the bovine *CSN1S1*B-b*, *CSN1S1*B-c*, *CSN1S1*C-c,* and *CSN1S1*C-b* haplotypes at the positions 17807 (corresponding to aminoacid 192) and 1957 of the sequence (numbering as published in GenBank Accession No. X59856) (Nukleotidsequenz der bovinen *CSN1S1*B-b, CSN1S1*B-c, CSN1S1*C-c* und *CSN1S1*C-b* Haplotypen an Position 17807 (entspricht Aminosäure 192) und 1957 der Sequenz Accession-Nr. X59856)

	Haplotype			
Sequence position	***CSN1S1*B-b***	***CSN1S1*B-c***	***CSN1S1*C-c***	***CSN1S1*C-b***
Nucleotide position 17807	*G**A**A*	*G**A**A*	*G**G**A*	*G**G**A*
Amino acid position 192	**Glu**	**Glu**	**Gly**	**Gly**
Nucleotide position 1957	***A***	***G***	***G***	***A***

Data analysis: Allele frequencies, observed and expected genotype frequencies, and deviations from Hardy-Weinberg equilibrium were evaluated by GENEPOP Software (RAYMOND and ROUSSET, 1995). Expected and observed haplotype frequencies were calculated and compared (χ^2-values) with EH software (XIE and OTT, 1993). Correlations between degree of latitude and allele frequencies and linear regressions of allele frequencies on geographic latitude were calculated.

Results

PCR-SSCP analysis for *CSN1S1* exon 17 showed two distinct fragment patterns for alleles *B* and *C* which was in agreement for all 58 DNA samples of known genotypes (Figure 1).

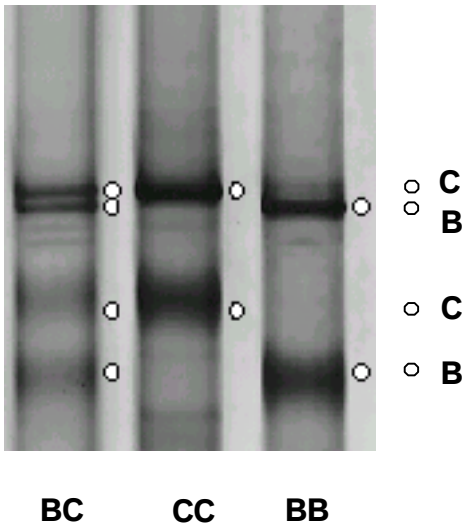

Fig. 1: PCR-SSCP based DNA test for *CSN1S1*B* and *CSN1S1*C*. Position of corresponding bands are marked by dots (PCR-SSCP DNA-Test für *CSN1S1*B* und *CSN1S1*C*. Die Lage der entsprechenden Banden ist durch Punkte gekennzeichnet)

Genotype frequencies

The observed and expected genotype frequencies and the probability-values for Hardy-Weinberg equilibrium are shown in Table 2. All populations were in Hardy-Weinberg equilibrium at *CSN1S1* locus for position 17807 and position 1957 except Pezzata Rossa and Piemontese for the first locus, where an excess of homozygous genotypes was observed. All of the expected six genotypes at the *CSN1S1* locus were observed in eight of the 17 breeds analysed while in Scottish Highland and Angler only two genotypes were found.

Table 2
Observed (obs) and expected (exp) genotypes and probability-values (p) for Hardy-Weinberg equilibrium at *CSN1S1* locus in European and Turkish cattle breeds (Beobachtete (obs) und erwartete (exp) Genotypen und Wahrscheinlichkeitswerte für Hardy-Weinberg Gleichgewicht am *CSN1S1* Genort in europäischen und türkischen Rinderrassen)

	***CSN1S1* Position 17807**							***CSN1S1* Position 1957**						
Breed	***BB***		***BC***		***CC***		**p**	***bb***		***bc***		***cc***		**p**
	obs	exp	obs	exp	obs	exp		obs	exp	obs	exp	obs	exp	
Aber. Angus	24	23.2	10	11.4	2	1.3	0.59	21	20.9	13	13.2	2	1.9	1.00
Anat. Black	2	3.1	9	6.8	2	3.1	0.30	6	6.1	6	5.8	1	1.1	1.00
Angler	46	46.0	0	0.0	0	0.0	-	46	46.0	0	0.0	0	0.0	-
Astur. Valley	23	25.1	19	14.9	0	2.1	0.09	35	35.3	7	6.5	0	0.3	1.00
Ayrshire	48	48.0	0	0.0	0	0.0	-	45	45.0	3	2.9	0	0.0	1.00
Brit. Friesian	35	35.0	3	2.9	0	0.0	1.00	35	35.0	3	2.9	0	0.0	1.00
Cas. Navarra	24	23.8	9	9.4	1	0.8	1.00	14	15.4	18	15.1	2	3.5	0.43
Charolais	40	40.4	11	9.9	0	0.6	1.00	42	42.4	9	8.3	0	0.4	1.00
Chianina	22	21.8	9	9.3	1	0.9	1.00	18	17.2	11	12.7	3	2.2	0.65
Fighting Bull	31	30.2	4	5.6	1	0.2	0.20	25	24.1	9	10.8	2	1.1	0.30
Hereford	34	33.0	10	12.0	2	1.0	0.26	42	42.1	4	3.9	0	0.1	1.00
Jersey	15	16.8	24	20.3	4	5.8	0.33	15	16.8	24	20.3	4	5.8	0.38
Maremmana	25	25.4	13	12.3	1	1.4	1.00	25	25.5	13	12.3	1	1.4	1.00
Pezz. Rossa	40	38.4	5	8.2	2	0.4	0.04	42	42.1	5	4.8	0	0.1	1.00
Piemontese	32	29.9	7	11.1	3	0.9	0.04	28	26.6	11	13.7	3	1.6	0.33
Sc. Highland	39	39.0	0	0.0	0	0.0	-	39	39.0	0	0.0	0	0.0	-
T. Grey Steppe	7	7.6	9	7.7	1	1.7	0.61	13	13.2	4	3.6	0	0.2	1.00

Arch. Tierz. **45** (2002) 1

Allele and haplotype frequencies

Table 3 gives the allele and haplotype frequencies at the *CSN1S1* locus and shows differences both in the occurrence and the frequencies of the alleles and the haplotypes in the cattle breeds studied. *CSN1S1*B* occurred in all breeds with frequencies varying from 0.50 in Anatolian Black to 1.0 in Ayrshire, Scottish Highland, and Angler. *CSN1S1*b* on the other hand varied from 0.63 in Jersey, 0.97 in Ayrshire to 1.0 in Angler and Scottish Highland.

In most breeds frequencies for *CSN1S1*b* are higher than for *CSN1S1*B*. In contrast, in Aberdeen Angus, Ayrshire, Casta Navarra, Chianina, Piemontese, and Turkish Grey Steppe *CSN1S1*b* occurs in lower frequencies than *B*.

From the four intragenic haplotypes (*B-b, B-c, C-c* and *C-b) B-b* is predominant in all breeds with frequencies varying from 0.3450 in Anatolian Black to 1.0 in Angler and Scottish Highland. Beside these two breeds, where only one haplotype occurs, in Ayrshire two haplotypes (*B-b* and *B-c*), and in Asturian Valley and Turkish Grey Steppe breed three haplotypes (*B-b, C-c* and *C-b*) are present, while four haplotypes occur in the other breeds. A χ^2-test comparing expected and observed haplotype frequencies of position 17807 and position 1957 shows highly significant disequilibrium ($p<0.01$) in Aberdeen Angus, Asturian Valley, Charolais, Jersey and Piemontese and a significant disequilibrium ($p<0.05$) in British Friesian.

Table 3
Allele frequencies, expected, and observed haplotype frequencies of *CSN1S1* in European cattle populations and corresponding χ^2 –value (Allelfrequenzen, erwartete und beobachtete Haplotypenfrequenzen von *CSN1S1* in europäischen Rinderpopulationen und entsprechende χ^2-Werte)

Breed	N	allele frequencies				haplotype frequencies: expected				haplotype frequencies: observed				χ^2
		B	***C***	***b***	***c***	***B-b***	***B-c***	***C-c***	***C-b***	***B-b***	***B-c***	***C-c***	***C-b***	
Aber. Angus	36	0.81	0.19	0.76	0.24	0.6156	0.1944	0.0456	0.1444	0.7342	0.0714	0.1648	0.0297	22.33
Anat. Black	13	0.50	0.50	0.69	0.31	0.3450	0.1550	0.1550	0.3450	0.3450	0.1550	0.1550	0.3450	0.00
Angler	46	1.00	0.00	1.00	0.00	1.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	-
Astur. Valley	42	0.77	0.23	0.92	0.08	0.7084	0.0616	0.0184	0.2116	0.7857	0.1429	0.0714	0.0000	12.00
Ayrshire	48	1.00	0.00	0.97	0.03	0.9700	0.0300	0.0000	0.0000	0.9688	0.0313	0.0000	0.0000	-
Brit. Friesian	38	0.96	0.04	0.96	0.04	0.9216	0.0384	0.0016	0.0384	0.9472	0.0133	0.0261	0.0133	8.14
Cas. Navarra	34	0.84	0.16	0.68	0.33	0.5712	0.2772	0.0528	0.1088	0.6397	0.2132	0.1101	0.0368	5.45
Charolais	51	0.89	0.11	0.91	0.09	0.8099	0.0801	0.0099	0.1001	0.8820	0.0101	0.0781	0.0298	25.96
Chianina	32	0.83	0.17	0.73	0.27	0.6059	0.2241	0.0459	0.1241	0.5781	0.2969	0.0000	0.1250	4.72
Fighting Bull	36	0.92	0.08	0.82	0.18	0.7544	0.1656	0.0144	0.0656	0.7723	0.1443	0.0362	0.0471	1.52
Hereford	46	0.85	0.15	0.96	0.04	0.8160	0.0340	0.0060	0.1440	0.8079	0.0399	0.0036	0.1486	0.06
Jersey	43	0.63	0.37	0.63	0.37	0.3969	0.2331	0.1369	0.2331	0.5232	0.1513	0.2674	0.0582	12.97
Maremmana	39	0.83	0.17	0.81	0.19	0.6723	0.1577	0.0323	0.1377	0.7120	0.1085	0.0966	0.0829	7.16
Pezz. Rossa	47	0.90	0.10	0.95	0.05	0.8550	0.0450	0.0050	0.0950	0.8813	0.0230	0.0302	0.0655	4.81
Piemontese	42	0.85	0.15	0.80	0.20	0.6800	0.1700	0.0300	0.1200	0.7472	0.0981	0.1043	0.0505	13.50
Sc. Highland	39	1.00	0.00	1.00	0.00	1.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	-
T. Grey Steppe	17	0.88	0.12	0.68	0.32	0.5984	0.2816	0.0384	0.0816	0.6765	0.0000	0.1176	0.2059	6.05

(χ2-limit for 1% significance level is 11.34 and for 5% level is 7.81. In breeds marked with "–" no χ2-analysis has been done due to fixation of one or two of the loci.)

Correlations and regression analyses with geographic data

Frequencies for *CSN1S1*B* and *b* could be shown to be increasing from southern sampling area (Turkey) to northern Europe (Scotland). Correlation of geographic

latitude in the range of the sampling area (37° - 58°N) with *CSN1S1*B* allele frequencies was r=0.417 (n.s.), and r=0.556 (p<0.05) for *CSN1S1*b.*
Regression analysis resulted in a regression equitation of y=0.00843*x+0.45308 for *CSN1S1*B* and of y=0.01103*x+0.31914 for *CSN1S1*b* allele frequencies and geographic latitude (Figure 2).

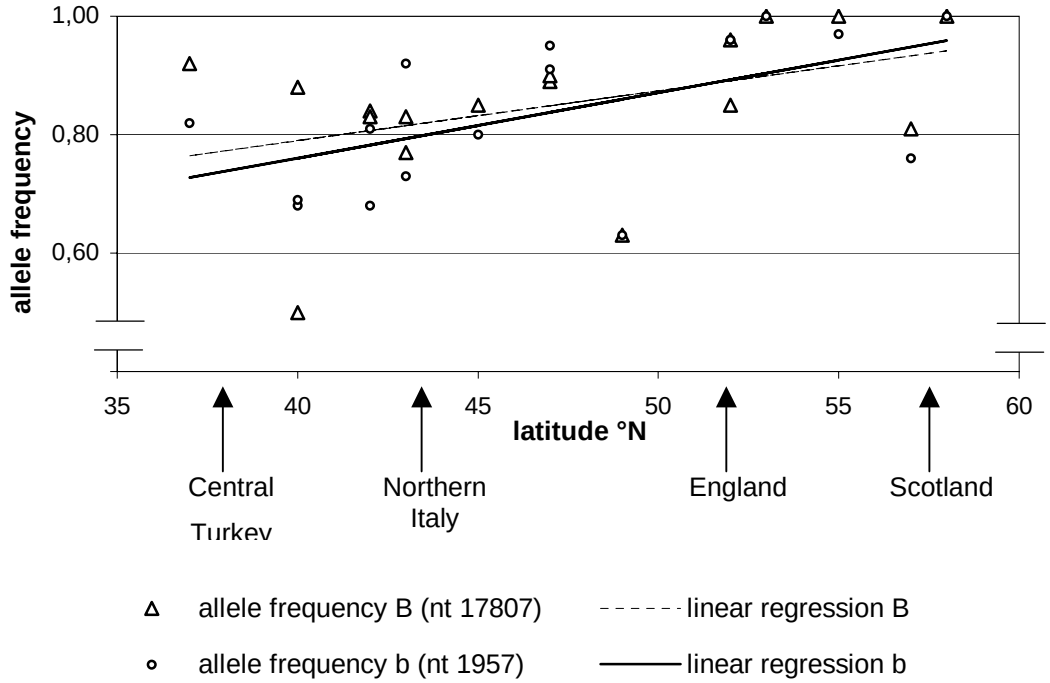

Fig. 2: Regression analysis of *CSN1S1*B* (y=0.00843*x+0.45308) and *b* (y=0.01103*x+0.31914) allele frequencies and geographic latitude (Regressionsanalyse von *CSN1S1*B* (y=0,00843*x+0,45308) und b (y=0,01103*x+0,31914) Allelfrequenzen und geographischer Breite)

Discussion

The PCR-SSCP test developed for the differentiation of *CSN1S1*B* and *C* offers a rapid and cost-effective alternative to the ASPCR described by SCHLEE and ROTTMANN (1992) and DAVID and DEUTCH (1992) and the ACRS method described by LIEN et al. (1993). Our genotyping results show, in agreement with TURECKOVÀ et al. (2001), that the promoter polymorphism described by KOCZAN et al. (1993) can not be used as a reliable genotyping method to infer *CSN1S1*B* and *C*-genotypes in all breeds. The small number of animals analysed by KOCZAN et al. (1993) belonged to Jersey, Holstein Friesian and German Simmental.
Aberdeen Angus, Ayrshire, British Friesian, Charolais, Turkish Grey Steppe, Pezzata Rossa, and Piemontese show low frequencies for *B-c* and *C-b* haplotypes, while frequencies of same haplotypes are high in Chianina and Anatolian Black.
Our results support the occurrence of different intragenic haplotypes in milk protein genes, that might contribute to variation of different milk production traits (SCHILD and GELDERMANN 1996; EHRMANN et al., 1997).
Expected and observed genotype frequencies at *CSN1S1 nt 17807* in Piemontese and Pezzata Rossa were not in Hardy-Weinberg equilibrium. Both breeds showed much lower frequencies of heterozygous animals than expected from the allele frequencies.

This may be a sampling artefact, however at position *nt 1957* all breeds were in Hardy-Weinberg equilibrium.

Despite of close genetic linkage χ^2-test for linkage disequilibrium does not show significant linkage in all populations. Linkage disequilibrium declines with increasing generations (FALCONER, 1984), so the level and extent of disequilibrium diminish in older populations. The British breed Ayrshire is a long established breed, as is the Hereford. Casta Navarra and Fighting Bull have also been maintained over many centuries. Maremmana represents a low-selected historic genotype, and Chianina is regarded to be the oldest Italian breed. Anatolian Black and Turkish Grey Steppe are very heterogenous breeds that remained without specific selection pressure over centuries (PORTER, 1991). Thus the limited linkage disequilibrium in these breeds points to the two mutations being very old. On the other hand Pezzata Rossa is a newly founded population (herdbook established in 1957) originating in crosses of Simmental with Friulana cattle. Simmental was planned to substitute Friulana by backcrossing, however its introgression stopped after a few generations. Haplotypes derived from both parental breeds are likely to be still present in the Pezzata Rossa and may cause the lack of linkage disequilibrium observed.

Frequencies of *CSN1S1*B* are in tendency higher and significantly higher for *CSN1S1*b* in northern than in southern European breeds, with highest values in dairy breeds of north western European origin. LIEN et al. (1999) reported an apparently contrary frequency gradient in Nordic breeds with high frequencies of *CSN1S1*C* in autochtone breeds of northern Scandinavia and lower frequencies to fixation in dairy breeds originated in southern Scandinavia. This indicates rather a selection gradient than a geographic gradient, supporting the suggestion made by LIN et al. (1986) that occurrence of *CSN1S1*B* variant is correlated with selection for improvement in milk production traits. Our study analysed northern European cattle breeds, that include a large proportion of dairy breeds, selection pressure thus is expected to lead to increasing fixation of alleles linked to production traits. Allele frequencies of *CSN1S1* show that in Anatolian Black both alleles *B* and *C* occur in equal frequency. LOFTUS et al. (1999) described a high admixture proportion of *Bos indicus* with 30.6% in Anatolian Black and BAKER & MANWELL (1980) pointed out that *CSN1S1*C* occurs in very high frequencies in zebu cattle. Therefore the high frequency of *CSN1S1*C* in Anatolian Black may have occurred by introgression of zebu genes.

However loss of haplotypes along a south-north gradient leading to fixation or nearly fixation of the *CSN1S1 B-b* haplotype in northern European cattle populations could be explained by drift. The loss of genetic diversity along a south-north gradient has already been described for a number of different loci. This may reflect distance from the center of domestication of cattle (MEDJUGORAC et al., 1994; TROY et al., 2001).

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