Short communication: Milk protein genetic variation and casein haplotype structure in the Original Pinzgauer cattle

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ABSTRACT

Milk protein genetic polymorphisms are often used for characterizing domesticated mammalian species and breeds, and for studying associations with economic traits. The aim of this work was to analyze milk protein genetic variation in the Original Pinzgauer, a dual-purpose (dairy and beef) cattle breed of European origin that was influenced in the past by human movements from different regions as well as by crossbreeding with Red Holstein. A total of 485 milk samples from Original Pinzgauer from Austria ($n = 275$) and Germany ($n = 210$) were typed at milk proteins α_{S1} -casein, β -casein, κ -casein, α -lactalbumin, and β -lactoglobulin by isoelectrofocusing to analyze the genetic variation affecting the protein amino acid charge. The Original Pinzgauer breed is characterized by a rather high genetic variation affecting the amino acid charge of milk proteins, with a total of 15 alleles, 12 of which were found at a frequency >0.05 . The most polymorphic protein was β -casein with 4 alleles detected. The prevalent alleles were $CSN1S1^*B$, $CSN2^*A^2$, $CSN1S2^*A$, $CSN3^*A$, LGB^*A , and LAA^*B . A relatively high frequency of $CSN1S2^*B$ (0.202 in the whole data set) was found, mainly occurring within the $C-A^2-B-A$ haplotype (in the order $CSN1S1$ - $CSN2$ - $CSN1S2$ - $CSN3$), which seems to be peculiar to the Original Pinzgauer, possibly because the survival of an ancestral haplotype or the introgression of *Bos indicus*.

Key words: milk protein, Original Pinzgauer cattle, α_{S2} -casein, haplotype

The analysis of milk protein genetic variation is a useful tool for characterizing domesticated mammalian species and breeds, with the aim of studying the evolution of animal resources and milk protein genes (Ceriotti et al., 2004; Jann et al., 2004; Ibeagha-Awemu et al., 2007). The 2 main whey proteins, α -LA and β -LG, are coded by the genes *LAA* and *LGB*, respectively,

whereas α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN are coded by the genes *CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3*, respectively. These 6 structural genes are affected by genetic variation that can modify the amino acid structure of the specific protein, possibly resulting in differences in the isoelectric points and electric charges of the genetic variants (Grosclaude, 1988; Farrell et al., 2004). Such biochemical variation can produce important effects on protein function as well as on milk composition and quality (Di Stasio and Mariani, 2000; Martin et al., 2002; Heck et al., 2009).

Another key aspect of milk protein variants is the tight linkage existing among the 4 CN genes within a 250-kb cluster (Ferretti et al., 1990; Threadgill and Womack, 1990). The CN alleles are thus most often inherited as a “haplotype” including, in order, *CSN1S1*, *CSN2*, *CSN1S2*, and *CSN3*. Local breeds often offer a reservoir of specific alleles and CN haplotypes, allowing deeper genetic data mining of milk protein variation.

The aims of this work were 1) to study the genetic variation affecting the amino acid charge of milk protein genes in the Original Pinzgauer cattle, with particular attention to the occurrence of rare variants and 2) to compare 2 different breeding areas in terms of the variability of milk protein.

The Original Pinzgauer is a dual-purpose (dairy and beef) red and white cattle breed of European origin. About 500 AD, Alpine herdsmen had begun to develop a breed of red and white cattle from the native red Bavarian cattle. These early cattlemen selected animals that could withstand the harsh conditions and produce meat and milk (<http://www.ansi.okstate.edu/breeds/cattle/pinzgauer/>). This medium-sized breed takes its name from Pinzgau in Salzburg, Austria, and the herd book dates back to the 1700s (Pinzgauer Cattle, 2008). The breed was influenced in the past by human movements from different regions and also by crossbreeding with Red Holstein, resulting in the current Pinzgauer breed. Original Pinzgauer are characterized by a Red Holstein contribution of less than 6%.

The census of the Original Pinzgauer population was 423 animals in Germany in 2006 (<http://www.genres>).

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Table 1. Frequencies of milk protein alleles, identified by isoelectric focusing, in Original Pinzgauer in the whole data set (overall) and in the Austrian and German samples

Locus ¹	Allele	Overall (n = 485)	Austria (n = 275)	Germany (n = 210)
<i>CSN1S1</i>	<i>B</i>	0.730	0.709	0.757
	<i>C</i>	0.270	0.291	0.243
<i>CSN2</i>	<i>A</i> ¹	0.357	0.342	0.376
	<i>A</i> ²	0.572	0.589	0.550
	<i>B</i>	0.015	0.011	0.021
	<i>C</i>	0.056	0.058	0.053
<i>CSN1S2</i>	<i>A</i>	0.798	0.755	0.855
	<i>B</i>	0.202	0.245	0.145
<i>CSN3</i>	<i>A</i>	0.728	0.722	0.736
	<i>B</i>	0.266	0.269	0.262
	<i>E</i>	0.006	0.009	0.002
<i>LAA</i>	<i>A</i>	0.002	0.004	0.000
	<i>B</i>	0.998	0.996	1.000
<i>LGB</i>	<i>A</i>	0.140	0.131	0.151
	<i>B</i>	0.860	0.869	0.849

¹*CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3* code for α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN, respectively; *LAA* and *LGB* code for α -LA and β -LG, respectively.

de/CF/tgrdeu/charakterisierung.cfm?idrasse=171), and 4,833 animals in Austria in 2007 (<http://www.oen-gene.at/index.jsp?catId=33>) compared with a world-wide population of 1 million Pinzgauers distributed in 25 countries, and mainly used for meat production (<http://www.pinzgauer-cattle.com/>). The endangered status of the breed is further motivation for the genetic characterization of the current population at milk protein genes.

A total of 485 individual milk samples were randomly collected from animals of the Original Pinzgauer breed reared both in Austria (n = 275) and Germany (n = 210) and analyzed by isoelectrofocusing (IEF) according to Erhardt et al. (1998). Allele frequencies were estimated by direct count. Deviations from Hardy-Weinberg equilibrium were estimated by the Genepop program (version 1.2; Raymond and Rousset, 1995). The *F*-statistics were estimated by FSTAT (version 1.2; Goudet, 1995). The haplotype frequencies (*CSN1S1-CSN2-CSN1S2-CSN3*) were estimated using the EH program (Xie and Ott, 1993). The program provides haplotype frequencies expected under the independence hypothesis (independent frequencies, **IF**) and estimated taking association into account (association frequencies, **AF**). The program uses an iterative maximum-likelihood algorithm under the assumption of Hardy-Weinberg equilibrium. In addition, it gives χ^2 values for this comparison, which were used to calculate *P*-values for the hypothesis that the estimated values (AF) differ from the expected values (IF).

After extraction of genomic DNA from milk somatic cells (Lühken et al., 2009), the coding region of exon IV of *CSN3* was sequenced in 59 Original Pinzgauer carrying *CSN1S2*B*, to differentiate the *CSN3* alleles *A*, *B*, *C*, *F*, *G*, *H*, *I*, and *J* (Prinzenberg et al., 2008). For this purpose, exon IV was amplified by PCR (Prinzenberg et al., 1999) using a modified reverse primer (5'-gtt-gtctctttgatgtctccttagag). Sequencing of PCR products from both sides with one of the PCR primers each and sequence analysis was done as described by Lühken et al. (2009).

Allele frequencies of milk protein genes are shown in Table 1. Fifteen alleles were found from the 22 alleles identifiable by IEF. This number is slightly larger than in other breeds analyzed by the same method and similar in sample size; that is, Italian Friesian (13 alleles), Italian Brown (14 alleles), and Reggiana (13 alleles; Caroli et al., 2004). Only one deviation from Hardy-Weinberg equilibrium was observed, namely at *CSN1S2* gene in the German population, which had an excess of homozygous genotypes. This deviation was not observed in the combined population. Individual genotypes for each locus are available on request.

Two variants were found at *CSN1S1*, *CSN1S2*, and *LGB*, whereas *LAA* was almost fixed for the *B* allele. The most polymorphic gene was *CSN2*, with 4 alleles detected (*A*², *A*¹, *C*, and *B*, in decreasing order of frequency). A rather high frequency was found for *CSN1S1*C*, which is usually more common in *Bos indicus* than in *Bos taurus* (Formaggioni et al., 1999).

Three alleles were detected at *CSN3*. The prevalent allele was *CSN3**A, whereas *CSN3**B, which is the most favorable *CSN3* variant from the point of view of milk rennet clotting properties (Di Stasio and Mariani, 2000), occurred at a frequency of 0.266. In addition, *CSN3**E was found at a very low frequency. The presence of *CSN3**E in the Original Pinzgauer is an indicator for the influence of Red Holstein genes in some animals, as already described for the Pinzgauer (Erhardt, 1996). Most of the former Original Pinzgauer underwent improvement by using Red Holstein semen in past breeding programs, although the influence of this improvement is difficult to quantify. Genetic markers can provide a useful tool for tracing Red Holstein introgression. In fact, a sample of animals from the current Pinzgauer was grouped with German and Austrian Holsteins in a phylogenetic study based on microsatellites, whereas animals of the Original Pinzgauer were clustered separately from the other breeds (Baumung et al., 2008).

Surprisingly, *CSN3**G was no longer present after its first discovery in the breed (Erhardt, 1996) and the detection of unfavorable effects on milk rennet clotting properties described (Erhardt et al., 1997). This rare variant occurred in one sire (unpublished results), whose genes were most probably lost or extremely diluted in the actual Original Pinzgauer population as a consequence of genetic drift.

A relatively large frequency was found for the *CSN1S2**B allele, which was more common in the Austrian sample. The F_{ST} statistics significantly differed from zero ($P < 0.001$) only for *CSN1S2*, as the consequence of the different distribution of *CSN1S2**A and *CSN1S2**B in the 2 populations; *CSN1S2**B is more common in *Bos indicus* breeds (Formaggioni et al., 1999). Grosclaude et al. (1976) found both *CSN1S2**A and B variants in Nepalese *Bos taurus* and *Bos indicus* populations. Merlin and Di Stasio (1979) described some unknown α_{S2} -CN bands with a smaller electrophoretic mobility than the ones produced by the predominant *CSN1S2**A in the Pinzgauer cattle reared in Italy. Possibly, such bands corresponded to *CSN1S2**B. Chianese et al. (1988) demonstrated the occurrence of this variant in the Podolian, an Italian *Bos taurus* breed. Ibeagha-Awemu et al. (2007) characterized *CSN1S2**B by identifying a C $\rightarrow$ T substitution in the 17th nucleotide of exon 3, leading to a Ser $\rightarrow$ Phe exchange in the eighth amino acid of the mature protein. The same authors found *CSN1S2**B to be widely distributed not only in *Bos indicus* breeds but also in European cattle breeds, particularly of southeastern origin, with the largest frequency (0.133) in the Turkish Gray Steppe. In the Original Pinzgauer, *CSN1S2**B frequencies were larger

than in the Turkish Gray Steppe, in both Austrian and German populations.

Another sign of *Bos indicus* introgression or conservation of ancient germplasm is the presence of *LAA**A, although limited, in the Austrian population. This allele was found in all zebu breeds at frequencies ranging from 0.082 to 0.405, whereas its frequency was <0.01 in taurine breeds except Turkish Gray Steppe and Anatolian Black (Ibeagha-Awemu et al., 2007). Unlike *CSN1S2**B, *LAA**A was mainly lost or not spread in the Original Pinzgauer, perhaps because of unfavorable or absent effects on the breed's performance, low frequency in the ancestral germplasm, or genetic drift.

Haplotype frequencies at the *CSN1S1*-*CSN2*-*CSN1S2*-*CSN3* cluster are reported in Table 2. A total of 48 possible haplotypes resulted from the combination of 2-4-2-3 alleles considered. Except for haplotype B-A²-A-A, a large difference was observed between IF and AF. In fact, the association test performed by the EH program was statistically significant ($P < 0.0001$), indicating high linkage disequilibrium (**LD**) at the CN cluster. This result can be a consequence, again, of the genetic drift in the small population, as well as of favorable effects of particular haplotypes on the breed performance and fitness. The predominant haplotype was B-A¹-A-A, followed by B-A²-A-A and C-A²-B-A. With regard to the least frequent CN alleles observed in the breed, *CSN2**C mainly occurred in B-C-A-B, but also in B-C-B-B haplotypes, whereas *CSN2**B was carried by B-B-A-B, and *CSN3**E by B-A¹-A-E.

Considering only haplotypes with frequency $>5\%$, 4 gametic types were observed between *CSN1S1* and *CSN3* (B-A, C-A, B-B, and C-B). These types were also observed among 411 individuals in which the gametic phase could be directly inferred, thus excluding an artifact of haplotype inference. The most likely explanation for this pattern is recombination within the casein cluster (Jann et al., 2002; Prinzenberg et al., 2003). Although it is unlikely that recombination occurred within Pinzgauer populations because of extensive LD, it is possible that recombination may have happened in the progenitors of the Original Pinzgauer or before the domestication event. Under this second scenario, recombinant haplotypes were captured during Pinzgauer breed formation.

Haplotypes carrying *CSN1S2**B, in particular C-A²-B-A, were predominant in the Austrian sample, reflecting the prevalence of this "*Bos indicus*" allele in this breeding area. In contrast, C-A²-A-A, which is the C-A²-B-A counterpart carrying the "*Bos taurus*" *CSN1S2**A, was predominant in Germany. The difference between the 2 breeding areas might be the consequence of a long breeding period when exchange of genetic material was

Table 2. Casein haplotype frequencies in the Original Pinzgauer in the whole data set (overall) and in the Austrian and German samples¹

CN gene ²				Overall (n = 485)		Austria (n = 275)		Germany (n = 210)	
<i>CSN1S1</i>	<i>CSN2</i>	<i>CSN1S2</i>	<i>CSN3</i>	IF	AF	IF	AF	IF	AF
<i>B</i>	<i>A</i> ¹	<i>A</i>	<i>A</i>	0.15	0.26	0.13	0.24	0.18	0.28
<i>B</i>	<i>A</i> ²	<i>A</i>	<i>A</i>	0.24	0.24	0.23	0.24	0.26	0.25
<i>C</i>	<i>A</i> ²	<i>B</i>	<i>A</i>	0.02	0.16	0.03	0.20	0.01	0.12
<i>B</i>	<i>A</i> ¹	<i>A</i>	<i>B</i>	0.06	0.08	0.05	0.07	0.06	0.09
<i>C</i>	<i>A</i> ²	<i>A</i>	<i>B</i>	0.03	0.07	0.03	0.07	0.08	0.06
<i>B</i>	<i>A</i> ²	<i>A</i>	<i>B</i>	0.09	0.05	0.08	0.05	0.09	0.04
<i>B</i>	<i>C</i>	<i>A</i>	<i>B</i>	0.01	0.04	0.01	0.03	0.01	0.05
<i>C</i>	<i>A</i> ²	<i>A</i>	<i>A</i>	0.09	0.04	0.09	0.02	0.03	0.07
<i>B</i>	<i>C</i>	<i>B</i>	<i>B</i>		0.01		0.03		
<i>B</i>	<i>A</i> ¹	<i>B</i>	<i>A</i>	0.04	0.01	0.04	0.02	0.03	0.01
<i>B</i>	<i>B</i>	<i>A</i>	<i>B</i>		0.01		0.01		0.01
<i>B</i>	<i>A</i> ²	<i>B</i>	<i>A</i>	0.06	0.01	0.07		0.04	0.01
<i>B</i>	<i>A</i> ¹	<i>A</i>	<i>E</i>		0.01		0.01		
<i>B</i>	<i>A</i> ²	<i>B</i>	<i>B</i>	0.02	0.01	0.03		0.02	0.01
<i>B</i>	<i>C</i>	<i>A</i>	<i>A</i>	0.02		0.02		0.02	0.01
<i>B</i>	<i>B</i>	<i>A</i>	<i>A</i>	0.01				0.01	0.01
<i>B</i>	<i>A</i> ¹	<i>B</i>	<i>B</i>	0.01		0.02		0.01	
<i>B</i>	<i>C</i>	<i>B</i>	<i>A</i>	0.01		0.01			
<i>C</i>	<i>A</i> ¹	<i>A</i>	<i>A</i>	0.06		0.05		0.06	
<i>C</i>	<i>A</i> ¹	<i>A</i>	<i>B</i>	0.02		0.02		0.02	
<i>C</i>	<i>A</i> ¹	<i>B</i>	<i>A</i>	0.01		0.02		0.01	
<i>C</i>	<i>A</i> ¹	<i>B</i>	<i>B</i>	0.01		0.01			
<i>C</i>	<i>A</i> ²	<i>B</i>	<i>B</i>	0.01		0.01		0.01	
<i>C</i>	<i>C</i>	<i>A</i>	<i>A</i>	0.01		0.01		0.01	

¹IF = independent frequencies; AF = association frequencies; only haplotypes with at least an AF or IF value >0.00 are shown. Haplotypes are reported in descending AF order in the overall group; blank = 0.00.

²*CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3* code for α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN, respectively.

limited because of geographic restrictions, genetic drift effect, or different selection strategies carried out by the breeders.

The prevalence of *CSN1S2*B* in the *C-A²-B-A* haplotype could indicate this combination as typical of the Original Pinzgauer, as a consequence of the survival of an ancestral haplotype or the introgression of *Bos indicus*. This hypothesis is supported by the high frequency (0.42) of the *CSN3*H* allele observed after the DNA sequencing of 59 Original Pinzgauer individuals extracted from the whole data set on the basis of the occurrence of *CSN1S2*B* in their genotype. In fact, *CSN3*H* is another “zebu-specific” allele, also found by Ibeagha-Awemu et al. (2007) in the taurine breeds carrying *CSN1S2*B*. Indeed, it would be more precise to define such alleles as “zebu-peculiar” instead of “zebu-specific,” because of their recurrent presence in *Bos taurus* germplasm. No further *CSN3* allele was detected at the DNA level in addition to the IEF identifiable variants and *CSN3*H*.

In addition, the hypothesis of ancestral polymorphism retention should be more carefully considered as an alternative to *Bos indicus* hybridization. In fact, *CSN1S2*B*, *LAA*A*, and *CSN3*H* might represent ancestral polymorphisms common to both *Bos indicus* and *Bos taurus*. In this case, genetic drift or selection

increasing the frequency of *CSN1S2*B* in the Original Pinzgauer breed could mimic *Bos indicus* introgression. However, whereas the occurrence of *CSN3*H* in the breed can be explained by LD at the CN haplotype, the simultaneous presence of *LAA*A*, even if at a low frequency, could be a further hint of *Bos indicus* introgression caused by the independent segregation of the *LAA* and CN cluster, harbored on chromosomes 5 and 6, respectively (Hayes et al., 1993; Popescu et al., 1996).

More information can be derived from the analysis of CN haplotype variability. Mahé et al. (1999) described the predominance of *CSN1S1*C-CSN2*A²-CSN3*A* haplotype in zebu breeds in an electrophoretic study at the protein level. Jann et al. (2004) confirmed these findings at the DNA level, allowing discrimination of the 3 alleles *CSN3*A*, *CSN3*H*, and *CSN3*A^I*, which co-migrate as *CSN3*A* if analyzed at the protein level by electrophoresis or IEF. The occurrence of haplotypes *CSN1S1*C-CSN2*A²-CSN3*A^I* and *CSN1S1*C-CSN2*A²-CSN3*H* indicated the introgression of *Bos indicus* in southern and eastern European cattle breeds (Jann et al., 2004). In the Original Pinzgauer, the high frequency (0.2 in the overall sample) of the haplotype *CSN1S1*C-CSN2*A²-CSN3*A* provides a further argument for *Bos indicus* introgression, as suggested

by previous reports on the basis of other markers in taurine breeds (e.g., Cymbron et al., 1999; Loftus et al., 1999; Beja-Pereira et al., 2006). However, further investigation is needed in the *Bos* genus at the CN haplotype level including the simultaneous detection of *CSN1S2*B*, *CSN3*H*, and *CSN3*A*¹.

Data on the Pinzgauer reared in Italy highlighted the distance from other *brachyceros* Italian breeds on the basis of milk protein genes (Pieragostini et al., 2002). Conversely, Astolfi et al. (1983) did not find particular differences for Italian Pinzgauer from the mountain Brown breed group on the basis of immunological and biochemical markers. The same authors proposed an introgression of *Bos primigenius* from the Middle East around the third millennium BC. The hypothesis of eastern introgression into Central Europe cattle is also supported by a work based on mitochondrial haplotypes, which indicated the introduction, during Roman times or earlier, of diverse female lineages from the domestication center or by later admixture in Switzerland (Schlumbaum et al., 2006). This introgression might have involved the progenitors of the Original Pinzgauer, too, with a particular effect on the CN haplotype. Nevertheless, the retention of ancient milk protein polymorphisms could be a possible alternative to the *Bos indicus* hybridization hypothesis.

In conclusion, the Original Pinzgauer breed is characterized by an interesting variation affecting the amino acid charge of milk proteins. The frequency of *CSN1S2*B* is relatively high in this breed and is mainly linked with the *C-A²-B-A* haplotype. The wide distribution of this allele in European *Bos taurus* breeds and its high frequency in the Original Pinzgauer demonstrate that *CSN1S2*B* has to be considered in milk protein studies in taurine breeds. Association analyses with milk production traits are necessary to investigate whether the maintenance of ancestral haplotypes provided the Original Pinzgauer with enhanced fitness or otherwise affected the productivity of this local breed.

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