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Genetic analysis of *CSN2* in local and international cattle breeds raised in Poland*

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The most common of the 12 known genetic variants of β-casein are A_1 and A_2. Unfortunately, the A1 allele is linked to a number of diseases in humans. Therefore we should consider whether it is possible to reduce the occurrence of the undesirable allele in milk. Hence the aim of the study was to determine the frequency of the A_1 and A_2 alleles and the degree of genetic variation in β-casein in cattle of two international breeds (Holstein-Friesian and Simmental) and two local breeds included in a genetic resources conservation programme (Polish Red and White-Backed), raised in Poland. The study was conducted on 386 cows from 29 farms. Genetic variation in the *CSN2* gene was determined by PCR-RFLP using the *Dde*I restriction enzyme. The cattle breeds, especially the White-Backed and Polish Red, were shown to have a relatively high frequency of the A_2 allele (over 0.5). In addition, an excess of heterozygous genotypes was observed in all the tested populations, but homozygous A_2A_2 individuals were clearly predominant in the local breeds.

The results show that local cattle breeds are particularly predisposed to produce 'A_2 milk' and suggest that steps should be taken towards selection of cattle producing milk with the A_2 variant of β-casein, which would increase the profitability of its breeding and meet the current requirements of both individual consumers and the medical profession, mainly pediatricians.

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Cattle are the most important species in world milk production and the most common breed is the Holstein-Friesian breed. In Poland this breed also has the largest share in the active population of dairy cattle (almost 93% of over 2 million cows) [FAO 2021, PFHBiPM 2020]. The Simmental breed ranked second, accounting for 1.26% [PFHBiPM 2020]. Both of these are international transboundary breeds. However, in recent years local, dual-purpose breeds have been gaining in popularity. Apart from being very well adapted to local environmental conditions, they are also characterized by good health and disease resistance, very high fertility and calf viability, longevity, the ability to reduce productivity in order to survive a temporary feed shortage and most importantly - high-quality raw milk [Barłowska and Litwińczuk 2006, Król *et al.* 2010, Litwińczuk *et al.* 2012, Prusak *et al.* 2015]. In Poland there are four breeds covered by a genetic resources conservation programme (Polish Black-and-White, Polish Red-and-White, Polish Red and White-Backed), which together account for 1.18% of the active population of dairy cows. The two latter cattle breeds are the oldest breeds currently raised in Poland [PFHBiPM 2020].

White-Backed cattle (PW) are bred mainly in eastern Poland, in a temperate climate zone. The breed is descended from primitive cattle originally found in northwestern Europe. In the second half of the 20th century these cattle were declared extinct. Work on the restitution of the breed began in the 1990s and the breed registry for this breed was opened in 2003 [Kasprzak-Filipek *et al.* 2020, Sawicka-Zugaj *et al.* 2018]. In 2020 a total of 813 White-Backed cows were assessed for dairy performance, 670 of which were kept on 55 farms implementing a genetic resources conservation programme [PFHBiPM 2020, Kasprzak-Filipek *et al*. 2020].

Polish Red cattle, which are among the oldest native European breeds of cattle, are found mainly in southern Poland. In the late 1960s the Polish Red population numbered about two million cows, which constituted about 18% of the Polish cattle population. As in the case of the White-Backed breed, activities aimed at intensifying agriculture, including increasing the milk yield of cows, resulted in a sharp decline in the population size of this breed [Litwińczuk *et al.* 2012, Sawicka-Zugaj *et al*. 2018]. In 1999, the breed was included in the genetic resources conservation programme. In 2020 a total of 2 820 Polish Red cows were assessed for dairy performance, 2 394 of which were kept on 242 farms implementing the genetic resources conservation programme [PFHBiPM 2020, Sawicka-Zugaj *et al.* 2018].

Milk is an excellent source of protein of high biological value, as well as lipids, carbohydrates, macro- and microelements, and vitamins [Barłowska *et al.* 2011]. Cow milk proteins are a common source of bioactive peptides, which are produced as a result of degradation of casein and whey proteins by gastrointestinal digestive enzymes of vegetable or microbiological origin [Kamiński *et al*. 2007]. These peptides can exhibit a wide range of health-promoting activity. However, milk may also contain substances inducing immune-mediated reactions (food allergies) [Pastuszka *et al*. 2016, Tsabouri

et al. 2014] or non-immunological reactions such as gastrointestinal disorders, e.g. due to lactase deficiency [Strzałkowska *et al*. 2018] or activity of the peptide beta-casomorphin-7 (BCM-7) released from beta-casein A_1 [Guantario *et al*. 2020].

Beta-casein is one of four casein fractions (αs1-, αs2-, β- and κ-) in cattle. Twelve genetic variants determining its synthesis have been identified in cattle, with the most common forms in dairy cattle breeds being A_1 and A_2 [Barłowska *et al*. 2012, Farrell *et al.* 2004]. The A_2 variant is the original form and is identified in old cattle breeds and in domestic buffalo raised in India [Duarte-Vázquez *et al*. 2017, Priyadarshini *et al*. 2018]. The A_1 variant, which evolved as a result of an unfavourable point mutation (C→A) occurring about 5,000-10,000 years ago in some European herds, is characteristic of contemporary breeds. It has histidine at position 67 of the amino acid chain, while the A_2 variant has proline. During digestion of milk protein in the small intestine the presence of histidine causes the release of the bioactive 7-amino acid peptide known as BCM-7 [Demirel and Bahattin 2018].

In the late 20th century the first reports appeared of the potential negative effect on human health caused by beta-casomorphin-7 released during digestion [Barłowska *et al.* 2011, Birgisdottir *et al.* 2006, Elliott 1992, Laugesen and Elliot 2003, McLachlan 2001]. Some studies indicate that this peptide increases the risk of autoimmune diseases such as type 1 diabetes [Birgisdottir *et al.* 2006, Laugesen and Elliot 2003, McLachlan 2001], allergic airway inflammation [Yadav *et al.* 2020], gastrointestinal disorders similar to lactose intolerance [Brooke-Taylor *et al.* 2017, Jianqin *et al.* 2016], schizophrenia and autism [Cade *et al*. 2000, Kost *et al*. 2009], cardiovascular disease [Laugesen and Elliot 2003, McLachlan 2001, Tailford *et al*. 2003] and sudden infant death syndrome [Sun *et al.* 2003]. A1 beta-casein can cause bloating and gastric discomfort in sensitive individuals, as in the case of lactose intolerance [Jianqin *et al*. 2016]. Although the results of many studies seem to confirm the negative effect of BCM-7 on human health, according to the 2009 Report of the European Food Safety Authority (EFSA) there is very limited evidence for a relationship between consumption of A1 milk and the aetiology or course of any of the diseases suggested. The report emphasised that most of these studies were conducted on laboratory animals, thus the results may not fully apply to humans, as studies carried out on humans in natural conditions have provided conflicting results. Recent studies, both in laboratory animals and humans, have nevertheless indicated the occurrence of the abovementioned gastrointestinal disorders following consumption of A1 milk [Brooke-Taylor *et al*. 2017, Cade *et al.* 2000, Chia *et al.* 2018, Demirel and Bahattin 2018, Elliott 1992, Guantario *et al*. 2020, Ho *et al.* 2014, Jianqin *et al*. 2016, Pal *et al.* 2015, Yadav *et al*. 2020].

The aim of the study was to determine the frequency and degree of genetic variation of beta-casein in cattle of two international breeds (Holstein-Friesian and Simmental) and two breeds covered by a genetic resources conservation programme in Poland (Polish Red and White-Backed).

Material and methods

Research material

The study was conducted on 386 cows belonging to four cattle breeds raised in Poland: 112 individuals of the Polish White-Backed breed (PW), 76 Polish Red (PR), 107 Polish Holstein-Friesian (PHF) and 91 Simmental (SM). The animals selected for analysis came from 29 farms located in southern and south-eastern Poland and keeping 10-30 cows, including PW from 18 farms, PR from 3, PHF from 4 and SM from 4, respectively.

The biological material, in the form of hair follicles, was obtained from animals selected on the basis of breeding documentation, taking into account their origin and relatedness. The cows selected were not upgraded and were unrelated going back three generations. The collected biological material was obtained from samples intended for genotyping, which did not require the approval of the ethics committee.

Analytical methods

DNA was isolated from the biological material using a Genomic Mini kit (A&A Biotechnology, Gdynia, Poland). Isolated DNA was stored at -20°C until the genotyping procedure. Quantitative and qualitative evaluation of the DNA solution was carried out in a 1% agarose gel using Eurx Simply Safe loading buffer (400 A, 80 V, 40 min). The gels were analysed in UV light (Transilluminator) and archived using an archiving kit with a CCD camera and Scion Image software (Syngen Biotech, Wrocław, Poland). The DNA was used to determine the beta-casein genotypes (CSN2) by the PCR-RFLP method (polymerase chain reaction - restriction fragment length polymorphism).

The genetic material was used to amplify a fragment of the beta-casein gene using the SensoQuest LabCycler (SensoQuest, Göttingen, Germany). Primer sequences described by McLachlan [McLachlan 2006] were applied for the amplification: forward primer 5'-CCT TCT TTC CAG GAT GAA CTC CAG G-3' and reverse primer 5'-GAG TAA GAG GAG GGA TGT TTT GTG GGA GGC TCT-3'. The reaction mixture consisted of 200 ng DNA, 2.5 µl 10x buffer, 200 µM of dNTPs, 25 pM of each primer, 25 mM $MgCl_2$; and 0.125 U AmpliTaq Gold DNA Polymerase (Life Technology). Thermal cycling conditions included an initial denaturation step at 94°C for 10 min, followed by 35 cycles of 94°C for 30 s, 58.3°C for 30 s, 72°C for 1 min and final extension at 72°C for 5 min.

Amplicons of the 121 base pair (bp) beta-casein gene fragment were subjected to restriction analysis using the DdeI restriction enzyme (Life Technology). The amplified DNA fragments were verified by staining with Simply Safe (Eurx) on a 3% agar-gel followed by visualization on a UV transilluminator. A 50 bp DNA ladder was lined up as a molecular size marker.

Statistical analysis

To show the differences in the genetic structure of the cattle breeds within the CSN2 gene, statistical analysis of the results was conducted in the POPGENE software

(version 1.32), determining the following: frequency of alleles and genotypes in individual loci, the observed (Na) and effective number (Ne) of alleles, the degree of observed (Heto) and expected (Hete) heterozygosity, the degree of observed (Homo) and expected (Home) homozygosity, the genetic diversity index (H) according to Nei [1973], the fixation index (FIS), Shannon's information index (I) [Wright 1965] and genetic distance according to Nei [1973]. The Hardy-Weinberg equilibrium in the cattle populations was tested using the chi square test ($\chi 2$).

Results and discussion

Identification of restriction fragment length polymorphisms of the CSN2 gene was based on the fact that the A1 allele is characterised by the presence of a single 121 bp fragment, while the A_2 allele has two restriction fragments, 86 bp and 35 bp in length (Fig. 1). A total of 386 individuals were analysed to determine variants of the CSN2 gene.

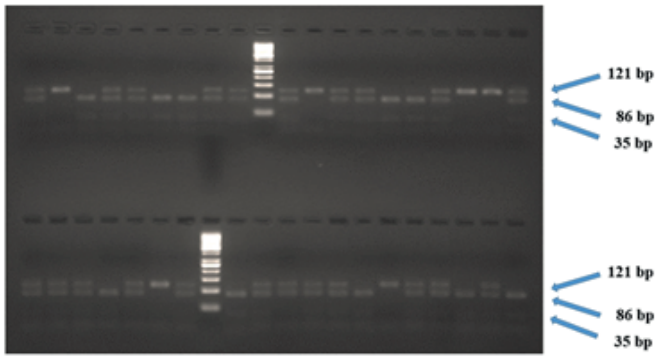

Fig. 1. Results of PCR-RFLP analysis for the *CSN2* gene by *Dde*I on a 3% agarose gel. Line 7 – DNA ladder; 5 – A_1A_1 genotype (121 bp); 3, 8 – A_2A_2 genotype (86 bp, 35 bp), others (1, 2, 4, 6, 9-12) – A_1A_2 genotype (121 bp, 86 bp, 35 bp).

In the 1980s studies were begun on the negative or positive effects of selected milk peptides on human health. After 10 years results were obtained on the relationship between A_1 and A_2 milk as well as links between the A1 allele and certain chronic diseases [Chin-Dusting *et al*. 2006, Jianqin *et al*. 2016, Sun *et al*. 2003, Tailford *et al.* 2003].

Table 1 presents the results obtained for the frequency of CSN2 alleles and genotypes in each of the breeds analysed. In the case of the PHF breed the distribution of A_1 and A_2 alleles was equal (50% each). In the other breeds the A_2 allele was more common, from 0.516 (SM) to 0.571 (PW).

In the present study the A_2 allele was found to be most frequent in the local breeds, i.e. White-Backed and Polish Red, which are the oldest breeds in Poland, in

Table 1. Frequency of alleles and genotypes of the *CSN2* gene in the analysed cattle populations

Breed	No. of cows	Genotype frequency			Allele frequency		P value
		A_1A_1	A_1A_2	A_2A_2	A_1	A_2	
PW	112	0.134	0.589	0.277	0.429	0.571	0.035
PR	76	0.198	0.526	0.276	0.461	0.539	<0.001
PHF	107	0.028	0.944	0.028	0.500	0.500	<0.001
SM	91	0.011	0.945	0.044	0.484	0.516	0.646
Mean		0.088	0.759	0.153	0.468	0.532	

P – value for Hardy-Weinberg equilibrium; PW – Polish White-Backed; PR – Polish Red; PHF – Polish Holstein-Friesian; SM – Simmental.

comparison to the high-production breeds. Miluchová *et al.* [2014] obtained a similar pattern as in the present study, finding that the local Slovak Spotted breed had a much higher frequency of the A_2 allele (0.7072) than the Pinzgau and Holstein breeds (0.4382 and 0.6322).

An A_2 allele frequency in Holstein-Friesians similar to that obtained in our study was reported by Gholami *et al*. [2016] in an Iranian population at 0.50; by Dai *et al.* [2016] in a Chinese population at 0.459; by Massella *et al*. [2017] in Italian populations at 0.546, and by Meier *et al*. [2019] in a German population at 0.562, respectively. In the case of SM, reported A_2 allele frequencies in world populations are somewhat higher: 48.43 in Iran, while in a German population it was 0.562. In the case of SM, reported A_2 allele frequencies in world populations are somewhat higher: 48.43 in Iran, [Gholami *et al*. 2016] 0.618 in Austria [Mayer *et al.* 2021], and 0.645 in Ukraine [Ladyka *et al*. 2021].

For Polish Red cattle, a different frequency of the A_2 allele from that obtained in our study was reported by Cieślińska *et al*. [2019] who conducted similar research in a single herd (24 bulls and 177 cows) in north-eastern Poland. The authors found that the A_2 allele was present with a frequency of 0.37 in cows and 0.58 in bulls. The differences in our results and those obtained by Cieślińska *et al*. [2019] may be due to the fact that Polish Red cattle kept in southern and north-eastern Poland form two subpopulations with differing genealogy, as shown by Klauzińska *et al.* [2004]. In the 19th and early 20th century there were three varieties of Polish Red cattle: mountain, lowland, and Silesian. Lubieniecka *et al.* [2000] used microsatellite DNA sequencing to demonstrate that the north-eastern subpopulation was significantly different from the southern subpopulation in terms of the number and frequency of alleles typical of the breed. In the case of White-Backed cattle these are the first published results in this regard, so comparison with the research of other authors is not possible.

However, Kamiński *et al.* [2006] claimed that the A_1 allele is predominant in breeds present in northern Europe, while in central and southern Europe its frequency is lower. Ho *et al.* [2014] and Jaiswal *et al*. [2014] indicated that variant A_1 is only the effect of an unfavorable point mutation which occurred about 5-10 thousand years ago. However, in global cattle populations the A_2 variant of CSN2 is found with the highest frequency in African zebu (100%) [Rangel *et al*. 2017], in the southern French

breeds Charolais and Limousin, and in the Channel Island breeds Guernsey and Jersey (70%) [Mishra *et al.* 2009, Truswell 2005]. In the local Indian breeds Nimari and Malwi the frequency of the A_2 allele was even 100% [Pandey *et al*. 2020].

Due to the negative effect of A_1 milk on human health there is great interest in milk from cows with the A_2A_2 genotype. For example, in 2000 the a2 Milk Company, which processes milk from specially selected cows from all over Australia, was founded in New Zealand [Bodnár *et al*. 2018]. In the present study the highest frequency of the A2A2 homozygous genotype among the four breeds analysed was found in cows of the local breeds PW and PR (0.276).

In our research the A1A2 genotype was predominant (>75% on average for all the breeds), especially in the PHF and SM breeds. The frequency of the A_2A_2 genotype was highest in the local breeds PW (27.7%) and PR (27.6). The A1A1 genotype was the least common – from 11% (SM) to 19.8% (PR). Only the local breeds, which are not subject to intensive selection, were in the Hardy-Weinberg equilibrium.

Many studies on the CSN2 gene have shown that local breeds have a higher frequency of the A_2A_2 genotype than the A_1A_1 genotype. These frequencies were 0.5406 and 0.1261 in Slovak Spotted [Miluchová *et al*. 2014]; 0.428 and 0.122 in Buša; 0.530 and 0.183 in Slavonian-Syrmian Podolian, and 0.558 and 0.093 in Istrian cattle [Ivanković *et al*. 2021]. Vast differences were observed between the expected and observed frequencies of genotypes in the PHF and SM breeds, indicating that these populations were not in the Hardy-Weinberg equilibrium. This may have multiple causes, such as the sample size, genetic drift and above all intensive selection in these breeds.

Table 2 presents indices of variation for the CSN2 gene in the cattle populations. The FIS index indicates an excess of heterozygotes in all populations, with the highest values obtained for PHF (-0.888) and SM (-0.892), due to the observed heterozygosity of these breeds, which was 0.945 for SM and 0.944 for PHF. The share of homozygous individuals was highest by far in the PR and PW breeds, at 47% and 41%. The combined average values for gene diversity (H) and Shannon's information index (I) for the CSN2 gene in the individuals belonging to the four cattle breeds were 0.498 and 0.691, respectively. The highest average H value was noted for the PHF breed, at 0.500, with Shannon's index of 0.693.

Table 2. Indicators of genetic diversity in the analysed cattle populations

Breed	Na	Ne	I	Hom_o	Het_o	Hom_e	Het_e	H	F_{IS}
PW	2.000	1.960	0.683	0.410	0.589	0.508	0.492	0.489	-0.203
PR	2.000	2.000	0.693	0.473	0.526	0.499	0.500	0.496	-0.059
PHF	2.000	2.000	0.693	0.056	0.944	0.497	0.502	0.500	-0.888
SM	2.000	1.997	0.692	0.054	0.945	0.497	0.502	0.499	-0.892
Mean	2.000	1.9916	0.691	0.240	0.759	0.501	0.498	0.498	-0.524

Na – observed number of alleles; Ne – effective number of alleles; I – Shannon's information index; Hom_o – observed homozygosity; Het_o – observed heterozygosity; Hom_e – expected homozygosity; Het_e – expected heterozygosity; H – Nei's genetic diversity index; F_{IS} – fixation index; PW – Polish White-Backed; PR – Polish Red; PHF – Polish Holstein-Friesian; SM – Simmental.

The present study showed a high frequency of A_1A_2 heterozygotes in all the four analysed populations. A high frequency of heterozygotes (0.82) was also reported by Hanusová *et al.* [2010] in a population of Slovak Holstein cows and by Gholami *et al.* [2016] in Iranian Holstein (83.2) and Simmental (65.62) populations. However, as a rule the frequency of heterozygotes of the CSN2 gene is lower, slightly over 0.5 [Dai *et al*. 2016, Ivanković *et al.* 2021, Ladyka *et al*. 2021, Miluchová et al. 2014, Sebastiani *et al*. 2020]. The high heterozygosity obtained in the PHF and SM populations in our study may be due to the fact that the individuals selected for the study were unrelated within three generations. In contrast, the studies cited above were conducted on individuals from one or a few herds, without regard to their relatedness. It should also be noted that in the case of the PHF and SM breeds the high heterozygosity may be due to the large population of these cattle in the country and the wide assortment of bulls used for breeding, in contrast to the local breeds. The high heterozygosity of the CSN2 gene may be a solid basis for undertaking steps towards selection of pairs for mating to obtain individuals producing A_2 milk. According to Sebastiani *et al.* [2020], population selection for the A2 allele is possible when its frequency is close to 50%. The most common genotype among the 386 individuals was the heterozygous A1A2 genotype, especially in the PHF (0.944) and SM (0.945) breeds. A similarly high result was reported for Slovak Holstein cattle [Hanusová *et al*. 2010]. In the present study the frequency of the homozygous A1A1 genotype conditioning A1 milk was low. In all the populations combined it was found in less than 9% of the population.

Studies have also been carried out to determine the relationships between the CSN2 genotypes and milk yield and milk chemical composition of cows [Bugeac *et al*. 2013, Dinc *et al*. 2013, Hanusová *et al*. 2010, Olenski *et al*. 2010]. According to Bugeac *et al*. [2015], Holstein-Friesian cows with the A_2A_2 genotype produced 2,564 kg more milk than A_1A_1 cows. These relationships were not confirmed by Gurcan [2011] in a study on Black-and-White cattle raised in Turkey.

Nei's unbiased measures of genetic distance in the four cattle breeds were calculated according to the allele frequency of each locus and the results are shown in Table 3.

Table 3. Nei's unbiased measures of genetic distance

Breed	PW	PR	PHF	SM
PW				
PR	0.002			
PHF	0.010	0.997		
SM	0.006	0.999	0.001	

PW – Polish White-Backed; PR – Polish Red; PHF – Polish Holstein-Friesian; SM – Simmental.

The genetic distance between the PR and SM populations was the largest (0.999). The genetic distance between the PHF and SM breeds was the smallest (0.001).

Conclusions

Given the health problems described in the literature associated with the consumption of milk containing A_1 beta-casein (the precursor of the peptide BCM-7), it seems essential to take measures towards selection for raw milk with the A_2 beta-casein variant. Due to the high frequency of this allele (over 0.5) in the analysed population of cattle raised in Poland, further research on the presence of the A_2A_2 genotype is needed and steps should be taken to modify breeding programmes to select for this genotype. Our study showed that among the populations analysed the local breeds White-Backed and Polish Red are particularly predisposed to produce 'A_2 milk'. This is an additional asset that may increase profitability of raising these cattle and chances of preventing their extinction. Promotion of 'A_2 milk' can make it possible to meet the current demands of both consumers and physicians, mainly paediatricians.

Conflict of interest: The authors declare that they have no conflict of interest.

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