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Analysis of genetic structure in Polish Red and Polish Black-and-White cattle using twelve marker *loci* potentially related to milk or meat production traits*

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Polish Red (PR) is the native Polish cattle breed included in the FAO National Rare Livestock Breeds Preservation Programme. The breed is characterized by high vitality and fertility, calving ease, resistance to diseases, and low requirements for feed. Milk yield is low, but fat and protein content of milk are high. The frequencies of alleles of commonly studied *loci* as well as the presence of new or rarely reported DNA polymorphisms were studied in a group of about 300 PR cows belonging to two subpopulations. Moreover, gene frequencies were compared between two PR subpopulations, and with a reference population of the Black-and-White (BW) cattle. The investigation was intended to reveal the genetic specificity of PR and provide new arguments for its protection.

Except for *loci Pit1* and *GH-Msp*I, the allele frequencies of *CSN3*, *LGB*, *GH-Alu*I, *GHRH*, *LEP* and *PRL* genes in PR were found different from those appearing in BW cattle. Although the estimated genetic distance between PR and BW populations appeared very short (0.0146, R=0.986), variants of several genes identified in PR were found absent or very rare in BW cattle. These included the *LGB*[I] variant, 11-bp deletion in *MSTN* gene, as well as several unique nucleotide sequence variants of 5'-noncoding regions of *CSN1S1*, *CSN1S2*, *GH*, and *PRL* genes. At *loci CSN3*, *LGB*, *IGF1* and *GH-Alu*I significant differences were found also between the two PR subpopulations studied.

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According to Czaja [1991] the documented breeding of the Polish Red (PR) cattle dates back to the end of XIX century. The present PR cattle, probably the only native Polish cattle breed that survived, emerged from Poland's local Podgorska Red breed, upgraded with Danish Red, German Red and Angler cattle. PR breed is characterized by high vitality and fertility, calving ease, resistance to diseases and low requirements for feed. Milk yield is relatively low, but fat and protein content of milk are high. Moreover, milk of PR cows is a highly valuable raw material for production of maturing cheeses [Żurkowski and Reklewski 1987].

Today, in Poland, two subpopulations of PR cattle exist. About 50 cows are maintained at the Polish Academy of Sciences Research Station for Ecological Agriculture and Preserve Animal Breeding, Popielno (north-east Poland), and about 220 animals are kept by farmers in the south of Poland (Subcarpatian region – *Podhale*). Both subpopulations are included in the FAO National Rare Livestock Breeds Preservation Programme, which is a part of the international strategy for global management of genetic resources [Żurkowski and Reklewski 1987]. There is also the third herd of about 60 PR cattle kept, however, abroad – at the State Farm near Zbaraż, Tarnopolski region, Ukraine.

Milk protein or milk protein gene polymorphism in PR cattle was studied by Michalak [1969], Feleńczak [1982], Erhardt *et al*. [1998], Klauzinska [2002], Čitek *et al*. [2001] and Oblap *et al*. [2002]. These authors, focusing mostly on casein and β-lactoglobulin polymorphism, have shown that PR cattle harbour some rare genetic variants, e.g. the breed-specific LGB^{I} variant [Erhardt *et al*. 1998]. Moreover, an analysis by Lubieniecka *et al*. [2001] of the polymorphism within 26 microsatellite *loci* showed that in PR cattle the high genetic specificity is retained.

In our earlier paper [Klauzińska *et al*. 2000] the frequencies were compared of several *loci* occurring in Polish Black-and-White (BW) cattle with those found in 87 PR cows. The present study aimed at analysing more PR cattle (about 300 animals) to widen the knowledge on their genetic structure, to reveal the unknown areas of their genetic specificity and to provide new arguments for their protection as a breed. The frequencies of selected 12 common alleles of previously studied, as well as newly considered *loci* were estimated.

Material and methods

Animals

Included were 87 Polish Red (PR) cows and heifers maintained at the Polish Academy of Sciences Research Station for Ecological Agriculture and Preserve Animal Breeding, Popielno, and 224 animals owned by small farmers in the southern region of the country (subpopulation North and subpopulation South, respectively). Cows and heifers were daughters of 16 and 80 sires, respectively. Only three sires

were common for both subpopulations. The total number of Black-and-White (BW) cattle included in this study was 650, kept at the Experimental Stations Jastrzębiec and Gajewo. However, for some gene variants smaller groups of randomly chosen PR or BW animals were genotyped. Numbers of animals analysed for each *locus* are given in Tables and/or in the text.

A total of 311 PR cows and heifers were genotyped with regard to the following *loci* potentially associated with dairy traits, as well as growth rate, reproduction and feed intake: leptin (*LEP*), prolactin (*PRL*), myostatin (*MSTN*), κ-casein (*CSN3*), αS1-casein (*CSN1S1*), αS2-casein (*CSN1S2*), β-lactoglobulin (*LGB*), growth hormone (*GH*), growth hormone releasing hormone (*GHRH*) and *Pit-1*. These are considered excellent candidates for markers of economically important quantitative traits [Mercier and Grosclaude 1999, Parmentier 1999]. Moreover, in both PR and BW cattle the recently found [Klauzińska 2000] polymorphisms within 5'-noncoding regions of *GH* and *PRL* genes were analysed. Gene and allele frequencies were compared between the two PR subpopulations and referred to those found in BW cattle.

Analytical

The blood samples were collected on K_2EDTA. Genomic DNA was isolated with a rapid method described by Kawasaki [1990] or, for some specific purposes (e.g. sequencing), by the more time-consuming but simultaneously more precise method of Kanai *et al*. [1994]. Single strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) techniques were used for detection of gene variants. Details are given in Table 1. All PCR reactions were performed using the MJ Research PTC-225 Thermal Cycler. To identify a type and a site of mutation precisely, some gene variants were automatically sequenced with fluorescent labelled (Cy5) dideoxynucleotides using ALFexpress sequencer (AMERSHAM PHARMACIA BIOTECH).

Statistical

The *Chi*-square test was used to evaluate associations of allele frequencies with PR and BW and with the two subpopulations of PR cattle. The exact probability test was employed to evaluate possible deviations from Hardy-Weinberg equilibrium, with the GENEPOP package, version 3.2. [Raymond and Rousset 1995]. GENEPOP option 1 used in the calculations employed here is a population genetics software package, which allows exact tests for Hardy-Weinberg equilibrium be performed to evaluate the population differentiation.

The genetic variation was evaluated through the observed number of alleles (N), the observed heterozygosity (H_o) for each *locus*, mean heterozygosity over all *loci*, and genetic distance between breeds. These parametres were calculated according to Nei [1978] using the ARLEQUIN 2.0 software package [Schneider *et al*. 2000]. Polymorphic information content (PIC) was estimated according to Botstein *et al*. [1980]. The Fisher's F value was used as estimator of differences between PR and BW cattle.

Table I. Genes analyzed in the study and exchanges used for the polymorphism detection

Gene	Polymorphic site, protein alteration	Method	Primers	Number of PCR amplification cycles	Amplified fragment (bp)	References
*1 (CYP1A1)	exon 7, exon 7	RFLP, MspI, HaeIII	F: GGAAGATCCCTATCCCATCAGTATCCC R: CCCATCTGACCTCTATCTTAGGATG	35	371	Kawamoto et al. 1991
*2A (CYP2A6)	5'-noncoding region, pos. -703 (del/-)	RFLP, PstI	F: TATGGCCATATCTCCGGGGGGTGGGC R: GTATGGGAGATGGGGCTGGCCGGG	33	648	Oscarson and Gelboin 1998
*3 (CYP2A6)	5'-noncoding region, pos. -48 (del/-), E-1	RFLP, BamHI	F: TCCCTGTCCATGTGGGCCTGTGGGAT R: GGTCCTGGGCTTGGGCTCCATTTG	35	267	Oscarson and Gelboin 1998
	5'-noncoding region, pos. -834 (del/-), E-1	RFLP, AluI	F: TATGGCCATATCTCCGGGGGGTGGGC R: TTGGGGCGGGTGGCTGTATTGGGTTT	34	370	Oscarson and Gelboin 1998
P-glycoprotein (MDR)	exon 4, exon 4	RFLP, HaeIII	F: TGTGCTGGGGCCCCGGCTGCGGGGGG R: GCTCCCGGTGTGTGTGGCCGGCTCT	31	317	Nakamura and Aigaki-Tantsui 1998
	exon 4	RFLP, PstI	F: CTCCCGGGGGGTGCCCTCTTCTGCCGGG R: GGTGTGTGGCCGGCTCTGCCGGG	35	326	Hirschberg and Eklund 1997
GH	exon 5	RFLP, AluI	F: CCCGTGTCTGTGGGGGGC R: GTCTCTGGGCGGCCGTCT	37	430	Lacy et al. 1991
	intron 3	RFLP, AluI	F: GGGGTGGGGGCCCGGGCGGGGGTC R: GTCCGTGGCTCCGCCGTGTTT	33	195	Ogawa et al. 1991
	5'-noncoding region, pos. -843 ... -837	sequencing Miller	F: GGTGGGTTTGCCTTTCTCTCT R: GCCAGCCCAGCTCCCAGTGTTCT	29	97	Yamaoka, 1998 Unpublished sequences
	5'-noncoding region, pos. -63 ... -33	RFLP, AgeI	F: GGGGTGGGCCCCGGGGGGGGG R: GCCTGGGGGGGGGGGCCGGGG	28	191	Rodriguez et al. 1999, Ogawa et al. 2003
GHRH	intron 1	RFLP, HaeIII	F: TTCCCGGGGCCTCTCTCGGGTGG R: GCCGTGCCGTGGGGTCCTGGT	30	397	Maruyama et al. 1995 Ogawa 2003
GH-R	5'-noncoding region -378, C-1, -133, G-1, -313, C-1	RFLP, dual, dual TaqI	F: TGCGTTGCGGGGGGCTCGGGCC R: GGCGGGGCGGTGCGGGGTTGGG	28	418	Ogawa et al. 1999
LEP	exon 2	RFLP, BsaHI	F: GTCTCCCGGGGGGTCGGGTCT R: GGCCCGGGGCGGGGTCCCGGG	31	328	Thompson et al. 1997
Prnd	intron 1	RFLP, HaeIII	F: CGGGTGGGGGGGGTTGGTGTC R: TCTGCGTTCCGGGGTGCTC	34	177	Maruyama et al. 1995

Table 1 Continued

Locus	Polymorphic site, position of mutation	Method	Primers	No. of PCR amplification programme	Amplified fragment (bp)	Reference
[illegible]	[illegible]	[illegible]	F: [illegible] R: [illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	F: [illegible] R: [illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	F: [illegible] R: [illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	F: [illegible] R: [illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	F: [illegible] R: [illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	F: [illegible] R: [illegible]	[illegible]	[illegible]	[illegible]

Amplification programmes: [illegible]

Results and discussion

Polymorphism at gene coding regions – exons and introns

Numbers of animals of different genotypes and allele frequencies are shown in Table 2. All genes analysed showed to be polymorphic in both breeds. Significant associations were found between PR and BW cattle in the allele frequencies for *CSN3*, *LGB-Hae*III, *PRL*, *GHRH* and *GH-Alu*I. The value for the frequency of *LEP* allele reached the level of significance (P≤0.04). The same variants of *loci* were found in PR and BW cattle. Therefore, it is the allelic distribution, rather than diagnostic alleles that characterize inter-breed differences. The only exception is RFLP-*Sma*I *LGB*^{I} allele which was not found in BW cattle. For all the *loci* studied predominant were the same alleles in both breeds.

The milk protein *loci* have been studied most extensively in cattle. Despite the still existing controversies, they are considered as marker *loci* for milk production traits in cattle and other ruminants.

The ***CSN3*** *BB* genotype is associated with a higher protein content of milk, shorter rennet clotting time, curd firmness, and higher cheese yield than are two remaining genotypes [Schaar 1984]. Milk of Holsteins of *CSN3 BB* genotype contained on average by 0.13 per cent units more protein than milks of *AA* and *AB* cows [Ng-Kwai-Hang *et al.* 1984]. In German H-F crossbreds, cows with *CSN3 BB* were shown superior in fat and protein content of milk, while those with *AB* genotype appeared best for milk, fat, and protein yielding [Freyer *et al*. 1999].

In the Polish BW cattle, frequency of *CSN3* allele *B* is generally low (<0.3) – Michalak [1969], Litwińczuk [1991], Kamiński and Figiel [1993]. According to Ng-Kwai-Hang *et al*. [1984] the frequency of homozygous *BB* genotype in Holsteins is even lower than theoretically expected value of 0.04, and may indicate its elimination in the course of intensive selection for high milk yield. In the present study the estimated frequencies of the *CSN3 B* allele and the *BB* genotype for the BW cattle were 0.23 and 0.04, respectively, and did not differ from those reported for Holsteins. The frequencies of CSN3 *B* allele and *BB* genotype (0.31 and 0.08, respectively) appeared higher in PR than in BW cattle. Frequencies of *A* and *B* alleles in PR cattle were found similar to those reported earlier for the breed [Michalak 1969, Feleńczak 1982, Erhardt *et al*. 1996], but significantly different (P≤0.0001) from respective values noted in BW cattle (Tab. 2).

LGB allele *B* in cattle is probably associated with elevated protein and fat content of milk and its higher resistance to heat [Lundén *et al.* 1997]. The present report reveals the highly significant differences (P≤0.0000) in the estimated frequencies of *LGB* alleles between PR and BW cattle – 0.72 and 0.59, respectively (Tab. 2). The frequencies of *LGB A* and *B* alleles remained within the range previously reported for other populations of the PR and BW cattle [Michalak 1969, Feleńczak 1982, Litwińczuk 1991, Zwierzchowski *et al*. 1998, Klauzińska *et al.* 2000, Čitek *et al*. 2001].

The *LGB*^{I} allele was first described by Erhardt *et al.* [1998] as original of the PR cattle. In this study we found the *LGB*^{I} allele (recognized by RFLP-*Sma*I) in six PR

Table 1. Gene frequencies in the Polish Red (PR) and Polish Black-and-White (BW) cattle

Locus	Genotype	Polish Red cattle		Black-and-White cattle		Chi-square test
		number of animals	allelic frequency	number of animals	allelic frequency	
CSN1 RFLP-MseIII	AA	157		164		
	AB	141	A - 0.69	246	A - 0.77	P<0.0001
	BB	116	B - 0.31	116	B - 0.23	
	total	414		526		
LGB RFLP-MseIII	AA	179		121		
	AB	95	A - 0.58	178	A - 0.61	P<0.0000
	BB	178	B - 0.42	119	B - 0.39	
	total	412		526		
LGB RFLP-BseLI	AA	101		60		
	AB	6	A - 0.99	0	A - 1.00	n.s.
	BB	0	B - 0.01	0	B - 0.00	
	total	107		60		
CN RFLP-AluI	AA	114		167		
	AB	88		87		
	BB	9	A - 0.85	8	A - 0.86	n.s.
			B - 0.15		B - 0.14	
	total	161		111		
CN RFLP-AluI	LL	185		161		
	LP	103	L - 0.76	197	L - 0.69	P<0.00
	PP	14	P - 0.24	96	P - 0.31	
	total	414		526		
CAMN RFLP-MseIII	AA	111		111		
	AB	91	A - 0.77	84	A - 0.74	P<0.0001
	BB	71	B - 0.61	134	B - 0.26	
	total	184		219		
PRNP RFLP-MspI	AA	116		111		
	AB	179	A - 0.41	151	A - 0.35	n.s.
	BB	109	B - 0.59	115	B - 0.65	
	total	414		419		
PRL RFLP-AluI	AA	118		111		
	AB	181	A - 0.67	179	A - 0.78	P<0.0000
	BB	115	B - 0.11	136	B - 0.11	
	total	184		219		
LEP RFLP-BsaHI	AA	110		111		
	AB	111		111		
	BB	17	A - 0.71	18	A - 0.80	P<0.0
	AC	55	B - 0.11	80	B - 0.10	
	BC	19	C - 0.18	18	C - 0.10	
	total	108		211		

n.s. – non-significant, n.c. – not calculated

cows from subpopulation South (Tab. 2 and 6). All cows carrying the allele were LGB^N/LGB^I heterozygotes and the combined LGB-*Hae*III/*Sma*I genotypes were always A/I, indicating the existence of preferred haplotypes within *LGB locus*. In this study LGB^I allele was found neither in PR subpopulation North, nor in BW cattle (Tab. 2 and 6).

Due to the essential role of GH in lactation and growth, ***GH*** gene as well as other genes related to the somatotropic axis, became candidate markers for performance traits in cattle [Parmentier *et al*. 1999]. In the present study two polymorphisms were analysed within a coding, and two within a 5'-noncoding region of the bovine *GH* gene. Many earlier studies [Lucy *et al.* 1993, Schlee *et al*. 1994, Lee *et al.* 1996, Chrenek *et al*. 1998, Grochowska *et al.* 2001, Zwierzchowski *et al.* 2002, Dybus 2002] showed significant correlation between L/V polymorphism of *GH* gene and dairy and meat traits in cattle. Lee *et al.* [1996] proved that allele *L* in Holstein cattle was associated with higher ability for milk production than allele *V*. In Polish Friesians allele *L* seemed to be associated with higher live body weight, but animals with allele *V* consumed less feed per unit of live weight gain [Zwierzchowski *et al*. 1998]. In the present study the frequency at *GH locus* appeared significantly breed-associated ($P \leq 0.004$). The estimated frequency of the *L* allele in BW was 0.69, differing significantly ($P \leq 0.004$) from that in the PR cattle (0.76) – Table 2. A very similar frequency of *GH* allele *L* was recently reported in the PR cattle by Čitek *et al*. [2000]. Significant differences between the PR and BW cattle were observed neither for another *GH locus* polymorphism – RFLP-*Msp*I – nor for *Pit-1 locus* (Tab. 2).

Although considered a candidate marker, the ***GHRH*** *locus* was not thoroughly studied in cattle. The only polymorphism described so far in the bovine *GHRH* gene is RFLP recognized by *Hae*III nuclease [Moody *et al*. 1995]. The frequency of allele *A* of *GHRH* gene found in this study in BW cattle (0.24) was higher than that earlier reported for Holsteins (0.07) by Moody *et al*. [1995], but still significantly lower than that found in PR cattle (0.37) – Table 2. In a preliminary study by Parmentier *et al*. [1999] carried on 89 Holstein-Friesian AI bulls the *GHRH AA* genotype was found favourable for fat content of milk and milk fat yield. However, no results were published of further research on the effect of this polymorphism on performance traits in cattle.

As shown in this study, the frequency of alleles *A* and *B* at the ***Pit-1*** *locus* did not differ between PR and BW cattle (Tab. 2) and was similar to that found in Italian Holsteins by Renaville *et al*. [1997]. Zwierzchowski *et al*. [2002] reported the daily milk yield of *Pit-1 AB* cows to be higher than those of *AA* and *BB* homozygotes. Moreover, BW cows of heterozygous *Pit-1* genotype yielded more protein and lactose than the *AA* and *BB* genotype animals.

Leptin, the product of the ***LEP*** (*i.e*. obese) gene may be an important regulator of energy metabolism, adiposity and reproduction, and is perhaps linked to meat quality determinants such as marbling [Hossner 1998]. The hormone is also involved in the regulation of body weight in mammals [Friedman and Halaas 1998] and probably can be considered as one of the best biological markers reflecting total body fat in both animals and humans. In spite of this, only few studies have been performed on the

effect of *LEP* gene polymorphism on performance traits in cattle. Leifers *et al*. [2002] reported that in H-F heifers, the *LEP AB* genotype was associated with higher milk yield. They concluded that individuals with *B* allele can yield more milk without negative effects on their energy balance and fertility. Polish BW cows carrying allele *C* at *LEP locus* showed better performance in most milk production traits – daily milk yield and milk composition [Zwierzchowski *et al*. 2002]. As shown in the present study the frequency of the *LEP* alleles differed between the two breeds studied, but at low level of significance ($P \leq 0.04$) – Table 2.

In the present study 15 PR individuals (eight from subpopulation North and seven from subpopulation South) were found carrying 11-bp deletion within the ***MSTN*** gene (*nt821del11*) – not tabulated. The mutation was originally identified in Belgian Blue cattle to be associated with the double-muscle phenotype [Grobet *et al*. 1997, Kambabur *et al*. 1997] and was also found in some other cattle breeds [Grobet *et al*. 1998]. However, in the present study, in mutation-carrying PR cows no phenotypic muscle "hypertrophy" was observed (all animals were heterozygous). The occurrence of *MSTN* gene *nt821del11* mutation in cattle in which double-muscle phenotype was never reported seems interesting.

Polymorphism at gene 5'-noncoding regions – promoters

Table 3 shows allele frequencies and inter-breed differences within genotypes in 5'-noncoding regions (promoters) of *CSN1S1*, *CSN1S2*, *PRL*, *GH*, *GH-R*, *IGF1* and *MSTN loci*. At *CSN1S2* (*Mae*II), *GH* (microsatellite), *GH-R* (*Alu*I and *Acc*I), and *MSTN*, highly significant differences were found in allele frequency between PR and BW cattle.

Rodrigues *et al.* [1999] revealed a simple repeat sequence variation of $(AAG)_n$, in promoter region of the bovine GH gene, upstream the TATA box. The polymorphism can be easily detected by RFLP-*Mbo*II analysis [Dybus *et al*. 2002]. Deletion of the AAG repeat was found in Nelore and Limousine cattle, and not in dairy breeds such as Holstein or Brown Swiss, suggesting that selection for milk yield has eliminated the "shorter" allele. In the present study no polymorphism in this site was found in the dairy BW cattle, and only one PR individual was identified carrying heterozygous (*AB*) RFLP-*Mbo*II genotype. Another tri-nucleotide (TGC) repeat in bovine *GH* gene 5'-noncoding region was described by Yao *et al*. [1996]. In the present study, in addition to previously found alleles with 5 (A) and 6 (B) TGC repeats, two novel alleles were found with 2 (C) or 3 (D) insertions of TGC (Tab. 3). All four alleles were found in both breeds, however, with different frequencies ($P<0.000$). Only three heterozygous genotypes were found in BW and eight in PR cattle.

Ge *et al*. [2001] identified the T→C transition in the promoter region of the bovine IGF-1 gene recognizable by RFLP-*Sna*BI. In Angus calves the frequency of allele T was 0.639. An association was shown of *CC* genotype with higher live weight gain over the first 20 post-weaning days. In the present study the estimated frequency of the *IGF-1* allele T was 0.55 and 0.47 in PR and BW cattle, respectively (Tab. 3). The frequencies of *IGF-1* alleles apeared weakly associated ($P<0.04$) with breed.

Table 3. Frequency of genotypes in 3′-noncoding region (promoter) of PGI-exon (*CSF1*), PGI-exon (*CSF1*), *CA*, *CA-1*, *PAL* and *ASTP* genes in Polish Redland Black-and-White cattle

Locus	Position of mutation	Polish Red		Black-and-White		CSF-equivalence
		genotype – number of animals	allele and genotype frequency	genotype – number of animals	allele and genotype frequency	
CSF1	RFLP-*Sau*I, pos. -718	(+/+) – 31 (+/–) – 7 total: 38	(+) – 0.87 (–) – 0.13	(+/+) – 57 (+/–) – 8 total: 65	(+) – 0.94 (–) – 0.06	n.s.
CSF1	RFLP-*Sca*RV, pos. -186	TT – 178 CT – 10 total: 188	T – 0.97 C – 0.03	TT – 60 CT – 0 total: 60	T – 1.00 C – 0.00	n.s.
	RFLP-*Hin*dII, pos. -109	CC – 171 CT – 11 TT – 0 total: 193	C – 0.94 T – 0.06	CC – 100 CT – 1.1 TT – 1 total: 1.1	C – 0.98 T – 0.12	PTD0001
PAL	SSCP, sequencing, pos. from -1006 to -846	A – 136 B – 8 C – 37 D – 0 total: 181	A – 0.81 B – 0.01 C – 0.18 D – 0.00	A – 1.1.2 B – 11 C – 39 D – 1 total: 1.1.	A – 0.77 B – 0.07 C – 0.15 D – 0.01	n.s.
	SSCP, sequencing, pos. from -315 to -10	AA – 60 total: 60	1	BB – 60 total: 60	1	n.s.
CA	RFLP-*Hin*dII, SSCP	AA – 10.1 AB – 1 total: 105	A – 0.998 B – 0.001	AA – 111 AB – 0 total: 111	A – 1.00 B – 0.00	n.s.
	microsatellite pos. -6.1 – -6.5	A – 3 B – 31 C – 111 D – 18 W – 8 total: 171	A – 0.01 B – 0.11 C – 0.70 D – 0.10 W – 0.05	A – 11 B – 3.1 C – 71 D – 11 W – 1 total: 17.	A – 0.07 B – 0.11 C – 0.1.1 D – 0.19 W – 0.01	PTD000
CA-1	RFLP-*Ava*II, pos. -1110	(++) – 17 (+–) – 19 (––) – 18	(+) – 0.1. (–) – 0.37	(++) – 11 (+–) – 1.7 (––) – 11	(+) – 0.61 (–) – 0.19	PTD0001
	RFLP-*Ava*II, pos. -1.91	(++) – 11 (+–) – 19 (––) – 12	(+) – 0.1.1 (–) – 0.18	(++) – 67 (+–) – 11 (––) – 1	(+) – 0.8. (–) – 0.1.	PTD000
	RFLP-*Sau*I, pos. -111	(++) – 87 (+–) – 1 total: 8.	(+) – 0.99 (–) – 0.01	(++) – 8. (+–) – 6 total: 91	(+) – 0.97 (–) – 0.01	n.s.
ACTA-1	RFLP-*Sca*RI, pos. -1.1.	AA – . AB – 17 BB – 1. total: 1.7	A – 0.1. B – 0.1..	AA – 51 AB – 10. BB – 1. total: 11.	A – 0.17 B – 0.11	PTD0.
ASTP	BTAPI, SSCP, pos. -7,100	A – 19 B – 3 C – 1. D – 10. total: 13.	A – 0.1. B – 0.01 C – 0.1. D – 0.7.	A – 1. B – 11 C – 11 D – 0 total: 11.	A – 0.10 B – 0.11 C – 0.19 D – 0.00	n.s.

n.s. – non-significance, n.c. – not concerned
*Homozygotes

In our earlier studies [Klauzinska *et al*. 2000] the SSCP polymorphism was identified within the 160-bp promoter region of bovine ***PRL*** gene. Four SSCP genotypes (A, B, C, and D) were found in BW, and only two (A, C) in PR cattle. The sequencing analyses showed that these variants differed by deletion TG_2 at position -877 as earlier reported by Hart *et al*. [1993]. Moreover two novel nucleotide substitutions: A→C at position -996 and T→C at -928 were found. Since in the present work studied was almost a whole PR population existing in Poland, the lack of *PRL B* and *D* variants seems specific to PR cattle. Moreover, Klauzińska *et al*. [2000] identified two SSCP variants in the region extending from -235 to +30 of the bovine *PRL* gene. Sequencing showed that the variants differ by two nucleotide substitutions: A→C (position -124) and A→T (position -104). The variants appeared breed-specific for BW and PR cattle, respectively.

The only polymorphism (BTAFJ1) recently described in the bovine myostatin gene 5'-noncoding region is located as far as 7,600 bp upstream transcription initiation site [Jeanplong *et al*. 2000]. In the present study this polymorphic site was analysed using SSCP. Four variants (genotypes) were identified, and arbitrarily named *A, B, C* and *D*. In PR cattle the most frequent was variant *D*, totally absent in BW cattle (Tab. 3).

The 5'-noncoding regions (promoters) of bovine casein genes contain polymorphic nucleotide sequences [Schild and Geldermann 1996]. They include deletion of T nucleotide at position -728 bp of the *CSN1S1*, and T→C substitutions at positions -186 and -1084 of *CSN1S2* gene. Appearance and frequency of different alleles in *CSN1S1* and *CSN1S2* genes was analysed in small groups of randomly selected BW and PR cattle (Tab. 3). Allele *T* of *CSN1S1* gene (position -728) appeared more frequent in PR than in BW cattle, and variant *C* of the *CSN1S2* gene promoter (position -186) was totally absent from the latter. Both breeds differed significantly by the frequency of RFLP-*Mae*II alleles of *CSN1S2* gene.

Hardy-Weinberg genetic equilibrium

The Hardy-Weinberg equilibrium (HWE) was tested separately and in combination for *loci* with adequate numbers and allele distribution, using the Fisher's F value [Guo and Thompson 1992, Rousset and Raymond 1995]. Over all the *loci* the PR and BW populations showed a significant departure from HWE ($P \leq 0.0000$). The BW cattle were not in HWE for *CSN3, LGB* (*Hae*III), *GH* (*Alu*I), *GH* (microsatellite) and *LEP* genes (Tab. 4).

In the dairy BW cattle, selection for milk yield, and milk fat and protein content could also influence the allele frequency of genes related to dairy traits. However, in the PR cattle, virtually not selected since 1960's, the departures from the Hardy-Weinberg genetic equilibrium for *LGB*, *GH* (microsatellite) and *Pit-1 loci* were found, as well Erhardt *et al*. [1998] have described Polish Red cows as a population generally maintaining the HWE, except for the *LGB locus*.

Genetic diversity, heterozygosity and genetic distance between PR and BW cattle

Table 4. Hardy-Weinberg equilibrium test.*

Locus	P-value	
	Polish Red cattle	Polish Black-and-White cattle
CSN3; RFLP-*Hin*dIII	n.s.	0.05
LGB; RFLP-*Hae*III	0.0000	0.01
GH; RFLP-*Alu*I	n.s.	0.0000
GH; RFLP-*Msp*I	n.s.	0.02
IGF1; RFLP-*Sau*3AI	n.s.	n.s.
GHRH; RFLP-*Hae*III	n.s.	n.s.
PIT-1; RFLP-*Hin*fI	0.0001	n.s.
PRL; RFLP-*Rsa*I	n.s.	n.s.
LEP; RFLP-*Sau*3AI	n.s.	0.02
CSN1S2; RFLP-*Mse*II; pos. -1084	n.s.	n.s.
GH-R; RFLP-*Alu*I; pos. -1182	n.s.	n.s.
GH-R; RFLP-*Acc*I; pos. -892	n.s.	n.s.
Whole population (Fisher's test.)	0.000	0.000

*Numbers of animals are given in tables 2, 3, and 4.
n.s. – non-significant; n.a. – not estimated.

Table 5. Heterozygosity and polymorphic information content (PIC) estimates in Polish Red (PR) and Black-and-White cattle

Locus	Number of animals		Heterozygosity (H_e)		PIC[1]		Fisher's value[2] PR/BW
	PR	BW	PR	BW	PR	BW	
CSN3; RFLP-*Hin*dIII	314	636	0.426	0.359	0.335	0.294	1.27*
LGB; RFLP-*Hae*III	312	635	0.401	0.485	0.320	0.367	10.95**
LGB; RFLP-*Sma*I	307	60	0.019	0.0	0.019	0.0	0.0
GH; RFLP-*Msp*I	183	222	0.232	0.244	0.220	0.214	1.22
GH; RFLP-*Alu*I	314	636	0.369	0.425	0.301	0.334	3.07*
GHRH; RFLP-*Hae*III	184	219	0.466	0.368	0.337	0.300	0.40**
PIT-1; RFLP-*Hin*fI	314	419	0.331	0.383	0.276	0.309	1.67**
PRL; RFLP-*Rsa*I	182	219	0.221	0.340	0.196	0.282	1.36*
LEP; RFLP-*Sau*3AI	208	211	0.535	0.353	0.475	0.324	0.41**
Mean	–	–	0.355	0.328	–	–	–

Genetic distance: 0.0146, R = 0.986.
[1] Analysed as such *locus* for linkage analysed with other *loci*.
[2] Heterozygosity variance quotient (*P≤0.05, **P P≤0.01).

Two parametres – heterozygosity (H_o) and Fisher's value (F) were used to assess the genetic variation of populations studied. The values obtained for PR and BW cattle based on nine markers are shown in Table 5. Unlike the PR, the BW cattle is a highly specialized dairy breed in which the intensive selection could decrease the genetic variation. However, the observed mean heterozygosity (H_o) was not found to be determined by the breed and amounted to 0.355 and 0.328 for PR and BW cattle, respectively. The H_o at *locus LGB* (RFLP-*Sma*I) was close to 0 for both breeds as the frequency of LGB^I allele was 0.01 in PR and 0.00 in BW cattle (Tab. 2). Low heterozygosity – from 0.24 to 0.25 – was found for *locus GH* (RFLP-*Msp*I). Only for *locus LEP* the H_o differed significantly between breeds (0.53 in PR and 0.35 in BW cattle). PIC values for nine markers were estimated at assessing the suitability of each *locus* for the linkage analysis (Tab. 5). The estimated genetic distance between PR and BW populations appeared really low (0.0146, R=0.986).

Gene and allele frequencies in two PR subpopulations

Allele frequencies are shown in Table 6. Significant inter-breed differences were found for *loci CSN3*, *LGB* (RFLP-*Hae*II), *GH* (RFLP- *Alu*I), and *IGF-1*, showing that the genetic structures of both subpopulations may be different. The two PR subpopulations have slightly different breeding history, and out of 96 sires contributing to both, only three were common. In addition to the differences between the two Polish PR subpopulations, some specificity was demonstrated of PR cattle kept in Ukraine [Oblap *et al*. 2002]. Differences between Polish (subpopulations pooled) and Ukrainian PR were demonstrated with the frequency of *LGB* and *MSTN loci*.

Lubieniecka *et al*. [2001], basing on estimation of 26 microsatellite *loci* (class II markers, non-coding) compared genetic variation in several European cattle breeds, including PR. They concluded that among nine cattle breeds studied, the PR appeared the most genetically variable; out of 221 alleles found at 26 *loci* five appeared characteristic of PR cattle. Moreover, subpopulation North appeared significantly different in the number and frequency of these "private" alleles from the subpopulation South [Lubieniecka *et al*. 2000].

The results presented here representing class I genetic markers (coding sequences) showed that the genetic distance between PR and BW was low (0.0146, R=0.986), thus proving genetic similarity of the breeds of interest. Nevertheless, highly significant breed-associated relations appeared of the common allele frequencies. Moreover, several unique genetic variants were found in both breeds when analysing polymorphism of other genes, both in coding sequences and in 5'-noncoding regions (promoters). Of the polymorphic gene variants studied some appeared exclusively or preferentially in one breed. These include variant LGB^I found in PR cattle only, variant *C* of the *CSN1S2* gene promoter (position -186) totally absent from BW cattle, and variants *PR* and *BW* of the *PRL* gene promoter, found exclusively in the relevant breeds, *i.e.* PR and BW.

The success of conservation depends on the understanding of the reasons for the

Table 6. Genotype frequencies in the two populations of Polish Red (PR) cattle

Locus	Genotype	Subpopulation North: number of animals	Subpopulation North: allele frequency	Subpopulation South: number of animals	Subpopulation South: allele frequency	Chi-square test
CSN3 RFLP-MnlI	AA	34		111		
	AB	41	A – 0.63	100	A – 0.72	P≤0.05
	BB	12	B – 0.37	13	B – 0.28	
	total:	87		224		
LGB RFLP-HaeIII	AA	24		15		
	AB	52	A – 0.46	65	A – 0.21	P≤0.000
	BB	31	B – 0.54	144	B – 0.79	
	total:	87		224		
LGB RFLP-BmeI	NN	87	N – 1.00	214	N – 0.99	n.s.
	N'	0	I – 0.00	6	I – 0.01	
	total:	87		220		
GH RFLP-AluI	LL	40		70		
	LV	31	L – 0.64	146	L – 0.81	P≤0.000
	VV	16	V – 0.36	8	V – 0.19	
	total:	87		224		
GH RFLP-MspI	AA	57		77		
	AB	28	A – 0.82	16	A – 0.89	n.s.
	BB	2	B – 0.18	3	B – 0.11	
	total:	87		96		
IGF-1 RFLP-BmeB1	AA	23		31		
	AB	38	A – 0.51	39	A – 0.60	P≤ 0.05
	BB	21	B – 0.49	7	B – 0.40	
	total:	82		87		
GHRHR RFLP-HaeIII	AA	10		12		
	AB	40	A – 0.40	42	A – 0.34	n.s.
	BB	28	B – 0.60	43	B – 0.66	
	total:	87		97		
PrP RFLP-AluI	AA	5		20		
	AB	24	A – 0.19	54	A – 0.21	n.s.
	BB	58	B – 0.81	150	B – 0.79	
	total:	87		224		
PRL RFLP-RsaI	AA	68		70		
	AB	17	A – 0.88	25	A – 0.87	n.s.
	BB	2	B – 0.12	0	B – 0.13	
	total:	87		95		
LEP RFLP-Sau3AI	AA	43		87		
	AB	15		16		
	BB	2	A – 0.72	1	A – 0.75	
	AC	24	B – 0.13	31	B – 0.10	n.s.
		3	C – 0.15	6	C – 0.15	
	total:	87		121		

n.s. – non-significant, n.c. – not calculated

breed to be conserved. While using only one type of molecular markers, e.g. type II markers (microsatellites), the uniqueness of a breed may be underestimated. Therefore, the need to consider all relevant characteristics exists in scientific establishing and identifying breeds for conservation.

In conclusion, significant differences were found in the frequency of alleles at *loci CSN3, LGB, GH, GHRH, LEP* and *PRL* between the PR and BW cattle. The differences may reflect the different history of selection for milk yield applied in both populations. The HWE analyses showed that indeed, the BW cattle, intensively selected for high milk yield were not in equilibrium for *LGB*, *GH*, and *LEP* genes. Interestingly, the PR cattle, being a traditional and conserved breed, were not in equilibrium for *LGB* and *Pit-1* that might suggest some natural selection for fitness traits. Moreover, in PR cattle unique variants of several genes were identified, such as LGB^{I}, mutations in caseins, *GH*, and *PRL* gene promoters, and the 11-nt deletion in the *MSTN* gene. These results show that native cattle breeds, such as PR, may be a reservoir of unique alleles that might have been lost during intensive selection of specialized breeds, such as BW cattle. This provides arguments for their protection. On the other hand, however, the demonstrated differences may just be due to random drift and may not reflect any functional distinctiveness. As suggested by Ajmone-Marsan *et al*. [2002] the process of breed formation has only a limited effect on the diversity at marker *loci*.

The differences in allele frequency exist between the two Polish subpopulations of PR, as well as between Polish and Ukrainian PR cattle. Although recently prevented from crossing with other breeds both Polish subpopulations of the PR cattle had slightly different breeding history, while the history of PR in Ukraine is not known. The differences might be advantageous in arranging preservation programmes for the breed, aiming, as suggested by Lubieniecka *et al*. [2000] at reconstituting a common, more uniform population of the Polish Red cattle.

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Genetyczne zróżnicowanie polskiego bydła czerwonego i bydła czarno-białego na podstawie polimorfizmu dwunastu *loci* markerowych, potencjalnie związanych z produkcją mleka i mięsa

S t r e s z c z e n i e

Bydło polskie czerwone (w tekście i tabelach – PR) to rasa rodzima, objęta programem hodowli zachowawczej FAO, charakteryzująca się dużą żywotnością, płodnością, łatwością ocieleń, odpornością na choroby i małymi wymaganiami pokarmowymi. Wydajność mleka krów PR jest niska, ale mleko cechuje wysoki procent tłuszczu i białka. Dokonano genetycznej charakterystyki bydła PR na podstawie polimorfizmu 12 *loci* markerowych, potencjalnie związanych z cechami produkcji mleka i mięsa. Nadto, na podstawie częstości występowania alleli wybranych genów, bydło PR porównano z bydłem cb (w tekście i tabelach – BW). Obok chęci uzyskania precyzyjniejszej niż dotąd informacji o genetycznej strukturze bydła PR autorzy pragnęli dostarczyć nowych argumentów przemawiających za ochroną tej rasy. Przebadano 311 krów i jałówek rasy PR, należących do dwóch subpopulacji – północnej i południowej.

Poza *loci GH-Msp*I i *Pit-1*, frekwencje alleli innych analizowanych genów (*CSN3, LGB, GH-Alu*I, *GHRH, LEP i PRL*) w rasie PR okazały się istotnie różne niż w rasie BW. Ponadto, u bydła PR znaleziono warianty niektórych genów nieobecne lub bardzo rzadkie u bydła BW. Dotyczyło to m.in. wariantu LGB^{I}, 11-nukleotydowej delecji w genie *MSTN* oraz niektórych unikatowych mutacji w rejonach 5' genów *CSN1S1, CSN1S2, GH* i *PRL*. Wykazano także różnice w częstości występowania alleli *loci CSN3, LGB*, *IGF-1 i GH-AluI* między dwiema subpopulacjami bydła PR.