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THE ASSOCIATION OF FOUR POLYMORPHISMS WITHIN THE INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR GENE WITH MILK PRODUCTION TRAITS IN SIMMENTAL COWS*

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Abstract

The aim of this study was to determine the frequency of alleles and genotypes of four polymorphisms located in exons 2, 10 and 16 of the gene encoding insulin-like growth factor type 1 receptor (*IGF1R*) in the tested herd of 242 Simmental cows and to search for the relationship between these polymorphisms and selected milk production traits. The study applied the following methods: PCR-RFLP and combination of nested PCR and ACRS-PCR. The presence of three genotypes was found for all SNPs. The frequency of alleles was as follows: *C* – 0.29 and *T* – 0.71 (*IGF1R/e2/Msp*I), *A* – 0.33 and *G* – 0.67 (*IGF1R/e2/Taq*I), *C* – 0.77 and *T* – 0.23 (*IGF1R/e10/Msp*I) and *C* – 0.53 and *T* – 0.47 (*IGF1R/e16/Rsa*I). In all lactations, cows with *TT* (*IGF1R/e2/Msp*I) and *GG* genotypes (*IGF1R/e2/Taq*I) produced the highest amounts of milk, fat and protein (P≤0.01), particularly individuals with the combined *TT/GG* genotypes. As regards the *IGF1R/e10/Msp*I and *IGF1R/e16/Rsa*I genotypes, the highest milk, fat and protein yields were observed in cows with separate and combined *CC/CC* genotypes, while the lowest in animals with combined *TT/TT* genotypes. Cumulative analysis of all genotype combinations showed that individuals with a potentially best combination of *TT/GG/CC/CC* might be characterized by the highest milk yield as well as fat and protein content in milk. Potentially unfavorable combinations (such as *CC/AA/TT/TT* and similar) have been almost completely eliminated from the herd tested. The *IGF1R* gene is proposed as a candidate gene for milk traits in cattle.

Key words: cattle breeds, *IGF1R*, milk yield, milk composition

Simmental cattle, with the world's population of 42 million heads, is bred on almost all continents and in all climate zones (Choroszy and Choroszy, 2014), which proves its good acclimatization to different climatic conditions. In the Member States of the European Federation of Simmental Cattle Breeders, this breed population is estimated at 9.5 million of dual-purpose individuals, with very good milk and meat production, and 0.6 million animals grown for meat (Choroszy and Choroszy, 2013).

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A precise, broader analysis of DNA markers at the genome level is required that will provide a more complete characterization of the actual genotype of animals and implementation of information obtained in molecular studies to assess the breeding value of animals (Krupiński, 2011). In recent years, studies covering the entire genome – genome-wide association study (GWAS) – have gained importance, as they allow detection of genomic loci associated with specific traits without initial assumption about the possible genes or a complicated segregation analysis (Raven et al., 2014 a). Another popular strategy is to select candidate marker genes based on their involvement in physiological and biochemical processes (Suchocki et al., 2010). Regardless of the strategy, only few polymorphisms were found to be significantly associated with strong quantitative trait loci (QTLs). Two of them are well described (K232A within the diacylglycerol O-acyltransferase 1 gene on BTA14 (*DGAT1*; Gautier et al., 2006) for fat yield and F279Y within the growth hormone receptor (*GHR*) gene on BTA20 (Blott et al., 2003) for milk and protein yields). In addition to polygenic effects, phenotypic variance in milk production can be also explained by the impact of other than these two significant genes (overall, they explained a small portion of variation), e.g., variation in the genes involved in milk fat and protein synthesis, including casein cluster or the somatotropic axis genes. As members of the somatotropic axis, insulin-like growth factor (IGF) and growth hormone (GH) pathways play a significant role in the regulation of proliferation and differentiation of mammary epithelial cells (Plath-Gabler et al., 2001). It has been shown that during lactation in primary bovine mammary epithelial cells, insulin-like growth factor 1 receptor (*IGF1R*) is expressed, and insulin-like growth factor I (IGF-I) can act both in an endocrine and paracrine/autocrine fashion (Baumrucker and Erondu, 2000). Bovine *IGF1R* gene is located on chromosome 21 and consists of 21 exons (http://www.ncbi.nlm.nih.gov/gene/281848). Among them, the most important parts are: exons 2 to 6 that encode the putative ligand binding pocket for IGF-I (aa 2–460) and exons 16 to 20 (aa 956 ÷ 1211) that encode the tyrosine kinase domain (Leroith et al., 2003).

The Cattle QTL Database (http://www.animalgenome.org/cgi-bin/QTLdb/BT/index) contains over 4,000 QTLs associated with milk production traits; of these 906 entries are related to milk production, 1,561 to the performance and composition of the fat fraction and 1,600 to protein yield and composition. Thus far they have not directly overlapped with the location of the *IGF1R* gene – 8.3 cM on BTA21.

The aim of this study was to detect variability within various regions of the *IGF1R* gene and to associate these polymorphic variants with milk performance traits.

Material and methods

Animals and data collection

The study included a total of 242 Simmental (SM) dairy cows maintained in two herds: SM-P=159 (Pomerania province) and SM-D=83 (West Pomerania province). All animals were milked twice a day. Both herds were housed in stanchion barns

and grazed on the pasture during the summer period (from May to October). During the winter, they were allowed to enter the outside run for one hour, where they had access to water and distiller's grains from rye or maize. Feeding was based on the partially mixed ration (PMR) system with cows grouped according to their lactation stage and milk yield. The diet was based on maize and grass silage and hay. Brewer's grains, barley, soybean and rapeseed meal were used as concentrates (fed manually).

Data for 305-day milk production from the first to third lactations including overall milk yield (kg), milk fat yield (kg), milk protein yield (kg) as well as contents of these components (%) were collected from 2007 to 2014 on the basis of monthly milking tests from the official farm records performed using the A4 method (Polish Federation of Cattle Breeders and Dairy Farmers (PFCB&DF)). All the cows analyzed in the study completed 305-day lactations. Because of culling, the number of cows decreased in consecutive lactations.

PCR analysis

Genomic DNA was isolated from blood samples using MasterPure™ kit (Epicentre Biotechnologies) according to the manufacturer's instructions.

Four SNPs within coding sequence were selected for the association study (Table 1). All SNPs have been preliminarily identified and submitted via sequencing in the 48 cattle, including three which were putatively novel and one SNP (e2A, rs134868883) that has been previously published. To date, there is no frequency data and validation status available on these polymorphic loci.

Table 1. Primers and methods used for SNP detection

SNP	PCR oligos (5'-3')	Ta (°C)	PCR fragment (bp)	Method
rs134868883 exon 2 silent	IGF1Re2AF: cctctcctgtgtccctgtgt IGF1Re2AR: gatgaccagggcgtagttgt	60	586	Nested-PCR
	IGF1Re2AmF: ggcagtcggtggtgagaa IGF1Re2AmR: gatgtcgatgc**T**cggcc**G***	60	174	ACRS-PCR
rs207542405 exon 2 silent	IGF1Re2BF: acaactacgccctggtcatc IGF1Re2BR: gaagccctgcattaccaaaa	60	664	Nested-PCR
	IGF1Re2BmF: aagccgctgtgtgagaag**T**c* IGF1Re2BmR: caatagagccacacccacct	60.5	179	ACRS-PCR
rs210778604 exon 10 silent	NEST-F: tggcgtcttgtgtctgtgtt NEST-R: ggcctgggtgcaaagatg	59.5	701	Nested-PCR
	HBe10-F: catcaggaagtacgccg**C***** HBe10-R: cttctcagcttcggttttgg	60	121	ACRS-PCR
rs208140993 exon 16 silent	IGF1Re16-F: gattccagagccagccata IGF1Re16-R: ggctcatggtgatcttctcc	59.5	205	PCR-RFLP

* mishmash is underlined.

The genotypes were assayed with polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) or amplification created restriction site (ACRS). Initially, nested PCR was used to reduce non-specific binding. Polymorphic

sites, primers, methods applied, annealing temperature and the length of PCR fragments are shown in Table 1. Conditions of restriction enzyme digestion for particular polymorphism are summarized in Table 2.

Table 2. Restriction enzymes and temperatures required, alleles detected and their sizes

Polymorphism	Restriction enzyme	Restriction temperature (°C)	Alleles	Size of alleles (bp)
*IGF1R/e2/Msp*I	*Msp*I	at +37°C (3 h)	*C*	156+18
	c/cgg		*T*	174
*IGF1R /e2/Taq*I	*Taq*I	at +65°C (3 h)	*A*	179
	t/cga		*G*	160+19
*IGF1R /e10/Msp*I	*Msp*I	at +37°C (3 h)	*C*	103 + 18
	c/cgg		*T*	121
*IGF1R /e16/Rsa*I	*Rsa*I	at +37°C (3 h)	*C*	157 + 48
	gt/ac		*T*	205

Amplification of genomic DNA was performed in a BIOMETRA™ (Germany) thermal cycler. Restriction fragments were separated electrophoretically in a 2% agarose gel with ethidium bromide.

In order to determine the genetic structure of the tested herd of cows, the frequency of specific alleles, genotypes and Hardy-Weinberg equilibrium (HWE) was evaluated on the basis of the *IGF1R* gene polymorphisms.

Statistical analysis

Association of the traits of interest was analyzed using the general linear model (GLM). The differences between genotypes were tested using Duncan's test implemented in Statistica software (STATISTICA 10.0 PL software package, Statsoft Inc. 2011). The model was fitted separately for each corresponding lactation and was used as follows:

$$Y_{ijklm} = \mu + G_i + S_j + YS_k + H_l + b(x_m - x) + e_{ijklm}$$

where:

Y_{ijklm} – analyzed trait;
μ – overall mean,
G_i – effect of *IGF1R* genotype or combination,
S_j – random effect of a sire,
YS_k – effect of calving year/season,
H_l – fixed effect of the herd,
b – linear regression coefficient for age at first calving,
x_m – calving age of m^{th} cow,
x – mean age at first calving,
e_{ijklm} – random error.

Results

Genotypes, alleles and Hardy-Weinberg equilibrium

For all polymorphisms located in bovine *IGF1R*, the presence of three possible genotypes was detected (Table 3). With respect to mutations located in exon 2, the frequency of the *C* allele (*IGF1R/e2/Msp*I) and *A* allele (*IGF1Re2/Taq*I) was lower than *T* and *G* alleles, respectively. For *IGF1R/e10/Msp*I and *IGF1R/e16/Rsa*I polymorphisms, two alleles were identified: *C* (more frequent at both loci) and *T* (lower frequency).

Table 3. Genotype and allele frequencies in the analyzed population

Polymorphism	Genotype	n	Frequency	Alleles	
*IGF1R/e2/Msp*I	*CC*	22	0.09		
	CT	96	0.40	*C* 0.29	*T* 0.71
	TT	124	0.51		
*IGF1R/e2/Taq*I	*AA*	36	0.15		
	AG	90	0.37	*A* 0.33	*G* 0.67
	GG	116	0.48		
*IGF1R/e10/Msp*I	*CC*	160	0.66		
	CT	53	0.22	*C* 0.77	*T* 0.23
	TT	29	0.12		
*IGF1R/e16/Rsa*I	*CC*	57	0.24		
	CT	141	0.58	*C* 0.53	*T* 0.47
	TT	44	0.18		

The present study found statistically significant differences between the expected distribution of alleles in the analyzed population and their actual distribution in the case of three polymorphisms: e2/*Taq*I ($\chi^2 = 6.58$; P=0.01), e10/*Msp*I ($\chi^2 = 35.0$; P<0.001) and e16/*Rsa*I ($\chi^2 = 6.88$; P=0.01). Comparing the observed and expected genotype frequencies in the e2/*Msp*I polymorphism, it was found that the population was in genetic equilibrium according to the Hardy-Weinberg principle ($\chi^2 = 0.3$; P=0.58).

Association analysis

Table 4 presents the relationships between the four polymorphic sites in the gene encoding *IGF1R* and production traits of Simmental cattle.

Table 4. Frequencies, means and standard errors (in parentheses) for the analyzed milk production traits in Simmental cows with different *IGF1R* genotypes

Polymorphism	Lactation	Genotype	n	Milk yield (kg)	Fat		Protein	
					kg	%	kg	%
***IGF1R/e2/Am/ IGF1R/e2/Msp*I**	I	*CC*	22	5068 A (133.55)	225.64 (7.75)	4.47 AB (0.12)	171.0 AB (4.45)	3.38 (0.03)
		CT	96	5402 (74.53)	233.14 (4.22)	4.36 A (0.05)	179.8 A (2.21)	3.34 a (0.02)
		TT	124	5598 A (71.97)	235.71 (3.82)	4.14 B (0.04)	190.3 B (2.57)	3.42 a (0.03)
	II	*CC*	22	6148 a (166.55)	276.82 (9.83)	4.50 A (0.10)	207.82 A (4.82)	3.39 (0.04)
		CT	96	6448 (78.21)	284.37 A (4.56)	4.42 B (0.06)	215.99 B (2.52)	3.36 A (0.02)
		TT	106	6522 a (85.41)	263.08 A (3.92)	4.05 AB (0.05)	227.63 AB (3.05)	3.49 A (0.02)
	III	*CC*	19	6238 A (157.64)	285.1 (5.56)	4.32 (0.06)	226.6 (4.20)	3.40 (0.05)
		CT	56	6352 B (117.29)	277.8 (7.39)	4.16 (0.06)	225.1 (6.73)	3.36 (0.03)
		TT	57	7038 AB (151.54)	288.1 (11.27)	4.39 (0.08)	224.2 (8.58)	3.41 (0.03)
***IGF1R/e2/Taq*I**	I	*GG*	116	5692 AB (74.28)	245.8 AB (4.15)	4.31 AB (0.05)	192.5 (2.52)	3.40 (0.02)
		AG	90	5359 A (62.30)	225.2 A (3.32)	4.20 A (0.05)	179.9 (2.11)	3.37 (0.02)
		AA	36	5048 B (143.64)	214.4 B (6.90)	4.26 B (0.08)	169.4 (4.61)	3.37 (0.04)
	II	*GG*	107	6661 AB (80.55)	278.4 (3.93)	4.20 (0.05)	227.8 AB (2.86)	3.42 (0.02)
		AG	83	6311 A (84.19)	270.8 (5.24)	4.29 (0.06)	215.3 A (2.67)	3.42 (0.02)
		AA	34	6146 B (136.80)	264.8 (7.33)	4.32 (0.09)	211.5 B (5.28)	3.44 (0.03)
	III	*GG*	69	6907 AB (121.91)	293.8 a (5.43)	4.29 (0.06)	233.9 A (4.23)	3.39 (0.03)
		AG	46	6476 A (151.25)	277.7 (7.52)	4.28 (0.06)	222.6 a (5.97)	3.43 (0.04)
		AA	17	5938 B (184.97)	259.2 a (9.75)	4.38 (0.14)	202.4 Aa (6.79)	3.41 (0.04)

***IGF1R*/e10/*Msp*I**	I	*CC*	160	5544 a (64.05)	237.6 a (3.47)	4.28 (0.04)	187.6 A (2.15)	3.40 (0.02)
		CT	53	5436 (84.61)	230.0 (4.51)	4.22 (0.06)	181.5 (2.83)	3.34 (0.02)
		TT	29	5142 a (139.37)	217.1 a (7.12)	4.22 (0.07)	171.9 A (4.70)	3.35 (0.03)
	II	*CC*	147	6483 (68.99)	273.5 (3.53)	4.24 (0.04)	222.5 (2.39)	3,44 (0.02)
		CT	49	6455 (95.38)	276.3 (6.97)	4.26 (0.08)	218.8 (2.93)	3.40 (0.03)
		TT	28	6293 (192.31)	268.9 (7.81)	4.29 (0.08)	214.4 (7.16)	3.41 (0.04)
	III	*CC*	81	6623 (116.01)	285.1 (5.56)	4.32 (0.06)	226.6 (4.20)	3.42 (0.03)
		CT	32	6692 (179.43)	277.8 (7.39)	4.16 (0.06)	225.1 (6.73)	3.36 (0.03)
		TT	19	6569 (251.14)	288.1 (11.27)	4.39 (0.08)	224.2 (8.60)	3.41 (0.05)
***IGF1R/e16/Rsa*I**	I	*CC*	57	5681 A (115.5)	237.6 a (5.43)	4.17 (0.06)	191.3 A (4.01)	3.39 (0.03)
		CT	141	5518 B (58.4)	238.5 A (3.51)	4.30 (0.04)	185.4 B (1.95)	3.37 (0.02)
		TT	44	5055 AB (11.55)	214.8 Aa (5.56)	4.23 (0.06)	172.2 AB (3.90)	3.42 (0.03)
	II	*CC*	53	6614 a (89.56)	275.4 a (4.86)	4.19 a (0.08)	228.0 (3.77)	3.44 (0.03)
		CT	129	6441 (75.55)	273.7 (4.15)	4.25 (0.05)	218.8 (2.44)	3.41 (0.01)
		TT	42	6287 a (139.36)	270.9 a (6.72)	4.33 a (0.08)	217.2 (4.91)	3.45 (0.03)
	III	*CC*	39	6873 (164.5)	287.9 (7.50)	4.22 (0.09)	234.0 (5.62)	3.42 (0.04)
		CT	68	6542 (124.9)	279.5 (5.80)	4.28 (0.06)	222.5 (4.68)	3.40 (0.03)
		TT	25	6500 (209.9)	289.0 (10.01)	4.45 (0.07)	222.5 (7.59)	4.42 (0.03)

Means within columns marked with the same letters in the same locus and lactation differ significantly at: small letters – P≤0.05; capitals P≤0.01.

Exon 2

In three consecutive 305-day-long lactations, cows with the *TT* genotype (*IGF1R/e2/Msp*I) and *GG* genotype (*IGF1R/e2/Taq*I) produced highest milk volumes compared to peers with *CC* (P≤0.01) and *AA* genotypes, respectively, as well as *AG* heterozygotes (P≤0.01, P≤0.05). For *IGF1R/e2/Msp*I, the difference in fat yield was observed only in the second lactation in cows carrying the *CT* genotype versus cows with the *TT* genotype (P≤0.01). With regard to the protein yield in milk, it was found that individuals with the *TT* genotype in the first and second lactation were characterized by significantly higher values (P≤0.01) of this parameter, while in the third lactation, this genotype did not play an important role.

As regards the *IGF1R/e2/Taq*I locus, it was found that in addition to higher milk production, cows with the *GG* genotype were also characterized by higher yield of fat and protein. Significant differences were observed with respect to the fat (P≤0.01) in the first lactation, and protein yield (P≤0.01) in the second, while for both of these milk components (P≤0.01; P≤0.05) in the third lactation.

Exon 10

For the *IGF1R/e10/Msp*I locus, it was found that individuals with the *CC* genotype showed the highest, and with the *TT* genotype the lowest milk, protein (P≤0.05) and fat (P≤0.01) yields in the first lactation. However, there were no statistically significant differences observed in subsequent lactations.

Exon 16

For polymorphic site located in exon 16 (*IGF1R/Rsa*I), the highest milk and protein yields were found in cows with the *CC* genotype, while the lowest in homozygous *TT* individuals. Significant differences were confirmed only in the first two lactations (P≤0.01 and P≤0.05, respectively) for milk yield and in the first lactation and for protein production (P≤0.01). The fat yield of milk varied depending on lactation, it was highest in the *CT* animals in the first lactation (P≤0.01), *CC* in the second lactation (P≤0.05), while in the third lactation no significant differences were recorded. The percentage of protein in subsequent lactations was similar.

Genotype combinations

The study also analyzed combined *e2-Msp*I/*e2-Taq*I and *e10-Msp*I/*e16-Rs*aI genotypes in relation to milk production (Table 5). However, due to the small size of the groups, not exceeding a value of 10, some combined genotypes were excluded from the statistical analysis.

e2-MspI/e2-TaqI combination

Analyzing combined *e2-Msp*I/*e2-Taq*I genotypes, it was found that cows with the *TT/GG* genotype produced the highest amount of milk protein and fat in the analyzed lactations, which was confirmed statistically for most of the traits (P≤0.05; P≤0.01) (Table 6). The results confirmed the observations obtained for each locus separately (Table 4). Due to the low number of cows excluded from analysis with the

potentially worst performance (consistent with the individual analysis it would be a combination of *CC/AA*), the lowest milk yield in the first two lactations was recorded in cows with the *TT/AA* genotype, and *CT/AG* in the third lactation. The differences were confirmed statistically (P≤0.05, P≤0.01). The yield of fat and protein in milk during the first lactation was lower in *CC/AG* cows, while in the second lactation in *TT/AA* (fat), and *CT/AA* (protein) animals. In the third lactation, in the absence of individuals with a combination of *TT/AA* genotypes, less milk with lower fat and protein content was produced by cows with *CT/AG* genotypes.

Table 5. Frequencies, means and standard errors (in parentheses) for the analyzed milk production traits in Simmental cattle with different genotype combinations at *e2-Msp*I/*Taq*I and e10-*Msp*I /e16-*Rsa*I polymorphisms in the *IGF1R* gene

Lactation	Combined genotypes	Milk yield (kg)	Fat		Protein	
			kg	%	kg	%
1	2	3	4	5	6	7
			***IGF1R/e2/Msp*I /*Taq*I [230]**			
I	*CC/AG*[10]	4776 A (160.68)	208.6 a (8.91)	4.40 (0.20)	163.30 A (5.91)	3.42 (0.05)
	CT/AA[11]	5062 B (266,68)	212.7 b (6.99)	4.26 (0.15)	169.82 B (8.10)	3.37 (0.05)
	CT/AG[28]	5494 a (113.26)	234.7 (6.43)	4.26 (0.07)	180.99 C (3.55)	3.30 a (0.03)
	CT/GG[57]	5423 C (99.50)	240.6 c (6.09)	4.43 Aa (0.07)	181.13 D (2.88)	3.35 (0.03)
	TT/AA[21]	4999 D (204.36)	209.3 Ac (10.79)	4.17 (0.09)	167.67 E (6.72)	3.37 (0.06)
	TT/AG[52]	5398 E (75.39)	223.2 B (4.09)	4.12 A (0.06)	182.54 F (2.73)	3.39 (0.03)
	TT/GG[51]	6048 ABCDEa (101.81)	253.1 ABab (6.02)	4.15 a (0.07)	207.61 ABCDEF (3.57)	3.46 a (0.03)
II	*CC/AG*[10]	6251 (209.67)	271.4 (15.50)	4.32 (0.14)	209.0A (6.40)	3.35 (0.06)
	CT/AA[11]	6243 (253.47)	280.4 (11.55)	4.51 a (0.12)	208.4 B (8.09)	3.34 (0.04)
	CT/AG[28]	6496 (116.89)	292.8 ab (8.30)	4.51 A (0.10)	217.5 C (3.43)	3.36 a (0.03)
	CT/GG[57]	6463 A (108.90)	281.0 (6.12)	4.35 B (0.08)	216.7 D (3.58)	3.36 b (0.03)
	TT/AA[19]	6153 B (194.66)	250.9 a (9.83)	4.08 (0.09)	215.3 E (8.16)	3.49 (0.05)
	TT/AG[45]	6209 C (128.10)	257.1 b (6.78)	4.14 (0.08)	215.3 F (4.22)	3.47 (0.03)
	TT/GG[42]	7024 ABC (102.66)	275.0 (4.63)	3.94 ABa (0.06)	246.4 ABCDEF (3.51)	3.51 ab (0.03)

Table 5 – contd.

1	2	3	4	5	6	7
III	*CT/AG*[11]	5724 Aa (223.88)	247.7 (12.42)	4.34 (0.16)	193.3 AC (7.38)	3.39 (0.08)
	CT/GG[40]	6565 B (135.79)	291.2 (7.75)	4.44 A (0.08)	219.5 B (4.61)	3.35 (0.03)
	TT/AG[27]	6864 a (204.40)	293.6 (10.36)	4.25 (0.08)	240.0 C (8.09)	3.48 (0.05)
	TT/GG[22]	7577 AB (232.74)	295.0 (9.09)	3.93 A (0.11)	264.3 AB (6.54)	3.52 (0.04)
		***e10-Msp*I/*e16-Rsa*I [n = 210]**				
I	*CC/CC*[40]	5868 AB (130.1)	243.70 A (6.76)	4.11 (0.07)	199.7 Aba (4.45)	3.43 (0.03)
	CC/CT[96]	5502 a (75.7)	239.17 B (4.62)	4.34 (0.06)	185.2 a (2.54)	3.38 (0.02)
	CC/TT[24]	5173 A (180.8)	221.17 (7.69)	4.30 (0.08)	177.3 A (5.83)	3.45 (0.05)
	CT/CT[37]	5545 b (94.1)	231.46 (5.49)	4.17 (0.07)	186.1 (3.21)	3.36 (0.03)
	TT/TT[13]	4797 Bab (137.8)	197.31 AB (9.21)	4.09 (0.09)	163.3 B (6.55)	3.34 (0.05)
II	*CC/CC*[36]	6767 (106.9)	277.2 (4.88)	4.13 (0.08)	237.1 a (4.19)	3.50 (0.03)
	CC/CT[87]	6393 (97.54)	271.7 (5.04)	4.26 (0.06)	217.7 a (3.20)	3.41 (0.02)
	CC/TT[24]	6383 (151.80)	274.9 (9.15)	4.32 (0.12)	218.2 (4.97)	3.42 (0.03)
	CT/CT[34]	6505 (112.62)	274.9 (8.29)	4.20 (0.08)	219.8 (3.38)	3.39 (0.03)
	TT/TT[12]	6071 (340.73)	257.0 (12.62)	4.25 (0.09)	212.8 (13.56)	3.49 (0.06)
III	*CC/CC*[25]	7226 a (197.03)	294.5 a (9.35)	4.11 a (0.12)	246.3 a (6.83)	3.42 (0.05)
	CC/CT[45]	6374 a (151.18)	277.7 (7.83)	4.36 (0.09)	218.9 a (5.79)	3.43 (0.04)
	CC/TT[11]	6274 (222.50)	293.7 ab (13.86)	4.67 ac (0.10)	213.0 (7.21)	3.41 (0.05)
	CT/CT[18]	6999 (247.76)	285.4 b (9.12)	4.10 c (0.07)	235.7 (9.19)	3.37 (0.04)

Means in the columns marked with the same letters for the same locus and lactation differ significantly at: small letters – P≤0.05; capitals P≤0.01.

*e10-Msp*I/*e16-Rsa*I *combination*

As regards the combined *e10-Msp*I/*e16-Rsa*I genotypes, the highest yield of milk, fat and protein in the first (P≤0.05, P≤0.01), second (P≤0.05 – protein) and

third lactation (P≤0.05) was demonstrated in cows with a combination of *CC/CC* genotypes, while the lowest in individuals with combined *TT/TT* genotypes, which confirmed and strengthened the observations for individual loci, where the presence of at least one *C* allele and the absence of *TT* genotype, conditioned higher yield of milk, protein and fat. The low number of individuals with the *TT/TT* combination in the third lactation did not allow confirming these observations.

Table 6. Frequencies, means and standard errors (in parentheses) for the analyzed milk production traits in Simmental cattle with different genotype combinations

Lactation	Combined genotypes	Milk yield (kg)	Fat		Protein	
			kg	%	kg	%
I	*CT/AG/CC/CT* [11]	5449 AB (249.96)	234.1 a (13.48)	4.27 a (0.12)	180.2 AB (7.42)	3.32 (0.05)
	CT/GG/CC/CT [22]	5483 Ca (135.59)	256.9 Abc (10.04)	4.67 ABCDab (0.12)	183.9 CD (3.98)	3.36 (0.04)
	TT/AA/CC/CT [10]	5182 DE (312.60)	213.1 ABC (15.57)	4.09 A (0.12)	170.1 EFa (8.64)	3.31 (0.11)
	TT/AG/CC/CT [26]	5426 FG (97.44)	224.0 Dbd (6.16)	4.14 B (0.10)	183.9 GH (3.54)	3.41 (0.04)
	TT/AG/CT/CT [15]	5617 bc (160.10)	225.3 Ece (7.65)	4.02 C (0.09)	189.9 IJa (4.68)	3.39 (0.05)
	TT/GG/CC/CC [23]	6229 ABCDEFb (138.33)	253.3 Bde (8.11)	3.99 D (0.08)	213.3 ACEGI (4.79)	3.47 (0.05)
	TT/GG/CC/CT [16]	6130 BEGac (172.17)	263.6 CDEa (11.88)	4.30 b (0.15)	210.3 BDFHJ (6.44)	3.43 (0.04)
II	*CT/AG/CC/CT* [11]	6499 (181.10)	290.9 ab (11.53)	4.49 Aab (0.16)	218.5 AB (5.72)	3.37 a (0.06)
	CT/GG/CC/CT [22]	6500 (210.35)	284.6 (11.36)	4.39 c (0.12)	213.7 CD (6.32)	3.30 ABbc (0.03)
	TT/AA/CC/CT [9]	6218 ab (241.90)	247.8 a (12.60)	3.98 a (0.11)	215.7 EF (9.62)	3.47 b (0.06)
	TT/AG/CC/CT [22]	6143 cd (214.77)	252.2 b (10.50)	4.11 (0.11)	213.1 GH (7.37)	3.48 c (0.04)
	TT/AG/CT/CT [13]	6400 (224.29)	262.0 (13.72)	4.08 (0.15)	218.7 IJ (6.58)	3.43 (0.05)
	TT/GG/CC/CC [19]	7004 ac (140.19)	270.7 (6.15)	3.90 Ac (0.09)	248.8 ACEGI (4.92)	3.56 Aa (0.04)
	TT/GG/CC/CT [12]	7049 bd (161.16)	279.4 (7.20)	3.99 b (0.15)	246.8 BDFHJ (3.87)	3.51 B (0.06)
III	*CT/GG/CC/CT* [16]	6269 (232.79)	275.8 (13.75)	4.39 (0.13)	210.4 (8.30)	3.36 (0.06)
	TT/AG/CC/CT [13]	6625 (345.51)	283.5 (17.80)	4.22 (0.13)	237.1 (13.44)	3.55 (0.09)
	TT/GG/CC/CC [14]	7598 (275.57)	290.6 (11.68)	3.86 (0.12)	265.1 (7.84)	3.52 (0.06)

Means in the columns marked with the same letters for the same locus and lactation differ significantly at: small letters – P≤0.05; capitals P≤0.01.

Simultaneous combination of all loci

Combinations of four polymorphic loci were constructed in order to check whether there is a possible additive effect of all the analyzed polymorphisms in *bIGF1R* with milk performance traits of Simmental cows (Table 6). Analysis of this combination allowed formulating some observations that may in the future help in the genetic marker-assisted selection of individuals with favorable milk yield and protein and fat content in milk. Cows with the *TT/GG/CC/CC* combination in the first and third 305-day lactation were characterized by the highest milk yield, and protein and fat content. In most cases the differences between the analyzed traits were significant ($P \leq 0.01$; $P \leq 0.05$). In the second lactation, cows with the *TT/GG/CC/CT* combination obtained the highest values of the analyzed milk traits, but only slightly higher than individuals with a combination of *TT/GG/CC/CC* genotypes.

In the absence of the potentially worst combination of *CC*/*AA*/*TT*/*TT* and related, the lowest milk yield as well as fat and protein yields and contents in the first lactation were recorded for a combination of *TT/AA/CC/CT* ($P \leq 0.01$; $P \leq 0.05$), individuals carrying *TT/AG/CC/CT* in the second lactation ($P \leq 0.01$; $P \leq 0.05$), while *CT/GG/CC/CT* in the third.

Discussion

Investigations on the basis of functional candidate genes or contiguous genes located close to previously identified QTLs – the so-called positional candidate genes – allow discovering and localizing genes of large effect for quantitative traits. The products of these genes may indirectly influence such traits as the yield of milk, protein and fat.

Several GWAS studies have repeatedly reported that BTA14 (*DGAT1*), BTA19 (*GH*), BTA20 (*GHR*) and BTA26 (stearoyl-CoA desaturase, *SCD*) genes harbor a large number of genetic variants associated with milk production traits (especially fat yield and composition) in dairy cattle (Buitenhuis et al., 2014; Fang et al., 2014; Fontanesi et al., 2014; Jiang et al., 2014; Li et al., 2014; Xu et al., 2014). Among them, GH/GHR are the main elements of the somatotropic axis and well-known missense mutations are incorporated into the DNA Chip. The majority of GWAS studies have not identified *IGF1R* as a strong QTL. Only Raven et al. (2014 b) have qualified *IGF1R* as one of the sixty-four genes associated with higher milk, fat and protein yields, as well as fat and protein percentages (in total, they explained up to 9% of genetic variation). Li et al. (2014) showed that 4 SNPs closely linked with *IGF1R* were associated with milk medium-chain saturated fatty acids. This suggests that *IGF1R* is not a major QTL for milk traits and may not be detectable at a genome-wide level. Most of the markers is more likely in linkage disequilibrium (LD) with causal variants, and therefore, those SNP may only explain a small portion of genetic variance.

As breeds can share ancestral mutations, applying a multibreed strategy in dairy cattle may be useful in order to improve the localization of significant QTLs (Raven et al., 2014 a). The mutations that cause genetic variation in quantitative traits could

have ancestral origin and segregate across many breeds or they could be recent and segregate only within one breed. The older QTLs tend to have high derived allele frequencies and often segregate across several breeds (Kemper et al., 2015). All SNPs under study seem to be ancestral within dairy breeds. Moreover, in a preliminary study, we also found them in several beef cattle breeds as Angus, Hereford or Limousin (data not shown). For this reason, the four analyzed SNPs may be incorporated into the BTA21 marker set of a custom SNP chip. In the next step, targeted sequencing of GWAS specific regions using next generation sequencing (NGS) technologies can efficiently capture all the variants in these regions and their potential effects can be assessed in a subsequent association study (Jiang et al., 2014).

Each polymorphism examined in this is a synonymous mutation changing only the DNA sequence without directly altering the sequence of the encoded protein. Both SNPs within exon 2 (e2-*Msp*I/*Taq*I) are closely associated with a putative ligand binding pocket for IGF-I (aa C3 and T165, respectively). It is impossible to refer the obtained genotype and allele frequencies to literature data, because thus far research on this topic has not been carried out in the breed analyzed here or other breeds of cows. The third SNP (e10-*Msp*I; aa D654) is located in the region encoding the C-terminus of the α subunit (more precisely, extracellular fibronectin type III domain; aa 799 ÷ 897), whereas e16-*Rsa*I (aa Y957) precedes the region encoding the most important part of the receptor: the tyrosine kinase domain. Their significance should be attributed rather to the mRNA molecule, which may be to various extent processed, edited and transported prior to translation and degradation. Many of these diverse biological processes are strongly dependent on the mRNA secondary structure, which is essentially determined by SNPs and other forms of variation (Bali and Bebok, 2015). Lack of Hardy-Weinberg equilibrium of the majority of analyzed SNPs in the study population could be caused by long-term selection towards production traits. The occurrence of all described alleles of *IGF1R/e10/Msp*I and *IGF1R/e16/Rsa*I polymorphisms was confirmed by Szewczuk (2015) in Polish Holstein-Friesian, Holstein-Friesian, Montbeliarde and Jersey breeds.

There are no data in the literature regarding the relationship between the performance and milk composition of Simmental breed cows and the analyzed SNPs, therefore, we cannot compare the results obtained in the present study with works of other authors. As regards the present results, there is potentially one identical pair of haplotypes (it can be described as *TGCC/TGCC* diplotype = *TT/GG/CC/CC* combination) that are beneficial for milk production. In contrast, it was observed that cows with the least frequent genotypes were characterized by the lowest milk, protein and fat yields, which allowed predicting the least favorable combination: *CC*/*AA*/*TT*/*TT* corresponding to the *CATT/CATT* diplotype. Among the tested animals (242 individuals), this combination has not occurred, and other related combinations, if present at all, were extremely rare (n = 1 ÷ 2). The reason for the low frequency of this allele may be its parallel association with low milk yield in cows. It seems plausible that they were gradually eliminated during the long-term selection in the herd towards high milk yields. The low incidence of such combinations in the population of dairy cows limits the statistical power necessary to confirm the connection between variation and the traits analyzed. At first, it might appear that this can also be due to the

fact that the detected effect is only lactation-dependent. For example, the lack of the *CC*/*AA* combination (*e2-Msp*I/*Taq*I) caused that only the average and favorable combinations were compared (Table 5, part I). Similarly, a low number of *TT*/*TT* individuals (e10-*Msp*I/e16-*Rsa*I) in the second lactation and no such individuals in the third lactation significantly limited the observations (Table 5, part II). The first lactation in cows is affected by the growth performance of the mammary gland (among others, locally produced IGF-I is involved) and nutrient distribution mechanism; then other sets of genes play the main role during galactopoiesis (including endocrine IGF-I and GH) and remodeling stage – involution (once again autocrine/paracrine role of IGF-I) (Plath-Gabler et al., 2001). In consequence, QTLs associated with milk yield vary depending on lactation even in GWAS studies (Cho et al., 2015). Therefore, only significant SNPs, common to all lactations, can be considered as "pure" SNPs associated with milk production that are not affected by external nutritional and environmental factors. In the current study, polymorphisms within exon 2, associated with the putative ligand binding pocket for IGF-I, seem to be such markers.

The variability between animals with regard to the genetic basis of a trait results from the different arrangement of alleles in the genome of these animals. In addition to the present results, the study performed by Szewczuk (2015) showed that the *TT*/*TT* combination (e10-*Msp*I/e16-*Rsa*I) may positively ($P \leq 0.01$) affect the yield of milk and its components in four dairy breeds (other than Simmental). Intronic polymorphism (*IGF1R*/i16/*Taq*I), located close to the *IGF1R/e16/Rsa*I polymorphism, was associated with a higher milk yield ($P \leq 0.05$), but no significant effect on fat and protein yield was found (Szewczuk et al., 2012). Szewczuk et al. (2011) described two polymorphic variants located in intron 4 in Polish Holstein-Friesian cows using *Hinf*I and *Mph*1103I restriction enzymes. Statistical analysis showed that the *IGF1R*/*Hinf*I polymorphism significantly affected milk yield, milk protein yield ($P \leq 0.01$) and milk fat yield ($P \leq 0.05$), and according to these authors, the *BB* genotype was the most beneficial one. No significant effect of the *IGF1R*/*Mph*1103I polymorphic site on the yield of milk, fat and protein was found.

The possible use of information on the polymorphisms obtained in this study for improving traits associated with cattle milk production should be supported by further research in other more numerous herds, with more uniform distribution of individual genotypes.

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