An analysis of *CYP19*, *CYP21* and *ER* genotypes in Polish Holstein-Friesian cows with regard to the selected reproductive traits

Iwona Szatkowska[1], Wilhelm Grzesiak[2], Magdalena Jędrzejczak[1], Andrzej Dybus[1], Daniel Zaborski[2], Dorota Jankowiak[3]

[1]Laboratory of Molecular Cytogenetics, [2]Laboratory of Biostatistics, Department of Ruminants Science, [3]Laboratory of Physiological Chemistry, West Pomeranian University of Technology in Szczecin, Szczecin, Poland

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Abstract

The aim of this study was to relate polymorphic variants of *CYP19*, *CYP21* and *ER1* genes to reproductive traits in 472 Polish Holstein-Friesian cows. High frequencies of one of the homozygous genotypes were found. The *ER1*/*Sna*BI^{AA} homozygotes were not identified. In the first and third lactation, an average calving-to-conception interval (CLVC) in cows of *ER1*/*Sna*BI^{GG} genotype was significantly shorter ($P \leq 0.05$) than in heterozygous cows. In the cows of *ER1*/*Bgl*I^{GG} genotype, significantly shorter CLVC ($P \leq 0.05$) was observed compared to heterozygotes in the first lactation, whereas in the third lactation, CLVC in homozygous cows was significantly longer ($P \leq 0.05$) than in heterozygous ones. It was also found that homozygous cows were characterized by significantly longer calving interval (CLVI; $P \leq 0.05$) compared to heterozygotes in the third lactation. Longer CLVCs in *CYP19*^{AA} cows were found, compared to heterozygotes, and this difference was significant in the first and third lactation ($P \leq 0.05$). Similarly, the average CLVIs were longer in *CYP19*^{AA} homozygotes than in heterozygous cows; however, significance was proven only in the third lactation ($P \leq 0.05$). Description of the molecular mechanisms regulating reproduction, and thus identification of the individuals of genotypes with optimal potential may facilitate the employment of selected reproductive model by a breeder.

Gene polymorphism, calving interval, calving-to-conception interval, pregnancy length, inseminations

Abbreviations: *CYP19* - cytochrome P450 aromatase gene, *CYP21* - steroid 21-hydroxylase gene, *ER* – oestrogen receptor gene, CLVC – calving-to-conception interval, CLVI – calving interval, INSEM – number of services per successful conception, PREG – pregnancy length.

Genetic improvement of the reproductive traits in dairy cattle is crucial not only for ensuring quantitative aspect of production but mainly for the efficient course of lactogenesis and lactopoesis. It is an extremely difficult process, regulated at many stages in a multifactorial manner. Moreover, coefficients characterizing reproductive traits are of low heritability and there is a small number of described genetic markers associated with reproduction that could be used as potential factors supporting the classical selection methods. Therefore, searching for such markers is admittedly important both for the application and research purposes. So, the analysis of the influence of different polymorphic variants of enzymes crucial for oestrogen synthesis on the reproductive traits or of protein factors mediating their actions seems fully justified.

The reproductive cycle of all mammalian females is regulated mainly by oestrogen hormones. They are synthesized through the aromatization of androgens, which, in females, are mainly of ovarian origin. In this complex process, a key role is played by the p450 aromatase, which is an enzymatic complex comprising two proteins – nonspecific microsomal flavoprotein reductase and specific haemoglycoprotein, that is, cytochrome P450 aromatase, coded for by the *CYP19* gene (Amarneh and Simpson 1996; Lewis and Lee-Robichaud 1998). With the former, steroid 21-hydroxylase

Address for correspondence:
Dr hab. Iwona Szatkowska,
prof. nadzw. Department of Ruminants Science
Faculty of Biotechnology and Animal Science
West Pomeranian University of Technology in Szczecin
ul. Doktora Judyma 10, 71-460 Szczecin, Poland,

Phone: +48914496805
Fax: +48914496800
E-mail: iwona.szatkowska@zut.edu.pl
http://www.vfu.cz/acta-vet/actavet.htm

(P450c21) is associated, which is an enzyme essential for the occurrence of both gluco- and mineralo-corticoid activity. The decreased P450c21 activity results in an insufficient synthesis of cortisol and aldosterone as well as excessive secretion of androgens (Chung et al. 1986). Steroid 21-hydroxylase is coded for by the *CYP21* gene (Buske et al. 2006).

The bovine *CYP19* gene, mapped to the long arm of chromosome 10 at position q2.6, utilizes six different tissue-specific promoters (Vanselow et al. 2001) of which P1.1 and P1.5 were analyzed for their transcriptional activity associated with different methylation patterns by Vanselow et al. (2008). In placenta, which is a main site of cytochrome P450 aromatase expression, *CYP19* gene expression is under the control of distal P1.1 promoter (Kalbe et al. 2000), in which only few CpG dinucleotides were identified (Vanselow et al. 2008). The transition A→G at position -1044 (GenBank no. Z69241) has been described in the above-mentioned region and is recognized by the *Pvu*II restrictase (Vanselow et al. 1999). On the other hand, gene coding for steroid 21-hydroxylase, that is *CYP21* as a candidate for the QTL for reproductive traits is highly polymorphic in mammals (Barg et al. 2003). The bovine *CYP21* gene, comprising 10 exons, was mapped to chromosome 23. Its level of polymorphism is closely associated with breed, and the region in which individual substitutions were identified. In the promoter region, Bov-A2 SINEs (short interspersed nucleotide element) sequences were localized, which consisted of 115 bp-long segments specific for the *Bovidae* family genomes (Damiani et al. 2000b). These specific retroelements may be very important, therefore, in the present study, polymorphic site (GenBank no. M11267) in the promoter region, recognized by the *Hpa*II exonuclease (Damiani et al. 2000a) was chosen for analysis.

Active forms of oestrogens constitute an intracellular lipophilic ligand for the nuclear ER receptors, which functionally are protein regulators of gene expression (McKenna et al. 1999), and there are two their forms: ER1 and ER2 coded for by *ER1* and *ER2* genes, respectively. Their activity, after binding oestrogens, is associated with the formation of homodimers, capable of association with the ERE response elements, located in the promoters of target genes. The bovine *ER1* gene was mapped to chromosome 6. It consists of 8 exons, and the 5' region contains additional non-coding exon, containing information about 5'UTR regions of different length. In the present work, the transition of A to G in 5' region (GenBank no. AY340597) upstream the exon C (Szreder and Zwierzchowski 2004; Szreder et al. 2008), and recognized by the restriction enzyme *Bgl*I, was analyzed. The second polymorphic site in the gene under discussion, i.e. transition A→G (GenBank no. AY332655), is presumably also located in the promoter region at position -1213 and recognized by the *Sna*BI restrictase.

The aim of this study was to associate the polymorphic variants of *CYP19*, *CYP21* and *ER1* genes with the reproductive traits in Polish Holstein-Friesian cattle.

Materials and Methods

The study comprised a total of 472 Polish Holstein-Friesian cows kept on one of the farms located in the West Pomeranian Province of Poland. The cows were kept in a confinement system and fed a total mixed ration. Four genotypes: *CYP19*/*Pvu*II, *ER1*/*Bgl*I, *ER1*/*Sna*BI (Vanselow et al. 1999; Szreder and Zwierzchowski 2004; Szreder et al. 2007) and *CYP21*/*Hpa*II were analyzed using the PCR-RFLP method. Genomic DNA was isolated from blood by means of Master Pure™ Kit (Epicentre Biotechnologies, USA). The analyzed fragments of the 3 genes were amplified using the primers described in Table 1. Primer sequences for *CYP21*/*Hpa*II were designed using Primer3 software. The PCR mixture contained approximately 90-100 ng of genomic DNA, 0.5 μM of each primer, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.5 units of Taq polymerase (MBI Fermentas) and deionized water up to 15 μl. The following numbers of cycles for PCR reactions were applied: 35, 30, 28 and 35 for *CYP19*, *CYP21*, *ER1*/*Bgl*I and *ER1*/*Sna*BI, respectively.

Table 1. Primers and PCR conditions used for genotyping of the bovine *CYP19, CYP21* and *ER1* genes

Genes - enzymes	Primer sequences	Tm (°C)	Length of amplified fragment (bp)	References
*CYP19-Pvu*II	forward 5'-CTCTCGATGAGACAGGCTCC-3' reverse 5'-ACAATGCTGGGTTCTGGACT-3'	59	405	Vanselow et al. 1999
*CYP21-Hpa*II	forward 5'-TGTAAGATGAGTGCCGGAGA-3' reverse 5'-TCTGTGCGACCCCATAGAT-3'	60	252	*
*ER1-Bgl*I	forward 5'-TTTGGTTAACGAGGTGGAG-3' reverse 5'-TGTGACACAGGTGGTTTTC-3'	53	242	Szreder and Zwierzchowski 2004
*ER1-Sna*BI	forward 5'-GTCAGGTATTCCGTCAGGT-3' reverse 5'-GCCTTTCTGTTCCTTTGG-3'	54	340	Szreder et al. 2007

* the primer sequences for *CYP21/Hpa*II were designed using Primer3 software
(GenBank - access number: M11267, AF163098, AF163767)

Reproductive traits in the first, second and third lactation were analyzed based on the following general model:

$Y_{ijkl} = \mu + G_i + s_j + YS_k + \alpha(h_i - h_m) + \beta(w_i - w_m) + e_{ijkl}$, where:

Y_{ijkl} – examined trait, μ – overall mean, G_i –effect of genotype, s_j – random effect of sire, YS_k – effect of calving year-season, α – regression coefficient for the proportion of HF genes in cow genotype, h_i – proportion of HF genes in the genotype of cow *i*, h_m – average proportion of HF genes in the studied population, ß –regression coefficient for cow age, w_i – age of cow *i*, w_m – average age of cows in the studied population, e_{ijkl} – random error.

The mean values of the examined traits were compared using Tukey's test for unequal sample sizes.

Results

Frequencies of individual alleles and genotypes in the analyzed herd of Polish Holstein-Friesian cows in the individual lactations are presented in Table 2. From the data presented in this table, it appears that the highest frequencies were observed for the *ER1*/*Sna*BI^{GG} and *ER1*/*Bgl*I^{AA} genotypes, lower for the *ER1*/*Sna*BI^{AG} and *ER1*/*Bgl*I^{AG} heterozygotes, and lowest for the homozygous *ER1*/*Bgl*I^{GG} genotype. The *ER1*/*Sna*BI^{AA} homozygotes were not identified in the examined herd. The above-mentioned values did not differ much during the consecutive production seasons, remaining on almost the same level.

Table 2. Genetic structure of the analyzed herd of Polish Holstein-Friesian dairy cows

Lactation	Genotype frequency			Allele frequency	
	*ER1/Sna*BI^{GG}	*ER1/Sna*BI^{AG}	*ER1/Sna*BI^{AA}	*ER1/Sna*BI‘	*ER1/Sna*BI^{A}
1	0.9195	0.0805	-	0.9597	0.0403
2	0.9298	0.0702	-	0.9649	0.0351
3	0.9301	0.0699	-	0.9650	0.0350
Total	0.9244	0.0756	-	0.9622	0.0378
	*ER1/Bgl*I^{AA}	*ER1/Bgl*I^{AG}	*ER1/Bgl*I^{GG}	*ER1/Bgl*I^{A}	*ER1/Bgl*I^{G}
1	0.9174	0.0805	0.0021	0.9576	0.0424
2	0.9193	0.0772	0.0035	0.9579	0.0421
3	0.9231	0.0699	0.0070	0.9580	0.0420
Total	0.9189	0.0778	0.0033	0.9578	0.0422
	*CYP19/Pvu*II^{AA}	*CYP19/Pvu*II^{AB}	*CYP19/Pvu*II^{BB}	*CYP19/Pvu*II^{A}	*CYP19/Pvu*II^{B}
1	0.8496	0.1462	0.0042	0.9227	0.0773
2	0.8456	0.1509	0.0035	0.9211	0.0789
3	0.8462	0.1399	0.0140	0.9161	0.0839
Total	0.8478	0.1467	0.0056	0.9211	0.0789
	*CYP21/Hpa*II^{AA}	*CYP21/Hpa*II^{AB}	*CYP21/Hpa*II^{BB}	*CYP21/Hpa*II^{A}	*CYP21/Hpa*II^{B}
	1.00	0.00	0.00	1.00	0.00

The individuals of *CYP19*/*Pvu*II^{AA} genotype were identified with a frequency of 0.8478, and just as for the aforementioned genotypes, these frequencies remained at a relatively stable level during the consecutive lactations. In the case of the analyzed polymorphic site of *CYP21* gene, recognized by the *Hpa*II restrictase, monomorphism was found in the examined 320 individuals, and for that reason no further analyses were performed. The described genotype frequencies, both with original form and substitution, were reflected in the allele frequencies – high for *ER1*/*Sna*BI^{G}, *ER1*/*Bgl*I^{A} and *CYP19*/*Pvu*II^{A} and low for *ER1*/*Sna*BI^{A}, *ER1*/*Bgl*I^{G} and *CYP19*/*Pvu*II^{B}.

Average values of the analyzed indicators characterizing reproduction in connection with a specific genotype in the analyzed herd are presented in Tables 3, 4 and 5.

Analysis of the findings indicates a difference in the length of calving-to-conception interval depending on the analyzed lactation and genotype of the cow. Cows of the homozygous *CYP19*/*Pvu*II^{BB} genotype in the first and second lactation were characterized by the shortest calving-to-conception interval; however, the small size of the group of the individuals of this genotype should be noted. Significant differences ($P \leq 0.05$) were found in the values of the discussed indicator between individuals of the homozygous *CYP19*/*Pvu*II^{AA} genotype and the *CYP19*/*Pvu*II^{AB} heterozygotes in the first, second and third lactation.

Table 3. Average values of the analyzed reproduction indicators in cows of different *ER1*-*Sna*BI genotype variants

	ER1/*Sna*BI^{GG}			*ER1*/*Sna*BI^{AG}		
	n	Mean	SD	n	Mean	SD
			1st Lactation			
CLVC	434	119.33[a]	36.64	38	121.68[a]	37.38
CLVI	434	414.98	53.72	38	413.97	51.87
PREG	434	279.47	5.32	38	278.21	4.30
INSEM	434	2.59	1.89	38	2.89	2.98
			2nd Lactation			
CLVC	265	121.08	35.79	20	118.15	40.99
CLVI	265	422.32	55.27	20	424.59	66.37
PREG	265	279.40	5.21	20	279.86	4.73
INSEM	265	2.78	1.82	20	2.27	1.61
			3rd Lactation			
CLVC	133	116.57[a]	36.85	10	131.50[a]	34.91
CLVI	133	413.31	52.26	10	429.81	63.45
PREG	133	277.34	5.18	10	279.09	5.55
INSEM	133	2.37	1.48	10	2.81	1.60

[a]Means with the same superscripts within rows differ significantly ($P \leq 0.05$) CLVC – calving-to-conception interval, CLVI – calving interval, PREG – pregnancy length, INSEM – number of services per successful conception

The length of calving-to-conception interval was the most favourable in the group of individuals of *CYP19*/*Pvu*II^{AB} genotype, which may indicate an advantage of *CYP19*/*Pvu*II^{B} allele over *CYP19*/*Pvu*II^{A} in determining the phenotype of reproductive traits, even in a heterozygous configuration. The opposite relationships were found in the group of cows of the *ER1*/*Bgl*I^{AA} and *ER1*/*Sna*BI^{GG} genotypes. In this case, a wild allele without mutation – *ER1*/*Bgl*I^{A} and *ER1*/*Sna*BI^{G} in a heterozygous form, influenced the length of calving-to-conception interval more favourably; differences in the values of this indicator were small (Table 3 and 5) but significant at $P \leq 0.05$.

Calving interval turned out to be significantly longer ($P \leq 0.05$) in the third lactation in cows of the *CYP19*/*Pvu*II^{AA} genotype compared to the *CYP19*/*Pvu*II^{AB} individuals, and in the third lactation in the group of homozygous *ER1*/*Bgl*I^{GG} individuals compared to heterozygous *ER1*/*Bgl*I^{AG} cows. However, there were only a few homozygous cows carrying alleles with substitution and they were not included in the statistical analysis.

The numbers of services per successful conception and pregnancy length differed little but non-significantly. Moreover, when analyzing the level of the above-mentioned

Table 4. Average values of the analyzed reproduction indicators in cows of different *CYP19/Pvu*II genotype variants

	*CYP19/Pvu*II^{AA}			*CYP19/Pvu*II^{AB}			*CYP19/Pvu*II^{BB}		
	n	Mean	SD	n	Mean	SD	n*	Mean	SD
				1st Lactation					
CLVC	401	121.72[a]	37.54	69	106.60[a]	27.79	2	95.50	22.02
CLVI	401	417.04	54.41	69	397.87	38.98	2	372.00	17.07
PREG	401	279.37	5.37	69	279.77	4.28	2	276.50	4.95
INSEM	401	2.67	5.05	69	2.16	6.05	2	1.00	5.31
				2nd Lactation					
CLVC	241	122.24	37.67	43	112.14	26.36	1	97.00	-
CLVI	241	425.68	58.65	43	407.05	40.68	1	376.00	-
PREG	241	279.35	5.05	43	279.00	6.05	1	287.00	-
INSEM	241	2.71	2.10	43	2.85	2.06	1	3.50	-
				3rd Lactation					
CLVC	121	120.40[a]	37.29	20	101.10[a]	32.76	2	143.50	23.44
CLVI	121	419.45[a]	53.60	20	383.44[a]	36.85	2	426.00	78.5
PREG	121	277.32	22.10	20	279.38	8.06	2	282.00	32.07
INSEM	121	2.42	1.51	20	2.38	1.75	2	2.00	3.53

[a]Means with the same superscripts within rows differ significantly ($P \leq 0.05$) CLVC – calving-to-conception interval, CLVI – calving interval, PREG – pregnancy length, INSEM – number of services per successful conception

*These animals were not included in the statistical analysis

Table 5. Average values of the analyzed reproduction indicators in cows of different *ER1-Bgl*I genotype variants

	*ER1/Bgl*I^{GG}			*ER1/Bgl*I^{AG}			*ER1/Bgl*I^{AA}		
	n	Mean	SD	n	Mean	SD	n*	Value	SD
				1st Lactation					
CLVC	433	117.90[a]	36.10	38	136.50[a]	38.89	1	141.00	-
CLVI	433	413.34	50.45	38	427.60	59.71	1	422.00	-
PREG	433	279.51	4.12	38	278.08	4.59	1	281.00	-
INSEM	433	2.55	1.86	38	3.22	3.04	1	2.00	-
				2nd Lactation					
CLVC	262	119.33	35.34	22	135.91	44.87	1	126.00	-
CLVI	262	421.98	56.40	22	432.65	61.36	1	405.00	-
PREG	262	279.39	5.27	22	278.96	5.13	1	279.00	-
INSEM	262	2.74	1.84	22	2.69	1.59	1	3.00	-
				3rd Lactation					
CLVC	132	116.97[a]	36.74	10	103.10[a]	32.42	1	92.00	-
CLVI	132	412.61[a]	49.84	10	396.86[a]	62.12	1	365.00	-
PREG	132	277.82	2.91	10	27.83	6.07	1	273.00	-
INSEM	132	2.33	1.41	10	0.75	1.87	1	1.00	-

[a]Means with the same superscripts within rows differ significantly ($P \leq 0.05$) CLVC – calving-to-conception interval, CLVI – calving interval, PREG – pregnancy length, INSEM – number of services per successful conception

*These animals were not included in the statistical analysis

indicators, it was impossible to find any trend, indicating potential advantageous effect of one of the genotypes; however, the insemination index was most favourable in the group of individuals of the heterozygous *CYP19*/*Pvu*II^{AB} genotype.

Discussion

Supervising reproduction and factors affecting its quality in an effective manner is one of the most difficult aspects of breeding work. In herds of dairy cows intensively exploited for their milk potential, a number of negative phenomena occur. Among them, metabolic diseases such as ketosis, rumen acidosis, parturient paresis, downer cow syndrome or displaced abomasum and the infectious diseases of reproductive system seem to be the most important, constituting more than a half of all the cases of cattle infertility. Additional problems negatively influencing cattle reproduction are inappropriate management and care, supervision and stress. Another important factor affecting reproductive process is the time of service, which is not always optimal. However, its effect may be difficult to assess, since it depends on subjective organizational factors. Identification of genetic markers that can potentially support reproduction control and aid the extension of the cow's productive life seems essential mainly for the breeding practice. Among the potential genetic markers analyzed in this experiment, *ER1*/*Sna*BI^{G}, *ER1*/*Bgl*I^{G} and *CYP19*/*Pvu*II^{B} alleles can play a role in the determination of the length of calving-to-conception interval, and, in some lactations, also the length of calving interval. Taking into account various functions and influences of oestrogens on the regulation of reproductive processes, development of the mammary gland, or the growth and differentiation of cells which is possible through the specific oestrogen receptors, it is not surprising that genes involved in their synthesis and coding for receptors are regarded as candidates for the markers of reproductive traits. It was found that substitution in the regulatory region of the bovine *ER1* gene (recognized by the *Sna*BI restriction enzyme) had effect on the expression level of this gene in the liver of young bull calves (Szreder et al. 2007; Szreder et al. 2008). However, these authors did not observe any association between the second analyzed polymorphism *ER1/Bgl*I and the expression level of the examined gene.

In the present study, the difference in the length of calving-to-conception interval favourable for homozygous *ER1*/*Bgl*I^{GG} cows was observed. The role of this allele in the determination of the other trait, such as calving interval, was proven by our experiments and those by Szreder et al. (2008). The potential role of substitution in the regulatory region of the bovine *CYP19* gene recognized by the *Pvu*II restrictase (Vanselow et al. 1999), which can significantly differentiate the transcription level of this enzyme, crucial for oestrogen synthesis, should also be emphasized (Lobo et al. 2009). It was noticeable especially in the first and third lactation in the heterozygous *CYP19*/*Pvu*II^{AB} individuals, in which the most favourable values of the length of calving-to-conception interval and calving interval were recorded.

In conclusion, the way in which the findings of the present study may be used in breeding practice must be considered. Dairy cattle breeders do not agree on the optimum length of calving-to-conception and calving intervals. On the one hand, their spontaneous lengthening in high-yielding cows can be observed. Also some breeders tend towards longer intervals. This is due to the improvement of fertility and health as well as increased milk yield of cows. On the other hand, the need for the shortening of the indices under discussion is suggested. The reason for this is the greater economic effectiveness resulting from reduced feeding and management costs as well as increased number of calves obtained from cows. The results of the present study may be useful for the supporters of both longer and shorter calving-to-conception and calving intervals. Breeders can select the preferred reproductive model due to the indication of the molecular mechanism regulating reproduction and the identification of animals with optimal genotypes.

Another important aspect of utilization of the defined genetic markers in breeding practice is their frequency. In our study, a very low frequency of advantageous alleles was observed. It is in agreement with the results of other studies analyzing frequency of

ER1/*Sna*BI, *ER1*/*Bgl*I and *CYP19*/*Pvu*II alleles and genotypes (Szreder and Zwierzchowski 2004; Jędrzejczak et al. 2006; Kowalewska–Łuczak 2009). Higher frequencies of polymorphic *ER1*/*Bgl*I alleles were found in Charolaise and Polish- Red cattle (*ER1*/*Bgl*I^{GG} – 0.68; *ER1*/*Bgl*I^{AG} – 0.32), but in the beef cows of breeds such as Aberdeen Angus or Hereford, values analogous to those in our study were obtained. Therefore, it may be difficult to support selection using the advantageous genetic markers that affect the breeding performance of cows entering the foundation stock. Thus, the specific breeding model supplemented by the knowledge of markers for reproductive traits cannot be defined at the current stage of research.

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