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Distribution of Allele Frequencies at 5′-Flanking Region of *CYP19* and *ERα* Genes between Iranian Simmental and Three Indigenous Cattle Breeds

Research Article

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ABSTRACT

This study was performed to investigate two polymorphic sites from *Cyp19* gene (*PvuII* and *MspI*) and one polymorphic site from *ERα* gene (*SnaBI*) in four cattle breeds including Mazandarani, Taleshi, Sistani and Simmental. In overall 278 samples for *CYP19* and 206 samples for *ERα* marker sites were genotyped using polymerase chain reaction single-strand conformation polymorphism (PCR-RFLP) procedure. For *CYP19*/*PvuII*, the frequency of A allele ranged from 0.89 in Mazandarani to 0.98 in Taleshi. For *CYP19*/*MspI*, Taleshi was the only monomorphic breed with just AA genotype. Other breeds were polymorphic with A allele frequency ranging from 0.93 (Simmental) to 0.98 (Sistani). No BB individuals were observed in the studied sample. Considering two loci in combination with each other, only 5 out of 8 theoretically possible combined genotypes were observed in the genotyped samples. For *ERα*/*SnaBI*, all populations showed polymorphism. The highest and lowest frequencies of A allele were observed in Sistani (0.55) and Simmental (0.96), respectively. Based on Nei's method, the most genetic distance was observed between Sistani and Simmental and the least genetic distance was observed between Mazandarani and Taleshi breeds. The results of the present study showed significant differences in genotypic and allelic frequencies among four investigated breeds. These findings may be used in further researches, such as association studies for performance traits in cattle breeding.

KEY WORDS cattle breeds, *CYP19* gene, *ERα* gene, estrogen, polymorphisms, promoter.

INTRODUCTION

Estrogen is one of the most important hormones involved in the regulation of male and female reproduction. This hormone influences growth, differentiation and function of reproductive tissues like mammary glands, uterus, ovary, testis and prostate (Szreder *et al*. 2007). Moreover, this hormone affects the cardiovascular system, central nervous network, immune system, fat deposition and bone metabolism (Inoue and Horie-Inoue, 2004). The only enzyme in vertebrates known to catalyse the conversion of androgens into estrogens is aromatase cytochrome P450 (Ghosh *et al*. 2009). The *CYP19* gene that encodes the aromatase enzyme in cattle has been mapped on chromosome 10 (Fürbass *et al*. 1997). Expression of this gene is regulated by several tissue-specific promoters through the mechanism of alternative splicing, which result in the generation of transcript variants with different 5' untranslated regions (UTRs) (Kalbe *et al*. 2000). However, the coding region and the translated protein products are identical in all tissues, be-

cause all of the untranslated first exons splice to a common splicing acceptor site in exon II (Simpson and Davis, 2001).

The expression of *CYP19* gene in bovine placenta, a main site of aromatase expression is controlled by promoter 1.1 region (Vanselow *et al*. 2004). Vanselow *et al*. (1999) identified three SNPs in P1.1 region of *CYP19* gene. In current research, two out of three identified SNPs were analysed. The first mutation was a G/A transition at position -1044 recognized by *PvuII* restriction endonuclease. The second one was an A/G transition at position -1179. To recognize the A/G transition, *MspI* endonuclease was used.

Estrogen exerts its effects on various tissues through its receptors. Estrogen receptors (*ERα* and *ERβ*) are ligand-inducible transcription factors that regulate the expression of target genes and their protein structures are composed of six functional domains, A to F (Szreder and Zwierzchowski, 2007). Estrogens bind to ERs via their E-F domains and the estrogen-bound ERs bind to estrogen response elements (EREs) on genomic DNA through their C domain, and thus control the transcription of downstream genes (Inoue and Horie-Inoue, 2004).

In cattle, *ERα* gene is located on chromosome 9 and contains eight protein coding exons (Szreder *et al*. 2011). In the 5' region, however, there are additional exons that are preceded by their own promoters and code for transcripts with different 5'-UTRs in various tissues or developmental stages (Szreder and Zwierzchowski, 2004). The alternative exons are spliced to the +85 acceptor site within coding exon 1, 68 nucleotides upstream the translation start codon ATG (Szreder *et al*. 2008). So far, several nucleotide sequence polymorphisms were identified within both coding and noncoding regions of bovine *ERα* gene. One of these mutations analyzed in the present study was A/G transition in the putative promoter for exon B identified by Szreder *et al*. (2007). This mutation, recognizable by *SnaBI* endonuclease, was located at position -1213 relative to the +85 acceptor site (Szreder *et al*. 2007).

The present study is based on two genes coding proteins closely related to estrogen, which are considered as candidates for probable markers of economic traits in cattle. The objective of this work was to detect polymorphisms in the 5′ regions of *CYP19* and *ERα* genes in four breeds of cattle in Iran, determine their allelic and genotypic frequencies and also compare the frequencies among different populations.

MATERIALS AND METHODS

The blood samples were collected from four breeds of cattle: Mazandarani (n=112), Taleshi (n=84), Sistani (n=48) and Simmental (n=34). The Mazandarani cattles are a zebu type breed found in northern Iran.

They are kept for meat and milk production and are seen in all colors. The blood samples for this breed were collected from herds around the Mazandaran forests. Taleshi cattles are native to northern parts of Iran and are a dual-purpose breed, too. The blood samples for this breed were collected from two breeding stations in Guilan province. Sistani cattles, a humped breed belong to *Bos indicus* group are a type of domestic cattle located in the eastern region of Iran. These animals are a heavy built breed and used as dual-purpose cattle breed. One of the most distinctive features of Sistani cattle is its great capability to resist diseases which makes it a potential resource of germplasm useful for putative breeding programs. The blood samples of this breed for the current study were collected from the animals kept in the Zabol university farm (Figure 1).

Figure 1 Geographic distribution of Iranian native breeds of cattle

The Simmental samples were provided by an industrial dairy herd in Amol, Mazandaran province. Sampling procedure from every breed was done randomly. Blood samples were kept in tubes containing EDTA. DNA extraction was done using modified salting out method (Miller *et al*. 1988).

After DNA extraction, PCR-RFLP method was used to genotype the cattle for three polymorphic sites: two SNPs in P1.1 region of *CYP19* gene (*CYP19*/*PvuII* and *CYP19*/*MspI*) and one SNP in the putative promoter for exon B of *ERα* gene (*ERα*/*SnaBI*). Briefly, the polymerase chain reactions were performed using a PCR mixture containing 2.5 µL of 10X PCR buffer, 160 µ*M* dNTPs, 1.6 m*M* $MgCl_2$, 2 units of Taq polymerase (Cinnagen), 10 pmol of each primer (Metabion), 50-100 ng of genomic DNA and H_2O up to 25 µL. Amplification of a 288 bp fragment from

the promoter region of the *CYP19* gene was carried out using primers proposed by Vanselow *et al.* (1999):

CYP19-F (5'-GCATGGGCACTTGCTCTCGAT-3')
CYP19-R (5'-TGATTTCCAGGTTGTTAAGTGAATGA-3')

For amplifying a 340 bp fragment from the promoter region of *ERα* gene, primers suggested by Szreder *et al.* (2007) were used:

ERα-F (5'-GTCAGGTATTCCGTCAGGT-3')
ERα-R (5'-GCCTTTCTGTTCCTTTGG-3')

The PCR reaction was performed in a TECHNE TC3000G thermocycler. In the next stage, the amplification products were digested overnight at 37 ˚C with appropriate restriction enzymes (Thermo Scientific). The 288 bp fragment was digested separately with *PvuII* and *MspI* enzymes and the 340 bp fragment of *ERα* gene was digested with *SnaBI* enzyme. The digestion products were electrophoresed through 3% agarose gel and stained with ethidium bromide. After staining, restriction patterns were observed under UV light. In overall 278 and 206 samples were genotyped for *CYP19* and *ERα*, respectively. For each breed, allelic and genotypic frequencies were assessed using POPGENE software (version 1.31). Furthermore, differences in genotypic and allelic frequencies among different populations were analyzed using Chi-square and Fisher's exact test which were performed with SAS software (version 9.1). Linkage disequilibrium measures (r^2) between two SNPs of *CYP19* gene were estimated according to the following formula (Hill and Robertson, 1968):

$$r^2= [f(A_1B_1)f(A_2B_2) - f(A_1B_2)f(A_2B_1)]^2 / f(A_1)f(A_2)f(B_1)f(B_2)$$

Where:
$f(A_1B_1)$: frequency of the A_1B_1 haplotype and likewise for the other haplotypes.
$f(A_1)$: corresponds to the frequency of the A_1 allele and likewise for the other alleles.

Additionally, the genetic distance among the studied breeds was calculated according to Nei's method using the POPGENE software.

RESULTS AND DISCUSSION

A 288 bp fragment from the promoter 1.1 region of *CYP19* gene was amplified and two polymorphic sites located in this fragment were analyzed separately by PCR-RFLP technique.

The first polymorphic site, G/A transition at position -1044, was detected by *PvuII* restriction endonuclease and restriction patterns for this enzyme were as follows: two restriction fragments with 197 and 91 bp for the BB genotype, three restriction fragments with 288, 197 and 91 bp for the AB genotype and one fragment of non-digested PCR product with 288 bp for the AA genotype (Figure 2).

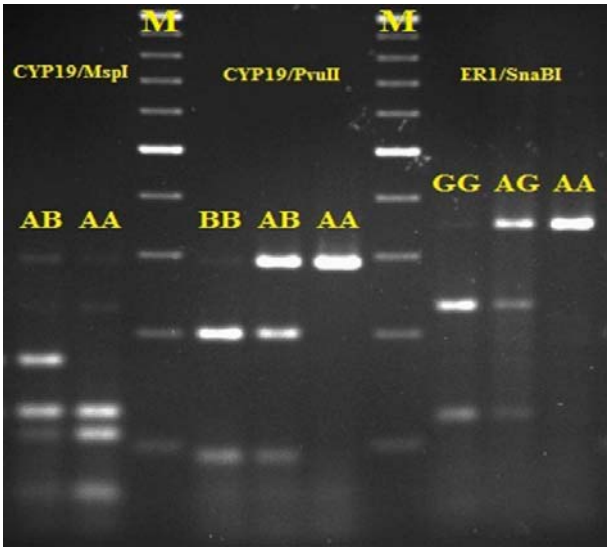

Figure 2 The results of the PCR-RFLP analysis. M: 100 bp molecular weight marker (Fermentas)

Genotypic and allelic frequencies for *CYP19*/*PvuII* related to the four studied breeds are presented in Table 1. The AA genotype was the most frequent genotype in all studied breeds. On the other hand, the BB genotype occurred very rarely, as one individual in both Mazandarani and Sistani breeds. Statistically significant differences in *CYP19*/*PvuII* genotype and allele frequencies were found between Taleshi and Mazandarani ($P<0.01$) and between Taleshi and Simmental ($P<0.05$) breeds (Table 2). The highest frequency of B allele was observed in Mazandarani breed, while the least frequency of this allele was observed in Taleshi breed. In Previous studies, frequency of A allele in German Holstein (Vanselow *et al.* 1999), Black and White (Jedrzejczak *et al.* 2006), Holstein-Friesian Black and White strain (Kowalewska-Luczak, 2009) and Holstein-Frisian (Szatkowska *et al.* 2011) was reported at 0.88, 0.947, 0.91 and 0.9211, respectively. However, in Jersey cattle, higher frequencies of A allele were found by Jedrzejczak *et al.* (2006) and Kowalewska-Luczak *et al.* (2013) 1.00 and 0.98, respectively. Zatkowska *et al.* (2011) investigated the effect of *CYP19*/*PvuII* polymorphism on reproduction traits in Holstein-Frisian cows. These authors showed that calving-to-conception interval and calving interval in heterozygote cows were signifcantly shorter than in AA homozygote ones.

Table 1 Genotypic and allelic frequencies of *CYP19*/*PvuII* in studied breeds of cattle

Breeds	n	Genotypic frequencies			Allelic frequencies	
		AA	AB	BB	A	B
Mazandarani	112	0.79	0.20	0.01	0.89	0.11
Taleshi	84	0.96	0.04	0	0.98	0.02
Sistani	48	0.92	0.06	0.02	0.95	0.05
Simmental	34	0.85	0.15	0	0.93	0.07

Table 2 Comparison of genotypic (above the diagonal) and allelic (below the diagonal) frequencies of *CYP19*/*PvuII* between studied breeds of cattle (P-value)

Breeds	Mazandarani	Taleshi	Sistani	Simmental
Mazandarani	-	0.0024	0.0889	0.6849
Taleshi	0.0002	-	0.3158	0.0293
Sistani	0.0513	0.0902	-	0.3234
Simmental	0.1417	0.0382	0.2173	-

In the study conducted by Kowalewska-Luczak (2010), milk production traits were significantly higher in the AA genotype. Other researchers didn't find any significant associations between *CYP19*/*PvuII* polymorphism and production traits in Black and White (Jedrzejczak *et al*. 2006) and Jersey cattles (Kowalewska-Luczak *et al*. 2013).

The second polymorphic site of *CYP19* gene analyzed in this study was A/G transition at nucleotide position -1179 in P1.1 regions. The transition situation at this marker site of *CYP19* gene was identified using sequencing technique by Vanselow *et al*. (1999). In the present study it was attempted to detect any polymorphism at this marker site by RFLP technique using restriction enzyme *MspI* for the first time. Due to the existence of two restriction sites in the 288 bp segment, the banding patterns should be as follows: two fragments (122 and 166 bp) for the B allele and three fragments (122, 103 and 63 bp) for the A allele. In this case, three fragments with 122, 103 and 63 bp indicate genotype AA and four fragments with 166, 122, 103 and 63 bp represent genotype AB (Figure 2). The allelic and genotypic frequencies of *CYP19*/*MspI* in the studied breeds are shown in Table 3. In all breeds the frequency of A allele was much higher than that of B allele. The BB genotype was not found in any of investigated breeds. For this locus, Taleshi was the only monomorphic breed with just AA genotype. Genotypic and allelic frequencies of *CYP19*/*PvuII* locus showed significant differences among the studied breeds (Table 4). Vanselow *et al*. (1999) reported the frequencies of A and B alleles in German Holstein cattle were 0.98 and 0.02, respectively. Until now we are not aware of further studies on this polymorphic site of bovine *CYP19* gene. The linkage disequilibrium between two SNPs of *CYP19* gene in all populations was estimated. Linkage disequilibrium which describes the non-random association of alleles at different loci within a population, plays an important role in evolutionary biology and association analysis (Wang *et al*. 2010) and can be caused by physical linkage, natural selection or random genetic drift (Barton, 2011).

The distance between these two polymorphic sites is 135 nucleotides. Interestingly, complete linkage between the studied sites was observed in Simmental breed (r^2=1), while relatively low LD (r^2) was obtained in Mazandarani (0.12) and Sistani (0.19) populations. Taleshi population showed a monomorphic banding pattern at *MspI* locus, so it was not included in the LD analysis.

Considering two loci in combination with each other, only 5 out of 8 were observed in the genotyped samples. This may probably be as a result of close correlation between loci and very low frequency of alternative alleles. The frequencies of combined genotypes are reported in Table 5.

The double homozygous AAAA was found to be the most frequent genotype in all breeds. The other four combined genotypes were much less frequent. Animals genotyped as AAAB were found only in Mazandarani population.

The polymorphism in *ERα* gene was analyzed using *RFLP*/*SnaBI* method. This SNP (A/G transition) was situated at position -1213 relative to the +85 acceptor site. Digestion of the 340 bp PCR product with the restriction enzyme *SnaBI* resulted in two bands (225 and 115 bp) for homozygote BB, three bands (340, 225 and 115 bp) for heterozygote AB and one band (340 bp) for homozygote AA (Figure 2).

Table 6 shows the frequencies of genotypes and alleles in four populations. In all studied breeds, the AA genotype was the most frequent. No homozygous genotype GG was found in Taleshi and Simmental breeds.

Based on Chi-square and Fisher's exact tests, genotypic and allelic frequencies for *ERα*/*SnaBI* polymorphism were found to be significantly different (P≤0.05) among the studied breeds (Table 7).

Considerably higher frequency of G allele was observed in Sistani cattle (0.45) compared to other studied breeds. In contrast, in Simmental population, the G allele was very rare (0.04).

Table 3 Genotypic and allelic frequencies of *CYP19*/*MspI* in studied breeds of cattle

Breeds	n	Genotypic frequencies		Allelic frequencies	
		AA	AB	A	B
Mazandarani	112	0.94	0.06	0.97	0.03
Taleshi	84	1.00	0	1	0
Sistani	48	0.98	0.02	0.99	0.01
Simmental	34	0.85	0.15	0.93	0.07

Table 4 Comparison of genotypic (above the diagonal) and allelic (below the diagonal) frequencies of *CYP19*/*MspI* between studied breeds of cattle (P-value)

Breeds	Mazandarani	Taleshi	Sistani	Simmental
Mazandarani	-	0.0196	0.2678	0.1159
Taleshi	0.0191	-	0.1842	0.0003
Sistani	0.1964	0.3636	-	0.0306
Simmental	0.0835	0.0018	0.0406	-

Table 5 Frequencies of combined genotypes of *CYP19* gene in studied breeds of cattle

Breeds	Combined genotypes				
	AAAA	AAAB	ABAA	ABAB	BBAB
Mazandarani	0.78	0.02	0.16	0.03	0.01
Taleshi	0.96	0	0.04	0	0
Sistani	0.92	0	0.06	0	0.02
Simmental	0.85	0	0	0.15	0

Table 6 Genotypic and allelic frequencies of *ERα*/*SnaBI* in studied breeds of cattle

Breeds	n	Genotypic frequencies			Allelic frequencies	
		AA	AG	GG	A	G
Mazandarani	64	0.65	0.30	0.05	0.80	0.20
Taleshi	60	0.75	0.25	0	0.87	0.13
Sistani	48	0.35	0.40	0.25	0.55	0.45
Simmental	34	0.91	0.09	0	0.96	0.04

Table 7 Comparison of genotypic (above the diagonal) and allelic (below the diagonal) frequencies of *ERα*/*Sna*BI between studied breeds of cattle (P-value)

Breeds	Mazandarani	Taleshi	Sistani	Simmental
Mazandarani	-	0.1783	0.0009	0.0198
Taleshi	0.0450	-	< 0.0001	0.0555
Sistani	< 0.0001	< 0.0001	-	< 0.0001
Simmental	0.0019	0.0409	< 0.0001	-

In Mazandarani and Taleshi breeds, the *ERα*/*SnaBI* allele frequencies were intermediate between those found in Sistani and Simmental breeds.

Some studies investigated the *ERα*/*SnaBI* polymorphism in ten *Bos taurus* breeds and one *Bos indicus* breed (Rathi Zebu). The overall frequency of A allele in *Bos taurus* and in *Bos indicus* breeds was 0.92 and 0.94, respectively. In another study carried out by Szatkowska *et al*. (2011) on *ERα*/*SnaBI* polymorphism, the frequencies of A and G alleles in Polish Holstein-Friesian cows were reported 0.96 and 0.04, respectively. In the cows of AA genotype, significantly shorter calving to conception interval was observed compared to heterozygotes. There were no associations between this polymorphism and milk production traits of the investigated cows (Jedrzejczak *et al*. 2011).

The genetic distance among different breeds was estimated based on allele frequencies.

The most genetic distance was observed between Sistani and Simmental. The least genetic distance was observed between Mazandarani and Taleshi breeds which may be reasonable due to short geographic distance between their rearing environments. Sistani, was genetically very distant from other breeds (Table 8). As mentioned before, estrogen is an important hormone involved in many physiological pathways. Due to various functions played by estrogenic hormones, enzymes crucial for estrogen synthesis and also protein mediators in estrogen actions are considered potential markers of production and functional traits in cattle (Szatkowska *et al*. 2011). The sampled populations in this study have different genetic origins and are raised in different environmental conditions. It is clear that the allelic and genotypic frequency significantly affected by breed factor. Therefore, differences in allelic and genotypic frequencies can be strongly due to distinct evolutionary pathways.

Both *CYP19* and *ERα* genes are under the control of several different promoter regions as different promoters are active in various tissues.

Table 8 Genetic distance between studied breeds of cattle

Breeds	Mazandarani	Taleshi	Sistani
Taleshi	0.0036	-	-
Sistani	0.0283	0.0395	-
Simmental	0.0094	0.0054	0.0676

Alternative promoter utilization may play a role in the posttranscriptional regulation of gene expression and influencing the efficiency of translation (Fürbass *et al*. 1997). All three mutations examined in the present study are located in the promoter regions of the genes, so they would not change the amino acid sequence of protein products. How these mutations affect the functions of estrogen can be explained if these mutations may be located in the transcription factors' binding sites and so alter the expression levels of the genes. Another explanation is that these mutations may have linkage with other mutations in the vicinity.

Based on our knowledge the studies regarding influence of mutations in these genes on economically important traits of cattle are limited and the few studies which were devoted to these polymorphisms didn't find any clear relationship.

Considering enough polymorphism in these loci in studied populations, planning for association studies regarding the relationship between the mentioned loci and important traits can be an interesting issue in cattle breeding.

CONCLUSION

The herds in our study differ with regard to the distribution of genotypic and allelic frequencies at studied mutations, which probably implied that these mutations played possible roles in production and fitness status of herds. Based on our knowledge the studies regarding influence of mutations in these genes on economically important traits of cattle are limited and the few studies which were devoted to these polymorphisms didn't find any clear relationship. Considering enough polymorphism in these loci in studied populations, planning for association studies regarding the relationship between the mentioned loci and important traits can be an interesting issue in cattle breeding.

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