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# GENETIC VARIABILITY IN EQUINE *GDF9* AND *BMP15* GENES IN ARABIAN AND THOROUGHBRED MARES*

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**Abstract**

**In horses, multiple ovulation resulting in implantation of multiple embryos is adverse. However, understanding the mechanisms underlying initiation of multiple ovulation (MO) is advantageous and is related to an increase in efficiency of embryo transfer techniques. It has been postulated that MO may have a genetic background. Two major genes: bone morphogenetic protein 15 (*BMP15*) and growth and differentiation factor 9 (*GDF9*) are considered to play a crucial role in folliculogenesis and controlling the ovulation rate. Thus, the aim of the presented study was to identify the variation within equine *BMP15* and *GDF9* genes to verify their potential role on spontaneous, repetitive multiple ovulations in mares. In addition, variation screening of investigated genes in population of Thoroughbred and Arabian breeds was performed together with establishment of transcript abundance of *BMP15* and *GDF9* genes in equine ovarian tissue. Sanger sequencing of Arabian and Thoroughbred mares divided according to ovulation rate, revealed occurrence of 3 SNPs in *BMP15* and STS in *GDF9* genes. The PCR-RLFP and statistical analysis indicated that none of the genotype frequencies were significant in any breeds and none of them were claimed as functional according to ovulation rate. Furthermore, evaluation of transcript abundance by RT-PCR of both genes in ovarian tissues showed that expression of both genes was similar but *GDF9* was significantly expressed in growing follicles with 21–30 mm diameter and in ovarian parenchyma, which suggest their potential role in folliculogenesis.**

**Key words: horse, ovary, ERE, *BMP15*, *GDF9***

In horses, multiple ovulation resulting in implantation of multiple embryos is adverse. However, understanding the mechanisms underlying initiation of multiple

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ovulation (MO) is advantageous and is related to an increase in efficiency of embryo transfer techniques. It has been postulated that MO may have a genetic background. Despite the low heritability rate there is convincing evidence that this trait is incorporated with segregation of single locus, caused by introduction of particular sire lines to maternal population (Bresińska et al., 2004; Wolc et al., 2006). The genetic aspects of mare fertility remain unknown. This is mainly due to inaccessibility of a good mammalian model and the fact that in equine breeding, male reproduction performance is inclined towards stallion research (Chowdhary, 2013). The most common mare problems are pregnancy failures (Coutinho da Silva, 2008) that may be caused by the multiple pregnancies arising by the multiple ovulation (MO). In fact, MO is disadvantageous (Ginther, 1982), but on the other hand artificial induced superovulation in mares is a main goal in breeding industry for increasing efficiency in advanced reproduction techniques e.g. embryo transfers (Roser and Meyers-Brown, 2012). It is well established that Thoroughbreds and TB crossbreds have higher incidence of MO than other breeds – 22.4% (Morel et al., 2005) and genetic background of this has been previously discussed (Mucha et al., 2012).

Horses are a mono-ovulatory species having one follicle which becomes dominant while subordinate follicles regress during the follicular wave of the estrous cycle before ovulation (Ginther and Bergfelt, 1992). Two major genes: bone morphogenetic protein 15 (*BMP15*) and growth and differentiation factor 9 (*GDF9*) are considered to play a crucial role in folliculogenesis and controlling the ovulation rate (Hanrahan et al., 2004; Otsuka et al., 2011).

Heterodimers of *GDF9* and *BMP15* having greatest affinity to the ligands, acting via SMAD2/SMAD3 pathways increase granulosa cells proliferation, reduce expression of the FSH receptor and steroid hormones (Peng et al., 2013; Mottershead et al., 2015). Furthermore, it is well established that expression ratio and activity of *BMP15: GDF9*, influencing granulosa cell proliferation and ovulation rate are specific and vary across poly- and mono-ovulatory species (Crawford and McNatty, 2011).

To date, numerous mutations have been found across species associated with hyperprolificacy or sterility (Hanrahan et al., 2004; Silva et al., 2011), premature ovarian insufficiency, twinning, infertility or ovarian hyperstimulation syndrome (Di Pasquale et al., 2006; Laissue et al., 2006; Monestier et al., 2014).

In humans and in cows, some rare mutations in *GDF9* had been described, which influenced the multiple ovulation resulting multiple pregnancy (Montgomery et al., 2004; Palmer et al., 2006; Marchitelli and Nardone, 2015),

Thus, the aim of the present study was to identify the variation within equine *BMP15* and *GDF9* genes to verify their potential role on spontaneous, repetitive multiple ovulations in mares. In addition, variation screening of investigated genes in population of Thoroughbred and Arabian breeds was performed together with establishment of transcript abundance of *BMP15* and *GDF9* genes in equine ovarian tissue.

## Material and methods

This work has been approved by the University of Agriculture in Kraków no. 34/2010**.**

### Animals and data collection

The peripheral blood samples from 126 mares belonging to two breeds: Arabian (n=73) and Thoroughbred (n=53) were collected into sterile tubes with EDTA anticoagulant by jugular venipuncture. For investigation of possible association between multiple ovulation and polymorphism of *BMP15* and *GDF9* genes, 10 mares have been selected: five mares with confirmed spontaneous, repetitive multiple ovulation (at least in 5 cycles, as determined by ultrasound scan) and five with no history of MO.

For gene expression study, ovary tissues were collected post mortem from 37 adult mares at slaughterhouse during short day period. After that, antral follicles were dissected free of stroma and sections of the follicular walls containing mural granulosa cells, corpus luteum and ovarian parenchyma samples (without visible antral follicles) were collected. One to three structures were isolated from each ovary, and allocated to one of the following groups: follicles according to diameter (A) ≤ 10 mm, (B) 11–20 mm, (C) 21–30 mm, (D) > 30 mm, (CL) corpus luteum and (OP) ovarian parenchyma (Młodawska and Słomczyńska, 2010). Tissues were stored in RNAlater solution (Ambion, ThermoScientific, USA) and then frozen in –80°C or submitted to hematoxylin and eosin (HE) staining.

### DNA and RNA preparation

DNA was extracted from equine blood using MasterPure Genomic DNA Purification Kit (Epicentre Technologies, USA). The RNA isolation was performed for all ovarian samples (corpus luteum*,* ovarian parenchyma and sections of follicles) collected at the slaughterhouse. The total RNA from samples was isolated using PureLink RNA Mini Kit (Ambion, ThermoScientific, USA) according to manufacturer's protocol. Evaluation of RNA and DNA quantity and quality was performed using the NanoDrop 2000 (Thermo Scientific, Wilmington, USA). Additionally, RNA was visualized by 2% agarose gel electrophoresis for examining its degree of degradation.

### Reverse transcription and quantification of *BMP15* and *GDF9* expression level

The 250 ng of total RNA was transcribed to cDNA using TranscriptMe reverse transcription kit with random primers (Blirt, Gdańsk, Poland), according to manufacturer's protocol.

The primers and probes for equine *BMP15* and *GDF9* genes were designed at the junction of exons to avoid contamination from genomic DNA, with the use of PrimerExpress 3.0 software, while the primers and probe for *SDHA* (endogenous control) were designed and synthesized as Assay (Applied Biosystems, ThermoScientific, USA) (Table 1). Furthermore, the genomic DNA contamination was checked by no reverse transcriptase control (no RT) to avoid adulteration of qRT-PCR results.

Table 1. Sequences of the primers and probes used for the analysis of gene expression by real-time qPCR

| Genes | Primers | Probe (label) | GenBank access number | Product size (bp) | PCR efficiency |
|---|---|---|---|---|---|
| *BMP15* | F: TCAGAGGCCCCTGGCATATA<br>R: TTTACCCGGTTGGATCTCAGA | AGACCCTGGACTTTC (MGB-FAM) | NC_009175.2 | 70 | 1.97 |
| *GDF9* | F: AATGTGTGTGCAAACCTGGTG<br>R: TTTTCTTGTTGGAGGCCACT | TCTCCCCGGAACTC (MGB-NED) | NC_009157.2 | 75 | 2 |
| *SDHA* | TaqMan gene expression assay ID: Ec03470479_m1(Applied Biosystems) | (MGB-VIC) | NC_009164.2 | 56 | 2 |

The *BMP15* and *GDF9* expression was evaluated by 7500 Real-Time PCR System using labeled TaqMan® probes and TaqMan® Gene Expression Master Mix (Applied Biosystems). Reaction for each sample was performed in three repeats in a total volume of 25 µl, containing: 12.5 µl TaqMan Gene Expression Master Mix (2X), 0.5 µl of specific probes (250 nM final concentration), 0.5 µl of primers (900 nM final concentration), 2.5 µl of cDNA (31.25 ng) and 9 µl of water. According to the TaqMan Gene Expression Master Mix protocol, reactions were performed in two initial steps of 50°C for 2 minutes (UNG incubation) and 95°C for 10 minutes (AmpliTaq Gold activation) and 45 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing/extending).

For all genes, the efficiency of real-time PCR reactions was defined by using the standard curve method, and the relative quantity of *BMP15* and *GDF9* mRNA abundance was calculated according to Pfaffl (2001).

**Identification and genotyping of SNPs**

DNA samples from five mares with confirmed spontaneous, repetitive multiple ovulation and five with no history of MO were Sanger sequenced on CEQ8000 Genetic Analysis System (Beckman Coulter, California USA), using DTSC Sequencing Kit (Beckman Coulter, California, USA) for entire (5'UTR, intron, exon's and 3'UTR) both *BMP15* and *GDF9* regions (EquCab 2.0, ChrX:40848723-40854413; Chr14:42748404-42752672; respectively).

For several identified SNPs, other methods (PCR-RFLP, Fragment Analysis) were applied to identify genotype frequency of the whole analyzed population. For PCR-RFLP method, PCR reactions were carried out in a final volume of 11 µl with use of AmpliTaq Gold 360 Master Mix (Life Technologies, California, USA) and annealing temperature assigned for each fragment. The digestions of PCR product were carried out in final volume of 10 µl, containing 5 µl of PCR product digested by 3U of restriction enzyme (Biolabs Inc,) 2.2 µl of buffer and 2.6 µl water. The Fragment Analysis application was performed for microsatellite *loci* in intron of *GDF9*, on sequencer CEQ8000 Genetic Analysis System with GenomeLab DNA Size Standard Kit-400 (Beckman Coulter, California USA). Primer sequences used for amplification, annealing temperatures and restriction enzymes used for digestion are presented in Table 2.

Table 2. Primers sequences used for screening and genotyping SNPs in equine *BMP15* and *GDF9* genes

| Gene region | *GDF9* | Annealing temperatures | *BMP15* | Annealing temperatures | Restriction enzymes/incubation temperatre |
|---|---|---|---|---|---|
| 5'UTR | P.1.F:AGGCCGGTCCCAAGATACTA<br>P.1.R:TCCATCCTCACCACTTCTCC | 56°C | P.1.F:TCAAGTTCCAACAGCCACAC<br>P.1.R:CCTGTTCTCATCCCACTTCC | 57°C | |
| | | | P.2.F:CCTTGTTCCACCTGCTGTTT<br>P.2.R:GGCTTAGCGTTCAGCAACAC | 56°C | |
| | P.2.F:CAATGCCAGCTGTGACTTTC<br>P.2.R:GAAACCACCCGTTTTCTGAG | 55°C | P.3.F:TTTTCCCATCCTGGTGGATA<br>P.3.R:AGGCAGGTAGGCCCTATGTT | 55°C | |
| | P.3.F:CACACCTTGACAGTTTCAGCA<br>P.3.R:TCTCACTTTTTCCTGGCATGT | 55°C | P.4.F:AGGAAGTGCTGCATGGAAGT<br>P.4.R:CAGGACTGGGGACAAGACAG | 55°C | |
| | P.4.F:CAGAAAACGGGTGGTTTCAT<br>P.4.R:CAAGGATGCCTAGCTGAAGG | 56°C | P.5.F:CACAGTGGTTTCCACATTTCA<br>P.5.R:AGTCCTTAAAGGGCCAGAGG | 56°C | |
| | P.5.F:AGGCCGGTCCCAAGATACTA<br>P.5.R:CAAGGATGCCTAGCTGAAGG | 55°C | P.6.F:TCACTGTTAAGGCACCTTCTTCA<br>P.6.R:TGACATCCTTTGGGCTTGGG | 56°C | |
| Exon I | E.1.F:TGCCAGGAAAAAGTGAGAGG*<br>E.1.R:AATCCGCTCCTACACACCTG | 55°C | E.1.F:ACCTGCCTGCCTTTCACTTT<br>E1.R:ACTGTTCTGGAGGGGAGAGA | 55°C | |
| Intron | I.F.1.F:AAGGCGTATGCTACCAAGGA<br>I.F.1.R:GCGCTGCTTTCTCTCTCTCT | 55°C | I.F.1.F:GGGATCTGGATTCCATTCCTA<br>I.F.1.R:TCAACTTATTCCACTCCCATTC | 56°C | HinfI/37°C |
| | I.F.2.F:TGGTGTGCTTTGTTAAACAGG*<br>I.F.2.R:CTTTTCCCACTTCCTCATGC | 60°C | I.F.2.F:AAGGAACGGAAAAGGGAGAC<br>I.F.2.R:AGATGGGCCCGGATGTTAG | 56°C | AvaII/37°C |
| Exon II | E.2.F.1.F:GCCTTTGTAGTTGCTGGGAGT<br>E.2.F.1.R:TCAGGACCCTGTGAAGGTCT | 55°C | E.2.F.1.F:AGAAGCTAACCCTCTGCTCT<br>E.2.F.1.R:AGTGATCCTCTGCCCAATCT | 57°C | |
| | E.2.F.2.F:TCACATGTGGGAAAGACCAG<br>E.2.F.2.R:GCCAAACAGGCTCAAGATGT | 55°C | E.2.F.2.F:AGAAGCTAACCCTCTGCTCT<br>E.2.F.2.R:AGTGATCCTCTGCCCAATCT | 57°C | |
| 3'UTR | UTR.F:CTCCTCAGTGCCAAGACCAT<br>UTR.R:CATGTGCTACAACAGAAATGGA | 56°C | UTR.F:TGATTGCCCAGTCCTGTACC<br>UTR.R:ACCTGGGGGAAAGTACCTGT | 56°C | HaeIII/37°C |

*D4 fluorescent labeled primer used for Length Fragment Analysis.

**Data analysis**

The data was analyzed by Power Marker V3.0 software. Estimation of MAF (minor allele frequencies) was carried for each *locus* (Barrett et al., 2005; Liu and Muse, 2005). Genotype frequencies were estimated for both breeds and compared using a $Chi^2$ test ($P<0.05$).

The statistical analysis of differences in gene expression pattern was performed using the ANOVA procedures, with significance level of 5% (SAS Institute, Cary, NC, v. 8.02, 2001).

The bioinformatics analyses of likely transcription factor binding sites were performed using prediction tool: MathInspector by Genomatrix (Cartharius et al., 2005). For investigation of sequence homology components, we performed screening against Repbase Censor software (Kohany et al., 2006).

## Results

**Expression patterns of *BMP15* and *GDF9* gene in equine follicles and ovarian tissue**

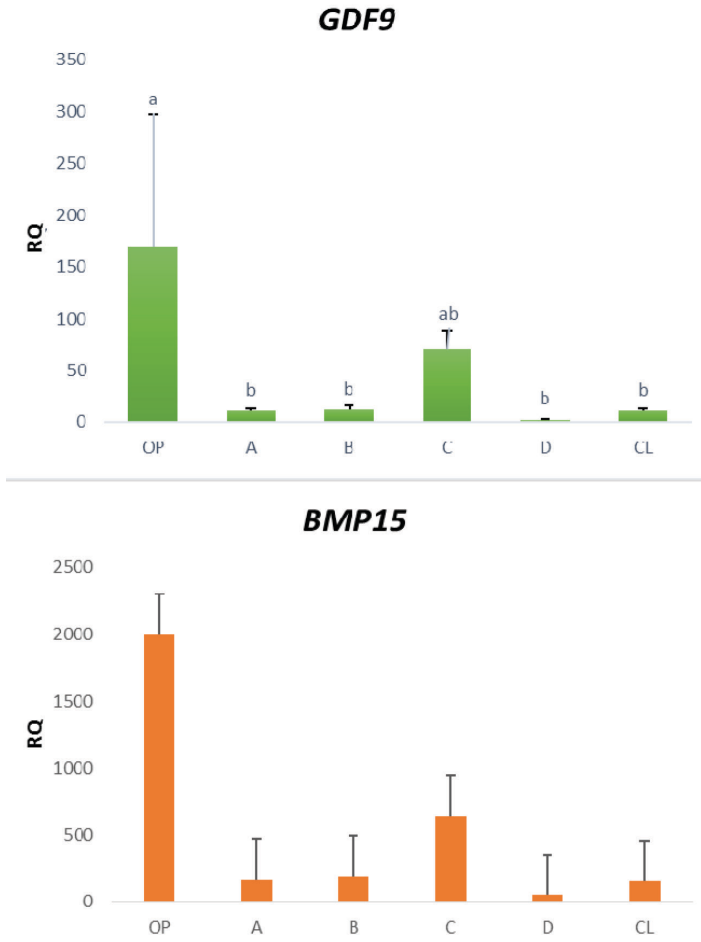
Our research showed that expression pattern of *BMP15* gene was very similar to expression of *GDF9* gene but *GDF9* was significantly more highly expressed ($P<0.05$) in growing follicles with 21–30 mm diameter (Group C) and in ovarian parenchyma (containing preantral follicles). The second structure showed a higher gene expression pattern of both *BMP15* and *GDF9*, differing significantly from the other groups. There was lower expression of both genes in smaller follicles (≥10 mm – Group A; 10–20 mm – Group B), followed by a marked decline in preovulatory follicles (≥30 mm – Group D) (Figure 1).

The presence of preantral follicles was confirmed with hematoxylin and eosin (HE) staining in control sections of ovarian parenchyma.

**Polymorphisms within *BMP15* and *GDF9* genes**

Sequencing of *BMP15* gene revealed the occurrence of 2 intronic SNPs (g.40851607T>C, g.40852502A>G) and one in 3'UTR region (g.40854998T>C). All polymorphisms were submitted to NCBI dbSNP databases and received accession numbers: ss# 1985399684; ss# 1985399685 and ss# 1985399686; respectively. The *GDF9* gene was highly conserved, however within the intronic region we identified STS sequence (accession numbers: KX139147; KX139148; KX139149): g.42750467GT[7]GA[9], g.42750467GT[8]GA[11], g.42750467GT[8]GA[12]. None of them was claimed as functional according to ovulation rate. To verify potential association of identified mutation with ovulation rate, we genotyped selected SNPs in mares grouped by breed. MAF for the SNPs and STS are presented in Table 3.

The genotype frequencies comparison between investigated breeds showed similar distribution pattern. However, in *BMP15* g.40851607T>C loci according to $Chi^2$ test, significant differences (P-value 0.029) between Arabians and Thoroughbreds (TB) occurred with higher frequency in TB mares.




Relative Quantity (RQ) of *GDF9* and *BMP15* transcripts, determined by Real-Time PCR System using labeled TaqMan® probes, in investigated ovary structures: follicles according to diameter (A) ≤10 mm, (B) 11–20 mm, (C) 21–30 mm, (D) >30 mm; corpus luteum (CL) and ovarian parenchyma (OP). Values with the same superscripts do not show significant differences between transcript levels (P>0.05) and with different superscripts show significant differences between structures investigated (P<0.05). Data are presented as means (± SE).

Figure 1. Expression pattern of *GDF9* and *BMP15* genes in growing equine follicles

Table 3. Minor allele frequencies for SNPs in *BMP15 loci* (g.40851607T>C, g.40852502A>G, g.40854998T>C) and STS (g.42750467GT [8]GA [11]) in *GDF9 locus*

| | | *BMP15* | | | *GDF9* |
|---|---|---|---|---|---|
| Horse breed | n | MAF g.40851607T>C | MAF g.40852502A>G | MAF g.40854998T>C | g.42750467GT [8]GA [12] |
| Arabians | 73 | 0.19 | 0.32 | 0.49 | 0.34 |
| Thoroughbreds | 53 | 0.28 | 0.48 | 0.49 | 0.39 |

| Gene | Name | Class | Sequence alignment | sim |
|---|---|---|---|---|
| *GDF9* | ***ERE1C*** | NonLTR/SINE/SINE2 | 186 AACATTGGC-CCTGAGCTAACATCTGTTGCCAATCTTCCT----------CTTTTTTATTTCCT<br>258 AAGATTGGCACCTGAGCTAACATCTGTTGCCAATCTTTCTTTTTCTTCTCCTTCTTCTTCTTCTTCT<br>CCCCGAAGCCTC--AGCACATAGTTGTATATCCTAATTGTAGGTTGTTCTAATTCTTCCATGTGGGATGC<br>CCCCAAAGCCCCCCAGTACATAGTTGTATATTCTAGTTGTAGGTCCTTCTGGTTCTGCTATGTGGGACGC<br>TGCCACAGCATGGCTTGATGAGTGGTACATATGTCCACGTGCAGGATCCCAACCAGTGAA-CCCCGGGGG<br>CGCCTCAGCATGGCTTGATGAGCGGTGC-TAGGTCCGCGCCCAGGATCCGAACCGGCGAAACCCCGGG--<br>GGCCACTGAAGCTGAGTGCCCTAACTTAACCACTGGGCCACAGG-CCGGTCCC 427<br>--CCACCGAAGCGGAGCGCGCGAACTTAACCACTCGGCCACGGGGCCGGCCCC 1 | 0.8156 |
| *BMP15* | ***ERE2*** | NonLTR/SINE/SINE2 | 1656 GGGGCCGGCCCGGTGGCGCAGCAGTTAAGTTTGCACGTTTGGCTTCTCAGCGGCCCGGGG-TTCTCCGGT<br>2 GGGGCCGGCCCGGTGGCANAGCGGTTAAGTTCGCGCGTTCCGCTTC--GGCGGCCCGGGGGTTCACCGGT<br>TCGGATCCCAGGTGTGGACATGGCACCGCATGGCAG--GCCATGCTGTGGTAGGCGTCCCACATGTGAAA<br>TCGGATCCCGGGTGCGGACATGGCACCGCTTGGCAAAAGCCATGCTGTGGTAGGCGTCCCACATATAAAG<br>TAAAGGAAGATGGGTATGGATGTTGGGTCAGGGCCAGTCCTCCTCAGCAAAAAGAGGAGGATTGGCAGCG<br>TAGAGGAAGATGGGCACGGATGTTAGCTCAGGGCCAGTCTTCCTCAGCAAAAAGAGGAGGATTGGCAGCA<br>GATGTTAGCTCAGGGCTAATCTTCCTCAAAGAAAAATTAAA 1903<br>G---TTAGCTCAGGGCTAATCTTCCTCAAAAAAAAAAAAAA 247 | 0.8785 |
| | ***ERE3*** | NonLTR/SINE/SINE2 | 4658 TTTTTTTTTTGAGGAAGATTAGCCCTGAGCTAACATCCGGGCCCATCTTCCTACACTTTGTATGTGGGAT<br>194 TCTTTTTGCTGAGGAAGACTGGCCCTGAGCTAACATCCGTGCCCATCTTCCTCTACTTTATATGTGGGAC<br>GCCTACCACAGCATGGCTT--GACAAGTGGTGCCACATCTGCACCTGGGATCTGACCCAGTGAATACT-G<br>GCCTACCACAGCATGGCTTTTGCCAAGCGGTGCCATGTCCGCACCCGGGATCCGAACCGGTGAACCCCCG<br>GGCCACCGAAGTGGAACGTGCACACTTAACTGCTGTGCCACCAGGCCGGCCCC 4847<br>GGCCGCCGAAGCGGAACGCGCGAACTTAACCGCTNTGCCACCGGGCCGGCCCC 2 | 0.8333 |
| | ***ERE2*** | NonLTR/SINE/SINE2 | 4503 GGGACCAGCCCAGTGGACGCAGCTGTTAAGTTCGCAGGCTCTGTTT-GGTGGCCCGGAGTTTGCCGTTTC<br>1 GGGGCCAGCCCAGTGG-CATAGTGGTTAAGTTCATGCGCTCCACTTCAGCGGCCTGGGGTTCGCCGGTTT<br>CAATCCTGGGTGCCAACCTATGAGCTGCTTATCAAGCCATGCTGTGGTAGGTGTC 4626<br>GGATCCCGGGCACAGACCTACGCACCGCTTATCAAGCCATGCTGTGGCAGGCGTC 124 | 0.7280 |

Figure 2. Sequence alignment to ERE elements in regions of *GDF9* and *BMP15* genes (Kohany et al., 2006)

Results of MatInspector *in silico* analysis of the transcription binding sites of the investigated genes indicated that occurrence of three polymorphisms in *BMP15* may create 8 new (HOXC, LEF, CLOX, PAX6, RXRF, GCMF, MOKF, GCMF) and disturb 1 (NFAT) of potentially existing transcription factor binding sites.

The screening against CENSOR tool for search of repetitive elements and "masks" revealed an occurrence of equine non-long terminal repeat (non-LTR) retrotransposons. Three of them are localized in *BMP15* intron and are classified to ERE2 and ERE3 elements with sim value ranging from 0.73 to 0.88 (Kohany et al., 2006)

In the predicted promoter region of *GDF9* gene the sequence matched to SINE element described as ERE1C (Tran and Gąsior, 2010). Further alignment to horse genome was utilized by Basic Local Alignment Search Tool (BLAST/BLAT) in Ensembl database and revealed that all found elements were complementary to numerous sequences located in various equine chromosomes in at least 86% (Figure 2).

## Discussion

It has been confirmed that both genes – *BMP15* and *GDF9* – may improve oocytes quality and their developmental potential by arousing M-phase-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) (Zhang et al., 2011; Lin et al., 2014). Furthermore, it has been shown that FSH regulates *BMP15* expression via Kit signaling pathways. The Kitl expression is inhibited by *GDF9* and stimulated by *BMP15* and regulation of oocyte growth is affected by differential regulation and activities of two Kit ligand isoforms (Gilchrist et al., 2008).

In this study, data showed the presence of equine *BMP15* and *GDF9* mRNA in follicles at all stages of development, corpus luteum, and ovarian tissue containing preantral follicles. It is well established that occurrence of transcript abundance in equine oocytes varies accordingly with age of mares and stage of follicle development (Campos-Chillon et al., 2015). Here we described expression patterns in granulosa cells distributed on the follicular wall (mural granulosa cells) in equine growing follicles. Most of growth of oocytes occurs in pre-antral follicles where granulosa cells are rather undifferentiated. At the end of follicular antrum formation granulosa cells differentiate into the mural granulosa cells, which are the wall of the follicle and that have principally a steroidogenic role and the cumulus cells. These, in turn, form connection with the oocyte known as cumulus oocyte complex (Albertini et al., 2001). In other species, it was previously shown that both genes are expressed in both oocytes and cumulus granulosa cells (Silva et al., 2005; Hosoe et al., 2011). Our findings suggest that both genes have similar pattern of expression during follicle development and furthermore both are detectable in ovarian tissue (OP) (without antral follicles), throughout primary stage onward. The observed results concord with those presented for goats (Silva et al., 2005), sheep and cows (Bodensteiner et al., 1999; Bodensteiner et al., 2000) and chickens (Han et al., 2015). In rodents and human, the earliest transcript abundance is noted at primordial follicles (Aaltonen et al., 1999; Erickson and Shimasaki, 2003).

The greater transcripts abundance (with significant differences for GDF9 $P<0.05$) in OP were demonstrated compared to other stages of follicles development.

These findings pinpointed that both genes may play an important role in recruitment of primordial follicles in horses. As there is evidence of striking similarities in antral follicle dynamics in both woman and mare at early folliculogenesis (Haag et al., 2013 a, b; Alves et al., 2016) this hypothesis will be further explored. The species differences in expression of *GDF9* is due to a single amino acid change in the receptor binding region which determines whether *GDF9* is secreted in an active or an inert form (Simpson et al., 2012). Furthermore, we detected expression of *BMP15* and *GDF9* in equine corpus luteum similarly as in humans (Sun et al., 2010) and goats (Silva et al., 2005) which may suggest their role in luteal activity.

Since it was established that growth and differentiation factor 9 and bone morphological protein 15 play a crucial role in reproduction processes across species, the genetic variation of them has been widely explored (Otsuka et al., 2011). To date several mutations in *BMP15* and *GDF9* influencing reproduction traits across mammals have been described (de Castro et al., 2016). In ruminants, mainly in sheep, mutations in both genes are a valuable tool for improving proliferation (Fabre et al., 2006). In humans, mutations in *BMP15* contribute to hypergonadotrophic ovarian failure (Di Pasquale et al., 2004), while mutations in both genes contribute to premature ovarian failure (POF) (Dixit et al., 2005; Laissue et al., 2006). Furthermore, polymorphisms found in the coding region of *GDF9* are significantly associated with twinning, but not in *BMP15* (Montgomery et al., 2004; Palmer et al., 2006; Zhao et al., 2008). However, there is still a lack of knowledge about the role and structure of *BMP15* and *GDF9* in horses. Here we describe for the first time the occurrence of 3 SNPs in *BMP15* gene and one STS in *GDF9* gene in equines. Both genes contain two exons and an intervening intron. Even though none of them are causative for MO in horses, intronic variants are worthy to explore as potentially affecting e.g. gene regulation, transcription factors binding sites and/or splicing. Recently, intronic variant in *MSTN* gene is considered as causal mutation and is recognized as speed marker in TBs (Hill et al., 2010). In our study, in *BMP15* g.40851607T>C *loci* TB have higher frequency of C allele.

The screening of entire investigated sequences against Repbase indicated occurrence of non-LTR retrotransposons known as LINEs, polyA retrotranspozons or target-primed retrotransposons. This group of non-autonomous retrotransposons does not contain long terminal repeats (LTR) but integrated mRNA instead (Han, 2010). These elements are ancient and preserved in eukaryotic genomes since prehistoric times (Eickbush et al., 2002). The perissodactyl-specific SINE family of Equine Repetitive Elements (ERE) is derived from tRNAser and occupies about 4% of the horse genome (Sakagami et al., 1994). To date, four main ERE subfamilies have been identified: ERE1-4 (Jurka et al., 2005) and the family of ERE1 has been frequently explored, as it was inserted most recently in the horse genome (Santagostino et al., 2015).

In our study, we localized equine repetitive elements within intron of *BMP15* in promoter region of *GDF9*. The occurrence of transposable elements inside genes or in the close region might disturb their structure and expression by alteration of

gene introduction of promoter sequences or splicing sites (Wheelan et al., 2005). Although there is lack of data about origin and functionality of all ERE elements, it is possible that those found in our study may play a role in establishment of ovulatory state of *Equus* during evolution. However, further analyses are required to better explore this hypothesis.

## Conclusions

Our results indicated that *BMP15* and *GDF9* may play a crucial role in the recruitment of ovarian follicles and regulation of follicular growth, similar as in other animal species. On the other hand, the analysis indicated that frequencies of g.40851607T>C genotype in *BMP15* were significant for Thoroughbred breed, but further investigations are necessary to analyze if identified variations in *BMP15* and *GDF9* are linked with ovarian performance in horses.

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