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DNA characterization and polymorphism of *KISS1* gene in Egyptian small ruminant breeds

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Genetic information especially of the Quantitative Trait Loci (QTL) which affect different performance traits is considered one of the most effective tools in the breeding programs of livestock. Several genes were reported as candidate genes that effect litter size performance and one of these genes is the *KISS1* which is considered as a regulator of puberty onset. The polymorphisms of *KISS1* gene have some relationships with high prolific and sexual precocity. The objective of this study was the detection of the restriction fragment length polymorphism (RFLP) and single nucleotide polymorphisms (SNPs) of *KISS1* gene in six major Egyptian small ruminant breeds. The primers used in this study flanked a 377 bp fragment from intron 1 of *KISS1* gene in sheep and goat. These PCR amplified fragments were digested with *Xmn*I endonuclease. According to the presence or absence of the restriction site (GAANN^NNTTC) at position 121^122, we genotyped the 122 tested animals as AT (54.92%) and TT (45.08) with the absence of AA genotype. The overall frequencies of alleles A and T were 27.46 and 72.54%, respectively. The sequence analysis of purified PCR products representing these two detected genotypes declared the presence of a SNP (T→A) at position 125 in the amplified fragment which is responsible for the elimination of the restriction site and consequently the presence of two different alleles T and A. The nucleotide sequences of sheep *KISS1* alleles T and A as well as goat *KISS1* alleles T and A were submitted to GenBank database and have accession numbers: KP835797, KP835798, KP835799 and KP835800, respectively. It is concluded that small ruminant breeds have high frequency of *KISS1* allele T which was associated with greater litter size. We recommend to increase this allele in Egyptian small ruminant breeds and also to select the animals which possess TT genotypes of *KISS1* gene and enter them in breeding programs of Egyptian small ruminants to increase their fecundity traits.

Key words: Sheep, goat, *KISS1*, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), single nucleotide polymorphisms (SNPs).

INTRODUCTION

Genetic information especially of the QTL which affect different performance traits is considered one of the most effective tools in the breeding programs of livestock. Several genes were reported as candidate genes that

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Abbreviations: RFLP, Restriction fragment length polymorphism; **LH,** luteinizing hormone; **FSH,** follicle-stimulating hormone; **PCR,** polymerase chain reaction.

effect on litter size performance and many studies were carried out to identify these genes and their relation with litter size and prolific status in different farm animals including small ruminants. Some of these genes are G protein-coupled receptor 54 (*GPR54*) gene (Cao et al., 2011), cocaine-amphetamine-regulated transcript (*CART*) gene (Wang et al., 2011), kit ligand (*KITLG*) gene (An et al., 2012) and bone morphogenetic protein receptor IB (*BMPR-IB*) gene (Chu et al., 2010). The *KISS1* gene encodes a family of neuropeptides called kisspeptins, which activate G protein-coupled receptor-54 and play a role in the neuroendocrine regulation of GnRH secretion (Smith et al., 2005). *KISS1* neurons in the hypothalamus has a critical role in reproductive maturation and function including brain-level sex differentiation, puberty onset and the neuroendocrine regulation of gonadotropin secretion and ovulation (Caraty et al., 2010). Also, kisspeptins are reported as regulators for luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion in different mammalian species (Gottsch et al., 2004; Dhillo et al., 2005).

According to the importance of *KISS1* as a regulator of puberty onset, there is a hypothesis that the polymorphisms of *KISS1* gene have some relationships with high prolific and sexual precocity. One novel nonsynonymous single nucleotide polymorphism (G/T) substituting one amino acid in kisspeptin (P/T) has been found to be statistically related to central precocious puberty in human (Luan et al., 2007). These findings indicate that the *KISS1* gene could be an excellent candidate gene for reproductive traits in humans and livestock. In view of the above considerations, the objective of this study was to detect the genetic polymorphism of *KISS1* gene in six major Egyptian small ruminant breeds; Barki, Rahmani and Ossimi sheep breeds as well as Barki, Baladi and Zaraibi goat breeds. Also, this work aimed to identify the single nucleotide polymorphisms in different *KISS1* genotypes which were detected in native small ruminant breeds.

MATERIALS AND METHODS

Blood samples and genomic DNA extraction

The whole blood samples were collected from 122 animals belonging to six native major small ruminant breeds; 32 from sheep Barki, 18 from sheep Rahmani, 24 from sheep Ossimi, 14 from goat Baladi, 16 from goat Barki and 18 from goat Zaraibi. Genomic DNA was extracted from the whole blood according to the method described by Miller et al. (1988) with minor modifications. Briefly, blood samples were mixed with cold 2x sucrose-triton and centrifuged at 5000 rpm for 15 min at 4°C. The nuclear pellet was suspended in lysis buffer, sodium dodecyl sulfate and proteinase K and incubated overnight in a shaking water bath at 37°C. Nucleic acids were extracted with saturated NaCl solution. The DNA was picked up and washed in 70% ethanol. The DNA was dissolved in 1x TE buffer. DNA concentration was determined, using Nano Drop1000 Thermo Scientific spectrophotometer and then diluted to the working concentration of 50 ng/µl which is suitable for polymerase chain reaction.

Polymerase chain reaction (PCR)

A PCR amplification reaction was performed using specific primer that was designed on the basis of DNA sequence of the *KISS1* gene (Accession: D00476) (An et al., 2013a): F: CCC GCT GTA ACT AGA GAA AG; R: CAT CCA GGG TGA GTG ATA CT

A PCR cocktail consisted of 1.0 µM of forward and reverse primer, 0.2 mM dNTPs, 10x of PCR reaction buffer and 1.25 units of Taq polymerase (Fermentas) was used. The cocktail was added into PCR tubes with 100 ng of sheep or goat DNA. The reaction was run at 94°C for 5 min, 35 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide to test the amplification success.

Restriction fragment length polymorphism (RFLP)

The PCR products were digested using restriction enzyme; *Xmn*I (Fermentas). 10 µl of PCR product was digested with 1 µl of FastDigest restriction enzyme for 15 min at 37°C. The restriction fragments were subjected to electrophoresis in 2% agarose ethidium bromide gel in 1x TBE buffer (0.09 M Tris-boric acid and 0.002 M EDTA). Gels were visualized under UV light and documented in FX Molecular Imager apparatus (BIO-RAD).

Sequencing analysis and single nucleotide polymorphism

The PCR products -representatives for each detected genotype of *KISS1* gene in different sheep and goat breeds were purified and sequenced by Macrogen Incorporation (Seoul, Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite to identify each single nucleotide substitution between different detected genotypes. Results of endouclease restriction were carried out using FastPCR. The nucleotide sequence of each genotype for Egyptian sheep and goat *GH* gene was submitted to GenBank (NCBI, BankIt).

RESULTS AND DISCUSSION

Kisspeptins, the product of the *KISS1* gene, play an essential role in the regulation of reproductive functions, acting primarily at the hypothalamic level of the gonadotropic axis (An et al., 2013a). Kisspeptins have been identified as the natural ligands for an orphan G-protein-coupled receptor, GPR54, and it is becoming clear that the hypothalamic KISS1/GPR54 system plays a crucial permissive role in controlling the onset of puberty by regulating the release of gonadotrophin-releasing hormone (GnRH) from hypothalamic neurons (Messager et al., 2005; d'Anglemont de Tassigny et al., 2007; Smith et al., 2007). In a view of the importance of *KISS1* as a regulator of puberty onset, there is a hypothesis that the polymorphisms of *KISS1* have some relationships with high prolific in small ruminant (Cao et al., 2010). So far, there have been some studies of the *KISS1* gene as a candidate gene for reproductive traits in animals, which revealed that the *KISS1* gene plays an important role in animal reproduction (Tomikawa et al., 2010). We aimed in this study to detect RFLP and SNP polymorphisms of KISS1 gene in three Egyptian sheep breeds; Barki, Rahmani and Ossimi as well as three Egyptian goat

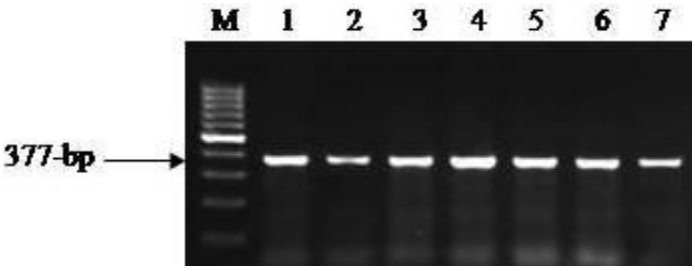

Figure 1. Ethidium bromide-stained gel of PCR products representing amplification of *KISS1 g*ene in Egyptian sheep and goat animals. Lane M: 100 bp ladder marker. Lanes 1 to 7, 377 bp PCR products amplified from sheep and goat DNA.

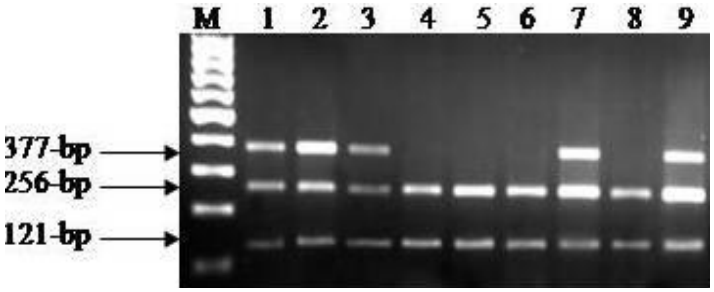

Figure 2. The electrophoretic pattern obtained after digestion of PCR amplified fragment of *KISS1* gene from sheep and goat DNA with *Xmn*I restriction enzyme. Lane M: 100 bp ladder marker. Lanes 1 to 3, 7 and 9, AT heterozygous genotype with three digested fragments at 377, 256 and 121 bp. Lanes 4 to 6 and 8, TT homozygous genotype with two digested fragments at 256 and 121 bp.

CCCGCTGTAACTAGAGAAAGCCCATGTGCAGAAGGCCCTGTGCTGCCAAAACTAAAATAAATTTTAAAAAGATAT
TCCAGTGCAAAGAGATCATGGACGCGGGTGTCCTTGGCCAAGAACT^TCTTCTCTCCTGGGATCGGGTGCTCTT
TCTGGGTAAGGGAGGATCCCCCGGAGAATAACAATGTCATCACCGCCCGGGGGGACGCTGAGCTCTTGGCTCT
TCTTGGCAAAATCTTTTAGGTGATGCTAAAAACAGGCATGCCTAAGTAGCGATCCACTTGCTGGGATGATGGCTG
TAGCTGGAAAAGAGGTCATCGTCCCTCCCATCTCTGCCAGGCCCAGGGCCCTCCTAGGAAGAGTATCACTACCC
CTGGATG

Figure 3. Endonuclease restriction of amplified fragment from sheep and goat *KISS1* using FastPCR. GAACT^TCTTC; the restriction site in red.

breeds; Barki, Baladi, and Zaraibi. The primers used in this study flanked a 377 bp fragment from intron 1 of *KISS1* gene in sheep and goat. The amplified fragments obtained from all tested sheep and goat animals were at 377 bp (Figure 1).

These PCR amplified fragments (377 bp) were digested with *Xmn*I endonuclease. Depending on the presence or absence of the restriction site (GAANN^NNTTC) (N = A or T or C or G) at position 121^122, we can easily differentiate between 3 different genotypes: AA with undigested one fragment at 377 bp, TT with two digested fragments at 256 and 121 bp and AT with three digested fragments at 377, 256 and 121 bp. The results showed the presence of two genotypes; AT and TT with the absence of AA genotype in 122 tested animals for this gene (Figures 2 and 3). The frequencies of AT and TT genotypes were 62.5 and 37.5% in sheep Barki animals (32 animals), 55.56 and 44.44% in sheep Rahmani animals (18 animals) and 45.83 and 54.17% in sheep Ossimi animals (24 animals), respectively, with the total frequencies of 55.41 and 44.59% for AT and TT genotypes, respectively, in 74 tested sheep animals for this gene. In tested goat animals, the frequencies of AT and TT genotypes were 57.14 and 42.86% for Baladi (14

Table 1. The genotype and allele frequencies of *KISS1* gene in Egyptian sheep and goat breeds.

Animal	Breed	Number of animals	Genotype frequencies		Allele frequencies	
			AT%	TT%	A%	T%
Sheep	Barki	32	62.50	37.50	31.25	68.75
	Rahmani	18	55.56	44.44	27.78	72.22
	Ossmi	24	45.83	54.17	22.92	77.08
	Total	74	55.41	44.59	27.70	72.30
Goat	Baladi	14	57.14	42.86	28.57	71.43
	Barki	16	62.50	37.50	31.25	68.75
	Zaraibi	18	44.44	55.56	22.22	77.78
	Total	48	54.17	45.83	27.08	72.91
	Overall	122	54.92	45.08	27.46	72.54

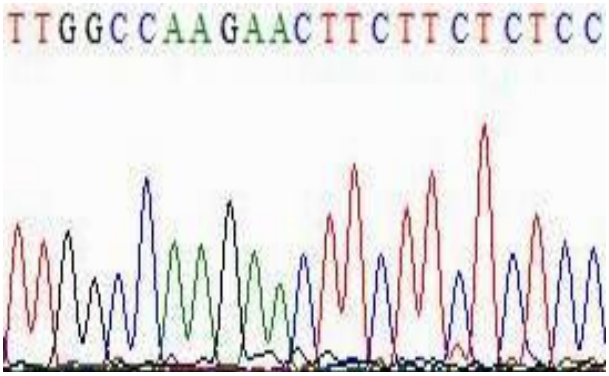

Figure 4. Genotype T/T.

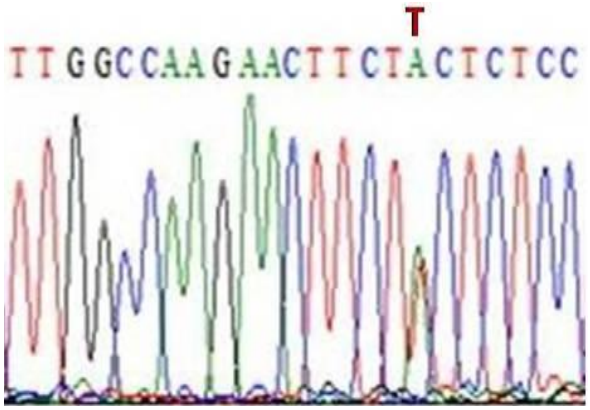

Figure 5. Genotype T/A.

animals), 62.5 and 37.5% for Barki (16 animals) and 44.44 and 55.56% for Zaraibi (18 animals), respectively, with total frequencies of 54.17 and 45.83% for AT and TT genotypes, respectively, in 48 tested goat animals for this gene. The overall frequencies for genotypes AT and TT as well as alleles A and T in all 122 tested animals were 54.92, 45.08, 27.46 and 72.54%, respectively (Table 1). The nucleotide sequences of two different genotypes T/T (Figure 4) and T/A (Figure 5) which were detected in this study declared the presence of one SNP substitution (T→A) at position 125 in the amplified fragments of sheep and goat *KISS1* gene (Figure 6) which is responsible for the elimination of the restriction site GAACT^TCTTC and consequently the appearance of two different alleles T and A. The nucleotide sequences of sheep *KISS1* alleles T and A as well as goat *KISS1* alleles T and A were submitted in GenBank database and have accession numbers: KP835797, KP835798, KP835799 and KP835800, respectively.

An et al. (2013a and b) detected polymorphisms of the goat *KISS1* gene in three Chinese goat breeds using PCR-RFLP and DNA sequencing methods. Two novel SNPs were identified in the intron 1 of the *KISS1* gene. The 2124T>A and 2270C>T SNPs were significantly associated with litter size where the combined alleles of T in both loci with greater litter size than the combined alleles of A and C. The frequencies of alleles T and A in first locus were 0.60 and 0.40, 0.59 and 0.41 and 0.55 and 0.45 in the three tested goat breeds. Whereas, the frequencies of alleles T and C in the second locus were 0.60 and 0.40, 0.59 and 0.41 and 0.64 and 0.36 in these tested goat breeds. On the other hand, Cao et al. (2010) used three pairs of primers to clone the goat *KISS1* and scan polymorphisms and four pairs to detect polymorphisms in sexual precocious and sexual late-maturing goat breeds. The genotype distribution did not show obvious difference between sexual precocious and sexual late-maturing goat breeds and no consistency within the sexual late-maturing breeds. This study preliminarily indicated an association between allele C in *KISS1* gene and high litter size in Jining Grey goats. Chu et al. (2012) analyzed SNPs in exon 1 of *KISS1* gene in high fecundity sheep breeds (small Tail Han and Hu breeds) and low fecundity sheep breeds (Dorset, Texel and Corriedale breeds) by PCR-SSCP. Polymorphisms in

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Allele T: 1   CCCGCTGTAACTAGAGAAAGCCCATGTGCAGAAGGCCCTGTGCTGCCAAAACTAAAATAA  60
Allele A: 1   CCCGCTGTAACTAGAGAAAGCCCATGTGCAGAAGGCCCTGTGCTGCCAAAACTAAAATAA  60
              ************************************************************

Allele T: 61  ATTTTAAAAAGATATTCCAGTGCAAAGAGATCATGGACGCGGGTGTCCTTGGCCAAGAAC  120
Allele A: 61  ATTTTAAAAAGATATTCCAGTGCAAAGAGATCATGGACGCGGGTGTCCTTGGCCAAGAAC  120
              ************************************************************

Allele T: 121 TTCTTCTCTCCTGGGATCGGGTGCTCTTTCTGGGTAAGGGAGGATCCCCCGGAGAATAAC  180
Allele A: 121 TTCTACTCTCCTGGGATCGGGTGCTCTTTCTGGGTAAGGGAGGATCCCCCGGAGAATAAC  180
              **** ********************************************************

Allele T: 181 AATGTCATCACCGCCCGGGGGGACGCTGAGCTCTTGGCTCTTCTTGGCAAAATCTTTTAG  240
Allele A: 181 AATGTCATCACCGCCCGGGGGGACGCTGAGCTCTTGGCTCTTCTTGGCAAAATCTTTTAG  240
              ************************************************************

Allele T: 241 GTGATGCTAAAAACAGGCATGCCTAAGTAGCGATCCACTTGCTGGGATGATGGCTGTAGC  300
Allele A: 241 GTGATGCTAAAAACAGGCATGCCTAAGTAGCGATCCACTTGCTGGGATGATGGCTGTAGC  300
              ************************************************************

Allele T: 301 TGGAAAAGAGGTCATCGTCCCTCCCATCTCTGCCAGGCCCAGGGCCCTCCTAGGAAGAGT  360
Allele A: 301 TGGAAAAGAGGTCATCGTCCCTCCCATCTCTGCCAGGCCCAGGGCCCTCCTAGGAAGAGT  360
              ************************************************************

Allele T: 361 ATCACTACCCCTGGATG 377
Allele A: 361 ATCACTACCCCTGGATG 377
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Figure 6. Nucleotide sequences and alignment between *KISS1* alleles T and A. T/A substitution at position 125.

exon 1 of *KISS1* gene were detected in prolific Small Tail Han sheep (AA, AB and BB genotypes) and in Hu sheep (AA and CC genotypes) and on the other hand, no polymorphism was found in low fecundity sheep breeds (only AA genotype). These results preliminarily indicated that the *KISS*1 gene may have some associations with prolificacy in sheep.

Our result matches with the previous results obtained by An et al. (2013a and b), where they studied the genetic polymorphism of *KISS1* gene in three Chinese goat breeds and recorded the association of T→A substitution with the litter size. They reported that frequency of allele T ranged from 0.55 to 0.60 and frequency of allele A ranged from 0.40 to 0.45 in the three tested Chinese goat breeds. These frequencies very close to the frequencies of T and A alleles in our animals. The allele T was reported to be associated with greater litter size, so we recommend to increase this allele in Egyptian small ruminant breeds and also to select the animals possess TT genotypes of *KISS1* gene and enter them in breeding programs of Egyptian small ruminants to increase their fecundity traits

Conflict of interests

The author(s) did not declare any conflict of interest.

REFERENCES

An XP, Han P, HouJX, Zhao HB, Yan Y, Ma T, Fang F, MengFX, Song YX, Wang JG, Cao BY (2013a). Molecular cloning and characterization of *KISS1* promoter and effect of *KISS1* gene mutations on litter size in the goat. Genet. Mol. Res. 12(4):4308-4316.

An XP, Hou JX, Li G, Song YX (2012).Polymorphism identification in the goat KITLG gene and association analysis with litter size. Anim. Genet. 43:104-107.

An XP, Ma T, Hou JX, Fang F, Han P, Yan Y, Zhao HB, Song YX, Wang JG, Cao PY (2013b). Association analysis between variants in KISS1gene and litter size in goats. BMC Genetics 14:63-68.

Cao GL, Chu MX, Fang L, Di R (2010).Analysis on DNA sequence of KiSS-1 gene and its association with litter size in goats. Mol. Biol. Rep. 37:3921-3929.

Cao GL, Chu MX, Fang L, Feng T (2011).Analysis on DNA sequence of GPR54 gene and its association with litter size in goats. Mol. Biol. Rep. 38:3839-3848.

Caraty A, Franceschini I, Hoffman GE (2010).Kisspeptin and the preovulatorygonadotrophin-releasing hormone/ luteinising hormone surge in the ewe: basic aspects and potential applications in the control of ovulation. J. Neuroendocrinol. 22:710-715.

Chu M, Xiao C, Feng T, Fu Y, Cao G, Fang L, Di R, Tang Q, Huang D, Ma Y, Li K, Li N (2012). Polymorphisms of KiSS-1 and GPR54 genes and their relationships with litter size in sheep. Mol. Biol. Rep. 39:3291-3297

Chu MX, Zhao XH, Zhang YJ, Jin M (2010). Polymorphisms of BMPR-IB gene andtheir relationship with litter size in goats. Mol. Biol. Rep. 37:4033-4039.

d'Anglemont de Tassigny X, Fagg LA, Dixon JP, Day K(2007). Hypogonadotropichypogonadism in mice lacking a functional Kiss1 gene. Proc. Natl. Acad. Sci. USA 104:10714-10719.

Dhillo WS, Chaudhri OB, Patterson M, Thompson EL (2005). Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J. Clin. Endocrinol. Metab. 90:6609-6615.

Gottsch ML, Cunningham MJ, Smith JT, Popa SM (2004). A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073-4077.

Luan X, Zhou Y, Wang W, Yu H (2007).Association study of the polymorphisms in the KISS1 gene with central precocious puberty in Chinese girls. Eur. J. Endocrinol. 157:113-118.

Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA (2005).Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc. Natl. Acad. Sci. USA 102:1761-1766.

Miller SA, Dykes DD, Polesky HF (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16:1215.

Smith JT, Clay CM, Caraty A, Clarke IJ (2007). KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology 148(3):1150-1157

Smith JT, Cunningham MJ, Rissman EF, Clifton DK (2005). Regulation of *Kiss1* gene expression in the brain of the female mouse. Endocrinology 146:3686-3692.

Tomikawa J, Homma T, Tajima S, Shibata T (2010). Molecular characterization and estrogen regulation of hypothalamic KISS1 gene in the pig. Biol. Reprod. 82:313-319.

Wang PQ, Deng LM, Zhang BY, Chu MX (2011). Polymorphisms of the cocaine-amphetamine-regulated transcript (*CART*) gene and their association with reproductive traits in Chinese goats. Genet. Mol. Res. 10:731-738.