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Relative abundance of tissue mRNA and association of the single nucleotide polymorphism of the goat NGF gene with prolificacy

Thomas Naicy[a,*], R. Thirupathy Venkatachalapathy[b], T.V. Aravindakshan[b], K.C. Raghavan[b], M. Mini[c], K. Shyama[d]

[a] Department of Animal Breeding, Genetics and Biostatistics, College of Veterinary and Animal Sciences, Mannuthy, Thrissur-680651, Kerala, India
[b] Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur-680651, Kerala, India
[c] Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur-680651, Kerala, India
[d] Department of Animal Nutrition, College of Veterinary and Animal Sciences, Mannuthy, Thrissur-680651, Kerala, India

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ABSTRACT

Nerve Growth Factor (NGF) promotes the development of pre-antral ovarian follicles through ovarian innervations and regulation of ovarian response to gonadotropins. The present study was conducted to study the tissue gene expression profile, to characterize the genetic variants, find associations of the NGF gene with prolificacy in the prolific Malabari and less prolific Attappady Black goats because NGF has an important role in reproduction by augmenting ovarian folliculogenesis. Relative abundance of NGF mRNA was greatest in reproductive tissues signifying its role in reproduction. The PCR-SSCP analysis of a 251 bp fragment of Exon 3 of the NGF gene from the 277 goats revealed four diplotypes (EE, EF, FF and EG) with respective frequencies of 0.76, 0.22, 0.01 and 0.01. Sequencing of the representative samples revealed one synonymous and one novel non synonymous mutations (g.705G > A and g.715C > T). Statistical analysis indicated that the SNP g.705G > A was associated with litter size in Attappady Black goats ($P < 0.05$) and a PCR-RFLP was designed using the restriction enzyme, *BpiI*, for rapid screening of the SNP. The results of the present study suggest that the NGF gene is a primary candidate gene affecting prolificacy in goats and may be used for Marker Assisted Selection (MAS) in goats, especially in lowly prolific Attappady Black goats.

1. Introduction

The livestock sector has an important role in the success of India's self-sufficiency in food production. Goats, as the second most important livestock species next to cattle, contribute substantially to this achievement. Reproductive traits in livestock are polygenic in nature and difficult to improve through conventional breeding methods. The identification of candidate genes and associations of these genes with reproductive traits in farm animals could assist in the genetic improvement of breeding stock. Prolificacy is one of the most important reproductive traits for the selection of multiparous animals, as the heritability of the reproductive traits are low, traditional selection methods can be complemented with gene assisted selection, to increase the intensity of selection (Bajhau and Kennedy, 1990; Menchaca et al., 2002; Li et al., 2010; Akpa et al.,

* Corresponding author.
E-mail address: naicy@kvasu.ac.in (T. Naicy).

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Table 1
Oligonucleotide primers used for PCR-SSCP of NGF 251 bp fragment and real time PCR.

Primer name	Primer sequence(5′-3′)	Product size (bp)	Annealing temperature (°C)	Accession Number
NGF-F	5′-TTGAGACCAAGTGCCGGG-3′	251	63	JQ308184
NGF-R	5′-GCAGGAGAGTGTAGAGGGGA-3′			
NGFRT-F	5′- GTCATCCCACCCCGTCTTTC – 3′	111	60	JQ308184
NGFRT-R	5′- ACCATCACCTCCTTGCCCTT – 3′			
GAPDHF	5′-TGGAGAAACCTGCCAAGTATG-3′	127	60	XM_005680968
GAPDHR	5′- TGAGTGTCGCTGTTGAAGTC –3′			

2011). Information about genes that influence fecundity is an important aspect in genetic improvement and extensive efforts are in progress for the identification of important candidate genes involved in the control of prolificacy (Li et al., 2011).

Nerve Growth Factor is a 26 kDa protein that belongs to the beta nerve growth factor family (Ebendal, 1992) that is required for the survival, maintenance and development of discrete neuronal populations in the nervous system. The major site of synthesis of NGF is the ovary, and NGF is responsible for ovarian innervation, follicular assembly and folliculogenesis (Ojeda et al., 2000). Nerve Growth Factor and its receptors have an important role in development of the mammalian ovary, oogenesis and folliculogenesis (Chaves et al., 2010). Significant increase in the expression of FSH and LH receptor genes in the oviduct after treatment with NGF also indicated the role of NGF in regulating the reproduction via the interactions with gonadotropins (Li et al., 2014). An Ovulation Inducing Factor (OIF), a potent component in the seminal plasma of alpacas and llamas (induced ovulators) which can elicit a surge in circulating concentrations of luteinizing hormone (LH) and induce ovulatory and luteotropic responses (Adams et al., 2005) have considerable sequence homology with β-NGF (Ratto et al., 2012), which has a luteotropic effect by altering the secretion pattern of LH, increasing the plasma LH concentration to induce ovulation and the subsequent development of a functional corpus luteum in llamas (Fernandez et al., 2014; Ulloa-Leal et al., 2014). Tribulo et al. (2015) documented the existence of OIF/NGF in seminal plasma of bulls which had an effect on both ovulation and luteal function in cattle.

Even though the role of NGF in reproduction is clear, evaluation of NGF as a candidate gene for genetic selection for reproduction in animals, especially prolific animals has not been conducted extensively and systematically. The literature concerning NGF and goat litter size is limited (An et al., 2013). Thus, the objectives of the present study are to determine the tissue expression profile of the NGF gene in reproductive tissues of goats, to characterize some genetic variants of the NGF gene and to ascertain associations with prolificacy.

2. Materials and methods

2.1. Animals and sample collection for DNA and RNA isolation

The tissue samples of the ovary, uterus, oviduct, muscle and liver were collected from six goats 4 to 5 years of age from the Kerala Veterinary and Animal Science University Meat Plant. Sufficient care was taken to collect the samples from regularly estrous cycling goats at the mid-estrus stage of the cycle. Approximately 100 mg of tissues were collected and immediately immersed in RNAlater® (Sigma-Aldrich) and stored at −80 °C until the isolation of RNA. Total RNA from the tissue samples was extracted using the Gen Elute mammalian total RNA miniprep kit (RTN10, Sigma Aldrich) and treated with *DNaseI* (DNase1 kit, Sigma–Aldrich) to prevent genomic DNA contamination. The RNA was quantified by NanoDrop spectrophotometer (Thermo Scientific, USA) and analyzed on 1% agarose gel. The cDNA was synthesized using the RevertAid first strand cDNA synthesis kit (Thermo Scientific, K1622) with 1 μg of RNA in a reaction volume of 20 μL and was stored at −80 °C until use.

A total of 277 adult female goats belonging to the Malabari ($n = 175$) and Attappady Black breeds ($n = 102$) were included in the present research from three farms (Thrissur, Malappuram and Palakkad districts) of Kerala, India. Selection of the goats was carefully conducted so animals were genetically unrelated to a maximum possible extent based on the pedigree records available at the respective farms. The genomic DNA from the white blood cells was extracted from venous blood (6 mL) collected from the jugular vein by the standard phenol chloroform method. The litter size data of these animals were collected for different parities from the records available at respective farms.

2.2. Reverse transcriptase-quantitative real-time PCR (qRT-PCR)

The relative abundance of NGF mRNA in the ovary, uterus, oviduct, liver and muscle tissues was measured by qRT-PCR using SYBR green chemistry (Thermo Scientific) in goats (n = 6). Three technical replicates per sample were analyzed. The fold change in the relative abundance of the mRNA was normalized by assessing the abundance of GAPDH mRNA. Primer pairs for goat NGF and GAPDH were designed using Primer3 software (Table 1). The PCR reaction efficiency assessment and analysis of relative abundance of mRNA were conducted using the Illumina Eco® Q- RT PCR system. The PCR efficiencies were determined by using standard curves with seven serial dilutions of the cDNA. Quantitative real time PCR was conducted in a 25 μL reaction volume containing 50 ng of cDNA and 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The reactions were performed using a pre-incubation of 95 °C for 10 min, 40 quantification cycles consisting of a denaturation of 95 °C for 30 s, annealing of 60 °C for 15 s and an extension of 72 °C for 30 s, followed by a dissociation curve analysis at 95 °C for 15 s, 60 °C for 15 s and

95 °C for 15 s to ensure the specificity of the PCR, which was confirmed by a single peak. The standard deviation of the crossing point value (CP) for the reference gene (GAPDH) was 0.41 and the coefficient of variance (CV) was 2.76% for the tissue samples under study. The relative abundance of NGF mRNA in different tissues was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and statistical comparisons between samples was performed using analysis of variance (ANOVA) and subsequently Duncan's multiple range test to find out the homogeneous subsets (SPSS V.21). *P*- value < 0.05 was considered as statistically significant.

2.3. PCR-SSCPof NGF gene 251 bp fragment and SNP identification

The goat NGF gene 251 bp fragment was amplified from genomic DNA using the primers designed from the published caprine Exon 3 sequences (GenBank accession No. KF724722) using Primer3 software (Table 1). The PCR was performed with these primers to amplify the region from the genomic DNA to identify the genetic variants. The PCR was performed in 25 μL reaction volume with 50 ng genomic DNA, 2.5 μL 10× reaction buffer, 200 μM dNTP (Fermentas), 10 pM of each primers (Sigma-Aldrich) and 0.5 U of Taq DNA polymerase (Sigma-Aldrich). The cycling protocol following the initial denaturation occurred at 95 °C for 3 min followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 63 °C for 20 s, extension at 72 °C for 30 s with a final extension at 72 °C for 5 min in a thermal cycler (Applied Biosystems). The PCR products were subjected to SSCP, for which initial denaturation was carried out by mixing 5 μL of the PCR product with a 10 μL denaturing buffer (9.5 mL formamide, 0.4 mL of 0.5 M EDTA, 2.5 mg xylene-cyanole and 2.5 mg bromophenol blue/10 mL), centrifuged, denatured at 95 °C for 10 min and then snap chilled on ice for 10 min. After which the contents were separated using 12% vertical polyacrylamide gel electrophoresis at 140 V for 18 h under refrigerated conditions. The SSCP patterns were visualized using silver nitrate staining, photographed and analyzed. Representative PCR products, which showed different banding patterns in SSCP analysis were sequenced to detect nucleotide variation and sequences were aligned by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

2.4. Statistical analysis

Allele frequency differences between Malabari and Attappady Black population were tested by Fisher's Exact and Chi-Square Test using SPSS V.21 (Table 2). The association between SNPs (genotypes) and litter size data were analyzed with the following fixed General Linear Model (SPSS V.21).

$$y_{ijklm} = \mu + p_i + c_j + g_k + w_l + e_{ijklm}$$

where Y_{ijklm} is the litter size measured on ijklmth animal, μ represents the overall population mean, p_i is the fixed effect associated with ith parity (i = 1, 2, 3), c_j is the fixed effect associated with jth centre (j = 1, 2, 3), g_k is the fixed effect associated with kth genotype (k = 1, 2) of *NGF* locus, w_l is the fixed effect associated with lth dam's body weight group, (l = 1, 2, 3, 4, 5, ie, 20–25 kg, 26–30 kg, 31–35 kg, 36–40 kg, 41–45 kg, respectively) and e_{ijklm} is the random error. The effects associated with age of dam, sire and season of birth were not included in the general linear model because the preliminary analyses indicated that the effect of these factors on litter size is non-significant in the goat population under study. Data were reported as least square means (LSM ± SE). *P*- value <0.05 was considered as statistically significant.

Table 2
Diplotype and genotype distribution and haplotype and allele frequencies of two single nucleotide polymorphism loci in the caprine NGF gene.

Locus	Genetic structure	Breed	
		Malabari	Attappady Black
	Diplotype		
(g.705G > A & g.715C > T)	EE	126	85
	EF	48	16
	FF	1	–
	EG Haplotype	–	1
	E	0.85	0.91
	F	0.15	0.08
	G	–	0.01
g.705G > A	Genotype		
	GG	126	86
	GA	48	16 –
	AA	1	
	Allele		
	G	0.86	0.92
	A	0.14	0.08
	Equilibrium χ^2 test	*P* > 0.05	*P* > 0.05
g.715C > T	Genotype		
	CC	126	101
	CT	–	1
	Allele		
	C	1.00	0.99
	T	–	0.01

3. Results

3.1. Tissue distribution of NGF mRNA

Quantitative real time PCR was performed to determine the relative tissue abundance of *NGF* mRNA in the ovary, uterus, oviduct, muscle and liver. Relative abundance was greater ($P \leq 0.01$) in the ovary, followed by oviduct, uterus ($P \leq 0.05$), liver and muscle (Fig. 1). This indicates a greater abundance of *NGF* gene in reproductive tissues. The melt curve analysis revealed a single peak, associated with the high specificity of the reaction without any non- specific amplification or primer dimer formation (Supplementary Fig. 1).

3.2. Characterisation of NGF Exon 3

A 251 bp fragment of *NGF* gene at Exon 3 was amplified by PCR in all the 277 goats belonging to the two breeds. Single strand conformation polymophism analysis revealed a polymorphic banding pattern with four distinct patterns,

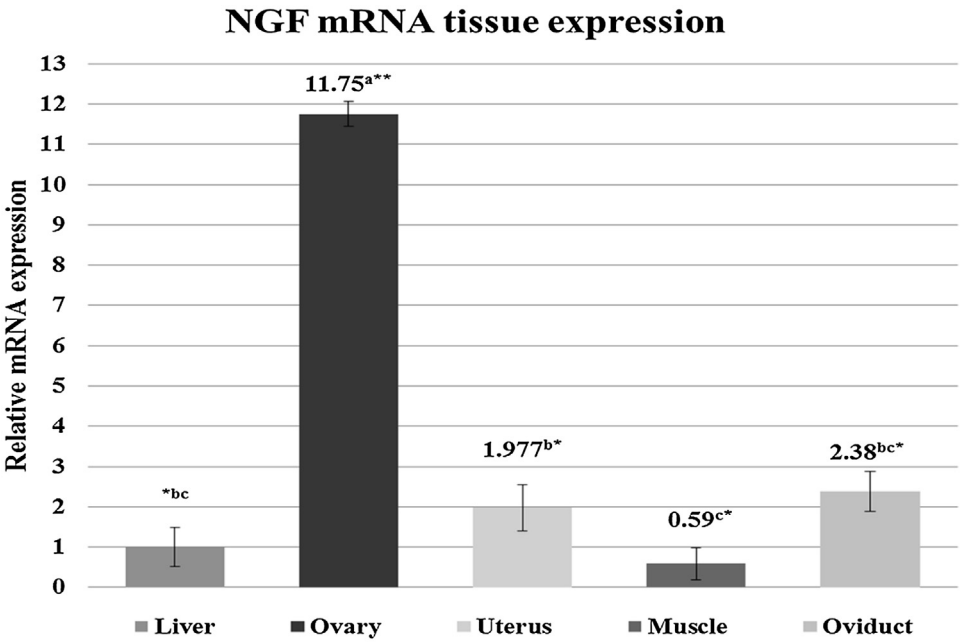

Fig. 1. Transcript relative abundance of NGF mRNA in different tissues of goats; Values with different superscripts differ (* $P < 0.05$, ** $P < 0.01$).

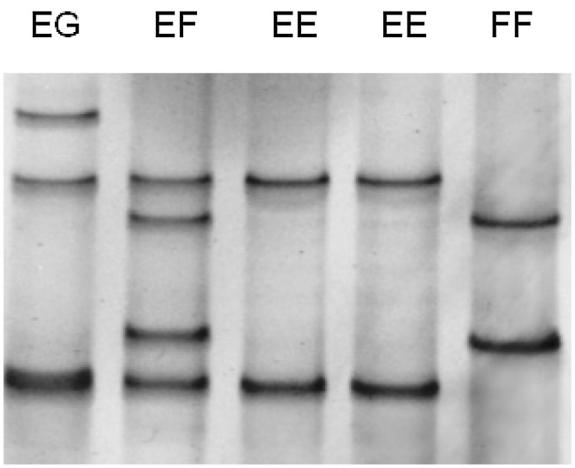

Fig. 2. Banding pattern of SSCP of 251 bp fragment of NGF gene with EE, EF, FF and EG diplotypes.

designated as EE, EF, FF and EG (Fig. 2). Sequencing of the PCR products from each type, revealed two SNPs at 182 (G → A transition) and 192 (C → T transition) positions of the 251 bp PCR product (705th and 715th positions of ORF) (Fig. 3). Data for diplotypic and genotypic distribution as well as haplotype and allele frequencies of two SNPs are included in Table 2. Sequences of the new genetic variants were submitted to GenBank as haplotypes E, F and G (Supplementary Fig. 2) with Accession Numbers KX029328, KX029329 and KX029330, respectively.

3.3. Association of NGF genotypes with litter size in goats

The least square means with standard error of litter size for different *NGF* haplotypes in prolific Malabari and low prolific Attappady Black are presented in Table 3. Litter size was less ($P < 0.05$) for the EF haplotype (1.25 ± 0.11), corresponding to the GA genotype, compared with the EE haplotypes (1.47 ± 0.08), corresponding to GG genotype at 705th position of the NGF ORF in Attappady Black goats. A similar observation was noted in the Malabari breed, but the difference was not significant. (Haplotypes FF and EG were not included in the analysis due to a lesser frequency in the population under study). A PCR-RFLP was designed using the restriction enzyme *BpiI* for the rapid screening of the population for the SNP, g.705G > A, which was found to be associated with litter size in goats (Fig. 4).

Table 3
Least square means with standard error of goat litter size for different *NGF* genotypes.

Locus	Genotype	Diplotype	Malabari	Attappady Black
g.705G > A	GG	EE	1.82 ± 0.08	1.47 ± 0.08
	GA	EF	1.72 ± 0.13	1.25 ± 0.11
	AA[a]	FF	–	–
	P value		$P > 0.05$	$P < 0.05$
g.715C > T[a]	CC	EE	–	–
	CT	EG	–	–

[a] Not included in the analysis, because number of observations was less than five.

4. Discussion

The reproduction traits are of great economic importance and need special attention to augment the production efficiency in goats. Improving litter size traits in goats could be an important aspect for enhancing the profitability of goat rearing. As the heritability of reproductive traits is low, the genetic improvement is difficult and time consuming task using conventional selection methods (Zhang et al.,

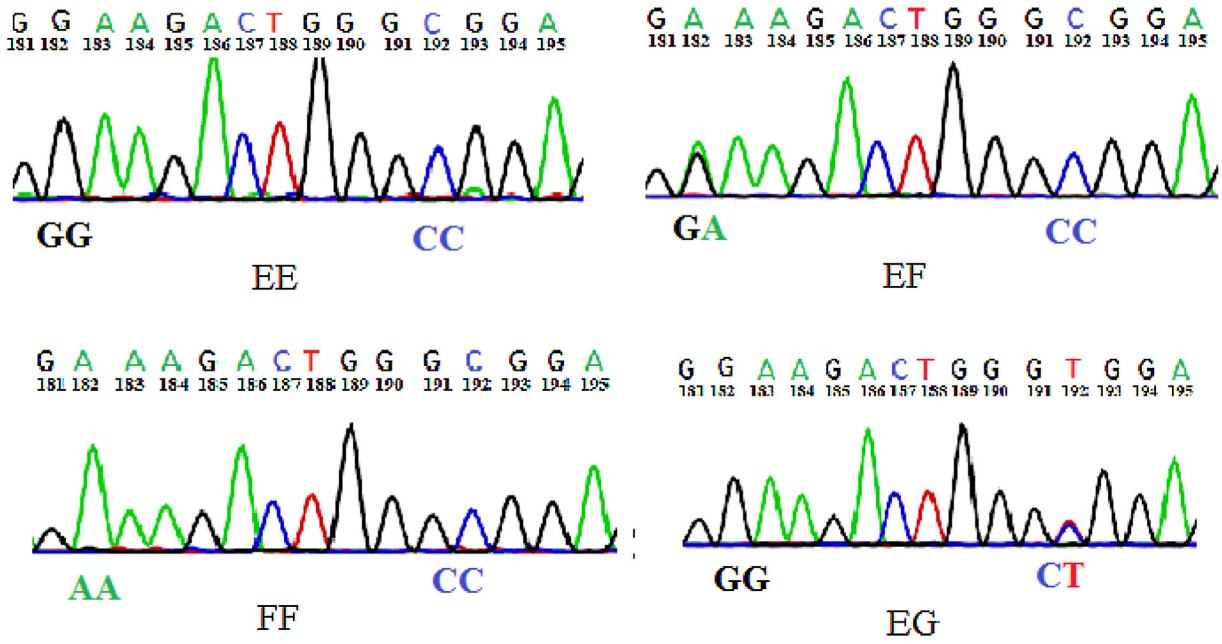

Fig. 3. Sequence maps of EE, EF, FF and EG diplotypes with g.705G > A and g.715C > T loci of the goat NGF gene.

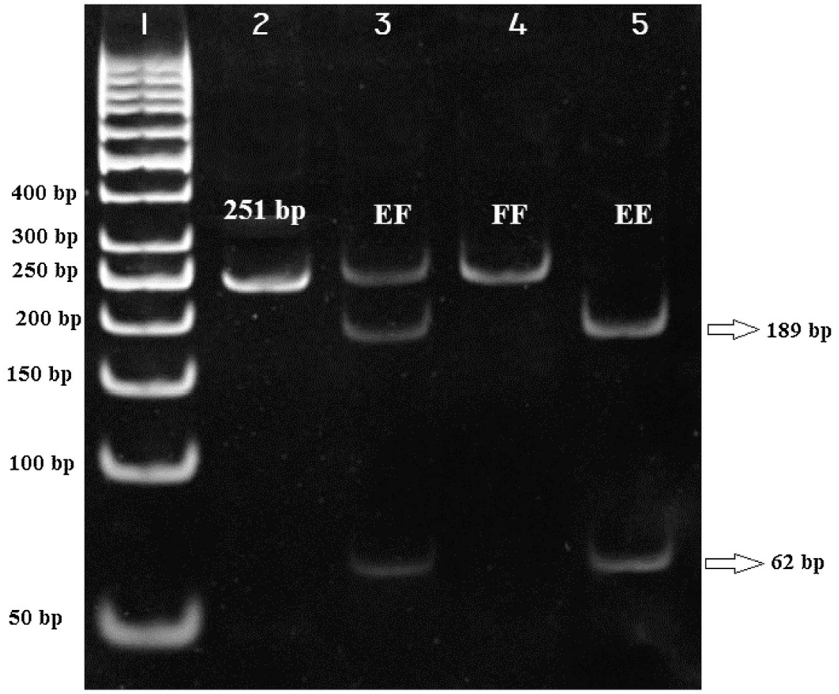

Fig. 4. Pattern of *BpiI* digestion of 251 bp with GG, GA and AA genotypes at g.705G > A locus – Lane 1: 50 bp marker; Lane 2: PCR product of 251 bp; Lane 3: GG genotype; Lane 4: AA genotype; Lane 5: GA genotype.

2009; Zhang et al., 2013). Nerve Growth Factor belongs to neurotrophin family of proteins and has an important role in the differentiation and survival of neurons of the nervous system, is involved in the development of the ovary and regulates the expression of FSH and LH receptor genes thus having a role in ovarian steroidogenesis and folliculogenesis (Zheng et al., 1996; Zhang et al., 2001; Dissen et al., 2002; Abir et al., 2005; Garcia-Rudaz et al., 2011). The present study was undertaken to elucidate the influence of the goat NGF gene on litter size through NGF transcript analysis and screening of the genetic variants.

The extent of expression of the NGF gene was quantified in different tissues by qRT-PCR. The relative abundance of NGF mRNA was greater ($P \leq 0.01$) in the ovary followed by the oviduct, uterus, liver and muscle. These observations were in agreement with that of the relative abundance of human NGF mRNA (www.proteinatlas.org/). The relative abundance of NGF mRNA was greatest in reproductive tissues, indicating NGF may have an important role in mammalian reproduction especially in follicle development and oocyte maturation. Mayerhofer et al. (1996) and Salas et al. (2006) reported the role of NGF in ovarian angiogenesis, cell proliferation and ovulation and the precise role of NGF as a factor responsible for follicular survival was also previously reported (Chaves et al., 2010). Because relative abundance of NGF mRNA was greatest in the ovary, the polymorphism detected may affect folliculogenesis and number of ova per estrus and indirectly the litter size in goats; further studies are required for the verification of these results.

An et al. (2013) reported that a single nucleotide polymorphism in the NGF gene of goats was associated with litter size in goats. To study the distribution of this SNP in the two breeds of goats, a set of primers was designed to amplify the region covering the identified SNP with an expected amplicon size of 251 bp. The genomic DNA from both breeds were subjected to PCR-SSCP to obtain genetic variants and representative PCR products from different banding patterns were sequenced and analyzed. In addition to the previously detected g.705G > A, another novel non synonymous mutation at g.715C > T was observed, which resulted in four haplotypes (EE, EF, FF and EG). Because haplotype frequencies of FF and EG were few in the population, these haplotypes were not included in the association analysis with goat litter size. The association analysis of the goat *NGF* gene with litter size in goats revealed that the SNP, g.705G > A had a significant effect on litter size as reported by An et al. (2013) by PCR-RFLP. Though in the present study, the mutation of g.705G > A is a synonymous mutation, this mutation could lead to the stability of the mRNA and affect the mechanism of mRNA deadenylation and degradation (Sauna and Kimchi-Sarfaty, 2011). As the allele frequency of novel non-synonymous mutation at the g.715C > T locus was few in number in the population (which changes positively charged Arginine to nonpolar Tryptophan), the association analysis with reproduction traits could not be conducted. The SNP should, however, be assessed further for gene expression and association studies in other goat breed types with large population size.

5. Conclusions

Together with the results of tissue distribution of NGF mRNA and the outcome of association analysis of NGF genotypes in the present investigation, it can be hypothesized that the genetic variants of the NGF gene may affect ovarian folliculogenesis and prolificacy in goats because individuals with the GG genotypes had a larger litter size than those with GA genotypes in the less prolific Attappady Black goats. The RFLP designed using *BpiI* could be used for rapid screening of individuals with favorable genotypes in goat populations. The novel SNP detected, g.715C > T, corresponding to a non synonymous mutation can be used for association studies in other goat populations.

Conflicts of interest

The authors report no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anireprosci.2016.08.009.

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