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Nerve Growth Factor gene ovarian expression, polymorphism identification, and association with litter size in goats

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ABSTRACT

The *Nerve Growth Factor* (NGF) plays an important role in reproduction by augmenting folliculogenesis. In this study, the coding regions of caprine *NGF* gene were analyzed to detect single-nucleotide polymorphisms (SNPs), their association with litter size, and the relative ovarian expression of *NGF* gene in the two indigenous goat breeds of South India *viz*., the prolific Malabari and less-prolific Attappady Black. The sequence analysis of the third exon containing the entire open reading frame of *NGF* gene was observed to be of 808 bp with one nonsynonymous mutation at 217th position. Later, polymerase chain reaction (PCR) was performed to amplify a region of 188 bp covering the region carrying the detected mutation. The genomic DNAs from the goats under study ($n = 277$) were subjected to PCR and single strand conformation polymorphism (SSCP). On analysis, four diplotypes *viz*., AA, AB, AC, and AD were observed with respective frequencies of 0.50, 0.22, 0.27, and 0.01. Sequencing of the representative samples revealed an additional synonymous mutation, i.e., g.291C>A. Statistical analysis indicated that *NGF* diplotypes and the SNP g.217G>A were associated with litter size in goats ($P < 0.05$). Relative expression of *NGF* gene was significantly higher in the ovaries of goats with history of multiple than single births ($P < 0.05$). The results of the present study suggest the significant effect of the *NGF* gene on litter size in goats and identified SNPs would benefit the selection of prolific animals in future marker-assisted breeding programs. The two novel PCR-restriction fragment length polymorphisms designed, based on the detected SNPs, would help in the rapid screening of large number of animals in a breeding population for identifying individual animals with desired genetic characteristics.

1. Introduction

Research on the identification of genes and their association with economic traits in farm animals could assist in the genetic selection of breeding stock. Litter size at birth is one of the most important reproductive traits for the selection of multiparous animals [1,2]. Superior kidding rate is a very important economic trait in goat production and ovulation rate is the precondition of kidding rate [3], and improving these traits in indigenous goat breeds may increase the profits from goat rearing. Because the heritability of the reproductive traits is low, traditional selection methods can be complemented with marker-assisted selection, to increase the intensity of

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selection [4]. The influence of candidate genes on reproductive traits differs considerably because these genes could affect the physiological pathways, metabolism, and phenotypic expression differentially with different paracrine or autocrine effects. The major focus of goat breeding for genetic improvement greatly relies on candidate genes influencing reproductive traits so as to ascertain the functions of genes causing changes in phenotypic values of the trait, molecular structure analysis, expression profile, sequence variability, and their association with phenotypes. The selection for favorable alleles of candidate genes could help in the development of a breeding stock of goats with high litter size [5–7].

Nerve Growth Factor (*NGF*) is a member in the neurotrophin family [8], whose major site of synthesis and secretion is ovarian cells [9,10], and can be considered as a key regulator of ovarian function because it promotes the development of preantral follicles by the cell-specific activation of TrkA receptors in the preovulatory follicles and influences the process of ovulation [11]. Its role in reproduction was demonstrated experimentally in *NGF* null mutant mice, which exhibited a markedly reduced numbers of primary and secondary follicles even at the normal serum gonadotrophin levels [12]. The *NGF* promotes the production of a principal angiogenic factor, vascular endothelial growth factor (VEGF) from the developing follicles, granulosa cells, and CL leading to enhanced ovarian follicular development and luteogenesis. It was also reported that any change in *NGF* expression could result in ovarian disorders related to impaired angiogenesis [13]. An upregulation of *NGF* in the ovary promotes steroidogenesis by enhancing the expression of enzyme genes involved in 17α-hydroxyprogesterone, testosterone, and estradiol synthesis [14]. In spite of the clear elucidation of the role of *NGF* gene in influencing the reproductive performance, the evaluation of this as a candidate gene for genetic selection of breeding animals has not been conducted extensively and systematically. Recently, it was reported that *NGF* gene demonstrated highest expression in caprine reproductive tissues, and a synonymous mutation in the coding region of this gene was associated with litter size in goats [15]. The present study envisages the characterization of coding region of caprine *NGF* gene, from two distinct south Indian indigenous goat breeds *viz*., the prolific Malabari and less-prolific Attappady Black, to detect the genetic variants and their association with prolificacy, if any. In addition, the expression of *NGF* gene in the ovaries of these two breeds was also quantified to know the *in vivo* influence of this gene in prolificacy.

2. Materials and methods

To characterize and identify the genetic variants of caprine *NGF* gene from Malabari and Attappady Black, genomic DNA was isolated from 277 goats, further subjected to polymerase chain reaction (PCR), molecular cloning, and SSCP. The total RNA was isolated from ovarian tissues from goats with multiple and single birth history for the three previous kiddings from Malabari and Attappady Black goats, respectively, for the *NGF* mRNA expression analysis by quantitative real-time (qRT) PCR.

2.1. Animals and sample collection for DNA and RNA isolation

A total of 277 female goats (2–5 years of age) belonging to the Malabari (n = 175) and Attappady Black breeds (n = 102) were included in the present research from four centers of three districts of south Indian state, Kerala, *viz*., Thrissur, Malappuram, and Palakkad. The goats were selected at random after verifying the farm records so as to make the individuals to be as unrelated as possible. The mean litter size of the selected animals belonging to each breed is provided in Supplementary Table 1. Venous blood (6 mL) was collected from the jugular vein of each animal and stored at 4 °C until processing. The genomic DNA from the white blood cells was extracted using the standard phenol chloroform method.

The ovarian tissue samples were collected from six goats each with multiple and single birth history for the three previous kiddings from Malabari and Attappady Black goats, respectively, from the Kerala Veterinary and Animal Science University Meat Plant. Approximately, 100 mg of ovarian tissues were collected, without including the follicles or their parts, 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 quantification of RNA was done by NanoDrop spectrophotometer (Thermo Scientific, USA). The mean ratios of RNA at A260/A280 and A260/A230 were 2.02 ± 0.17 and 2.13 ± 0.15, respectively. Furthermore, the integrity of extracted RNA was verified using 0.8% agarose gel electrophoresis to observe clear bands of 28S and 18S rRNA, indicating its high quality. Subsequently, cDNAs were synthesized using the RevertAid first-strand cDNA synthesis kit (Thermo Scientific, K1622) with 1 μg of RNA in a reaction volume of 40 μL and were stored at −80 °C until use.

2.2. NGF gene exon 3 PCR amplification, molecular cloning, and sequence analysis

The caprine *NGF* gene exon 3 was amplified from genomic DNA using the published primers [15]. A 50 μL PCR reaction was performed with 100 ng of the extracted genomic DNA, 5 μL of 10X Buffer, 1 μL of 10 mM dNTP, 10 pM each of forward and reverse primers, and 1 μL of JumpStart AccuTaq LA DNA Polymerase (2.5U/μL) with proofreading activity (Sigma–Aldrich). The cycling protocol was 96 °C for 2 minutes, 35 cycles of 95 °C for 30 seconds, 58 °C for 20 seconds, 68 °C for 1 minute, and a final extension at 68 °C for 7 minutes. The amplicons were run on 1% agarose gel, purified by gel extraction kit (Life Technologies, USA), cloned into the pGEM-T Easy Vector (Promega, USA), and transformed into the *Escherichia coli* (DH5α strain). The clones harboring the *NGF* gene were selected by blue white screening and confirmed with colony PCR. The plasmids were extracted from the positive clones using the PureLink Quick Plasmid Miniprep kit (Invitrogen) and sequenced

(Xcelris, Ahmedabad) using T7 and SP6 primers. Sequence data obtained were edited using the Chromas Lite Ver.2.33, (http://www.technelysium.com.au/chromas.html).

2.3. PCR-SSCP, single-nucleotide polymorphisms identification, and novel PCR-RFLPs

The derived caprine *NGF* gene sequences (GenBank accession numbers KF724722 and KF914670) were analyzed, and primers were designed using Primer3 software for amplifying a 188-bp fragment encompassing the detected novel single-nucleotide polymorphism (SNP) at 217th position of the open reading frame (ORF) (Table 1). Subsequently, PCR was performed with these primers to amplify the region from the extracted genomic DNA from the goats under study (n = 277) for enabling their rapid screening in SSCP. PCR was performed in 25 μL reaction volume with 50 ng genomic DNA, 2.5 μL 10X reaction buffer, 0.2 mM dNTP, 10 pM of each primers, and 0.5 U of Taq DNA polymerase (Sigma–Aldrich). The cycling protocol followed with initial denaturation at 95 °C for 3 minutes followed by 35 cycles of denaturing at 94 °C for 30 seconds, annealing at 66 °C for 20 seconds, and extension at 72 °C for 30 seconds with a final extension at 72 °C for 5 minutes in a thermal cycler (Bio-Rad, USA). The PCR products obtained were subjected to SSCP, for which initial denaturation was carried out by mixing 5 μL of 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, incubated at 95 °C for 10 minutes, and then snap chilled on ice for 10 minutes. After this, the contents were separated using 12% vertical polyacrylamide gel electrophoresis at 130 V for 16 hours under refrigerated conditions. The SSCP patterns were visualized using silver nitrate staining, photographed, and analyzed. Representative samples from different genotypes were sequenced to detect nucleotide variation and sequences where aligned by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) to detect two SNPs, i.e., g217G>A and g.291C>A. PolyPhen-2 score was used to predict the possible impact of an amino acid substitution on the structure and function of a protein as reported earlier [16].

Furthermore, the derived sequences when analyzed using *NEBcutter* tool, two restriction enzyme sites for the enzymes *AvaII* and *SacII* corresponding to the above SNPs, were observed. This would enable the rapid screening of the large number of DNA samples using the PCR-restriction fragment length polymorphisms (RFLP) for detection of these SNPs, surpassing the tedious SSCPs. The PCR-RFLPs were performed in 20 μL reaction volume containing 5 μL of PCR product, 2.5 U of restriction enzyme, 2 μL 10X buffer (Thermo Scientific), and nuclease-free water; incubated at 37 °C for 2 hours, and the digested products were subjected to 8% polyacrylamide gel electrophoresis.

2.4. Statistical analysis

Allele frequency differences between Malabari and Attappady Black population were tested by Fisher's exact and the chi-square test using SPSS V.21 (Table 2). Data were analyzed with the following general linear model to find the association between SNPs (genotypes and diplotypes) and litter size (SPSS V.21):

$$y_{ijklmn} = \mu + b_i + p_j + c_k + g_l + (bg)_{il} + w_m + e_{ijklmn}$$

where y_{ijklmn} is the litter size measured on $ijklmn^{th}$ animal, μ represents the overall population mean, b_i is the fixed effect associated with i^{th} breed ($i = 1, 2$), p_j is the fixed effect associated with j^{th} parity ($j = 1, 2, 3$), c_k is the fixed effect associated with k^{th} center ($k = 1, 2, 3$), g_l is the fixed effect associated with l^{th} diplotype (genotype in the case of analysis for individual SNPs) of *NGF* locus, $(bg)_{il}$ is the interaction between the i^{th} breed and l^{th} genotype, w_m is the fixed effect associated with m^{th} dam's body weight group (m = 1, 2, 3, 4, 5, i.e., 20–25, 26–30, 31–35, 36–40, 41–45 kg, respectively), and e_{ijklmn} is the random error. Data were analyzed for Malabari and Attappady Black goat populations separately with the general linear model, excluding the factors, b_i and $(bg)_{il}$. 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 there was no significant effect for these factors on litter size in the goat population covered in the study. Post hoc test (Duncan multiple range test) was used to identify the homogeneous subsets (SPSS V.21). A value of $P \leq 0.05$ was taken to indicate a significant difference between means.

Table 1
Oligonucleotide primers used in molecular cloning of *NGF* exon 3, PCR-SSCP, and real-time PCR.

Primer name	Primer sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Accession number
NGFCD-F	5′-ATAGCGTAATGTCCATGTTG-3′	808	58	JQ308184
NGFCD-R	5′-ATTTACAGGTTGAGGTAGGG-3′			
NGF1-F	5′-GACACAGTCCTCCGCAGA-3′	188	66	JQ308184
NGF1-R	5′-GCCTCGAAGTCCAGATCCTG-3′			
NGFRT-F	5′-GTCATCCCACCCCGTCTTTC-3′	111	60	JQ308184
NGFRT-R	5′-ACCATCACCTCCTTGCCCTT-3′			
β-actin-F	5′-CCACACCTTCTACAACGAGC-3′	105	60	JX046106
β-actin-R	5′-ATCTGGGTCATCTTCTCACG-3′			
GAPDHF	5′-TGGAGAAACCTGCCAAGTATG-3′	127	60	XM_005680968
GAPDHR	5′-TGAGTGTCGCTGTTGAAGTC-3′			
18sRNA F	5′-TGCAATTATTCCCCATGAACG-3′	101	60	DQ149973
18sRNA R	5′-GTAGTAGCGACGGGCGGTGT-3′			

Abbreviations: NGF, *Nerve Growth Factor*; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

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

Locus	Restriction enzyme	Genetic structure	Breed	
			Malabari (175)	Attappady Black (102)
Diplotypes (g.217G>A and g.291C>A)		Diplotypes		
		AA	88	39
		AB	38	23
		AC	47	37
		AD	2	3
		Haplotypes		
		A	0.75	0.69
		B	0.11	0.11
		C	0.13	0.18
		D	0.01	0.02
g.217G>A	*AvaII*	Genotypes		
		GG	134	76
		GA	41	26
		Allele		
		G	0.88	0.87
		A	0.12	0.13
		He	0.21	0.24
		Equilibrium χ^2 test	P = 0.08	P = 0.03
g.291C>A	*SacII*	Genotype		
		CC	126	62
		CA	49	40
		Allele		
		C	0.86	0.80
		A	0.14	0.20
		He	0.22	0.32
		Equilibrium χ^2 test	P = 0.14	P = 0.01

Abbreviation: NGF, *Nerve Growth Factor*.

2.5. Reverse-transcriptase qRT-PCR

The relative ovarian expression of *NGF* gene was measured by qRT-PCR using SYBR green chemistry (Thermo Scientific) in Malabari and Attappady Black goats with the history of multiple and single births. Six samples from each group with three technical replicates per sample were analyzed. The *BestKeeper* software was used to validate the three housekeeping genes, *viz*., *GAPDH*, *β-actin*, and *18S rRNA* selected for the current research. Primer characteristics are presented in Table 1. After analyzing the descriptive statistics for the individual candidate, the expression stability was calculated based on the inspection of calculated variations, i.e., SD and coefficient of variation values (Supplementary Table 2). According to the variability observed, housekeeping genes were ordered from the most stably expressed, exhibiting the lowest variation to the least stable one, or exhibiting the highest variation. The gene *18S rRNA* with the SD higher than 1 was observed inconsistent [17] and hence excluded from calculations. Hence, the fold change in the relative expression of the gene was normalized by the geometric mean of the *GAPDH* and *β-actin* genes. The PCR reaction efficiency checking and relative mRNA expression analysis were conducted using Illumina Eco Q- RT PCR system. The R^2 values and PCR efficiencies were detected using standard curves with seven serial dilutions of the cDNA. qRT-PCR was conducted in a 25 μL reaction volume containing 50 ng of cDNA and 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The reactions were performed using a pre-incubation of 95 °C for 3 minutes, 40 quantification cycles consisting of a denaturation of 95 °C for 30 seconds, annealing of 60 °C for 15 seconds, and an extension of 72 °C for 30 seconds. The amplification program was followed by a melt curve analysis at 95 °C for 15 seconds, 60 °C for 15 seconds, and 95 °C for 15 seconds. This analysis was performed to ensure the purity of the PCR product, which was confirmed by a single peak. The relative expression of *NGF* mRNA was calculated by using the $2^{-\Delta\Delta Ct}$ method [18], and statistical analysis was performed using SPSS V.21.

3. Results

Sequencing of the cloned *NGF* exon 3 revealed that the 808 bp fragment contains 5′ untranslated region (1–8 bp), ORF (9–734 bp), and 3′ untranslated region (735–808 bp) with 60.27% GC content. The sequences were submitted to the GenBank (accession numbers KF724722 and KF914670 for Attappady Black and Malabari breeds, respectively). Multiple sequence alignment of this sequence with other caprine sequences revealed one SNP at 217th position (g.217G>A).

A 188 bp fragment of *NGF* gene exon 3 was amplified by PCR in all the 277 goats belonging to the two breeds. SSCP revealed a polymorphic pattern with four distinct diplotypes, designated as AA, AB, AC, and AD (Supplementary Fig. 1). Haplotypic and genotypic distribution and allelic frequencies of two SNPs are provided in Table 2. It was observed that low-prolific Attappady Black goats were in Hardy–Weinberg disequilibrium at the two SNP loci ($P < 0.05$; Table 2). The greatest diplotype and genotype frequencies were detected for the homozygotes. Sequencing of the PCR products from each banding pattern revealed them as diplotypes with two novel SNPs

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Haplotype A   GACACAGTCCTCCGCAGAGCCCACAGCGCCCCGGCTGGGCCGATAGCCGCCAGGGTGGCA 60
Haplotype B   GACACAGTCCTCCGCAGAGCCCACAGCGCCCCGGCTGGGCCGATAGCCGCCAGGGTGGCA 60
Haplotype C   GACACAGTCCTCCGCAGAGCCCACAGCGCCCCGGCTGGGCCGATAGCCGCCAGGGTGGCA 60
Haplotype D   GACACAGTCCTCCGCAGAGCCCACAGCGCCCCGGCTGGGCCGATAGCCGCCAGGGTGGCA 60
              ************************************************************

Haplotype A   GGGCAGACCCACAACATCACTGTGGACCCCAAACTTTTTAAAAAGCGGCGACTGCGTTCA 120
Haplotype B   GGGCAGACCCACAACATCACTGTGAACCCCAAACTTTTTAAAAAGCGGCGACTGCGTTCA 120
Haplotype C   GGGCAGACCCACAACATCACTGTGGACCCCAAACTTTTTAAAAAGCGGCGACTGCGTTCA 120
Haplotype D   GGGCAGACCCACAACATCACTGTGAACCCCAAACTTTTTAAAAAGCGGCGACTGCGTTCA 120
              ********************** ***********************************

Haplotype A   CCTCGCGTGCTGTTCAGCACCCAGCCCCCACCTGTGGCCGCGGACACTCAGGATCTGGAC 180
Haplotype B   CCTCGCGTGCTGTTCAGCACCCAGCCCCCACCTGTGGCCGCGGACACTCAGGATCTGGAC 180
Haplotype C   CCTCGCGTGCTGTTCAGCACCCAGCCCCCACCTGTGGCAGCGGACACTCAGGATCTGGAC 180
Haplotype D   CCTCGCGTGCTGTTCAGCACCCAGCCCCCACCTGTGGCAGCGGACACTCAGGATCTGGAC 180
              ************************************ ********************

Haplotype A   TTCGAGGC  188
Haplotype B   TTCGAGGC  188
Haplotype C   TTCGAGGC  188
Haplotype D   TTCGAGGC  188
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Fig. 1. Sequences of the new genetic variants/alleles A, B, C, and D of *Nerve Growth Factor* (NGF) haplotypes with Accession Numbers KU695803, KU695804, KU695805, and KU695806.

(g.217G>A and g.291C>A), 85th (G→A transition; Supplementary Fig. 2) and 159th (C→A transversion) positions of the 188 bp product (Supplementary Fig. 3). Sequences of the new genetic variants were submitted to GenBank as alleles A, B, C, and D (Fig. 1) with Accession Numbers KU695803, KU695804, KU695805, and KU695806, respectively. Two novel PCR-RFLPs were designed for the rapid screening of the genotypes for the loci, g.217G>A and g.291C>A using *AvaII* and *SacII*, respectively (Fig. 2A, B).

The least-square means with standard errors of litter size for different *NGF* genotypes and diplotypes are presented in the Table 3. Litter size was lower ($P < 0.05$) for the AB diplotype (1.35 ± 0.10) compared to the AA, AC, and AD diplotypes. Individuals with the GG genotype had greater litter size than those with the GA genotype at the g.217G>A locus in Malabari ($P < 0.05$) and Attappady Black goat breeds ($P = 0.07$). PolyPhen-2 score for the non-synonymous mutation g.217G>A (p.73D>N) was 0.99 indicating it as probably damaging with high confidence (Supplementary Fig. 4).

Quantitative real-time PCR was performed to determine the relative expression of ovarian *NGF* mRNA in goats with single births in Attappady Black and multiple births in Malabari goats. Specificity of the reaction was confirmed by the melt-curve analysis. The results were obtained by $2^{-\Delta\Delta Ct}$ method after normalization with geometric means of *GAPDH* and *β-actin* genes. When comparing the expression of *NGF* gene in ovaries of goats with multiple births against single birth, the relative

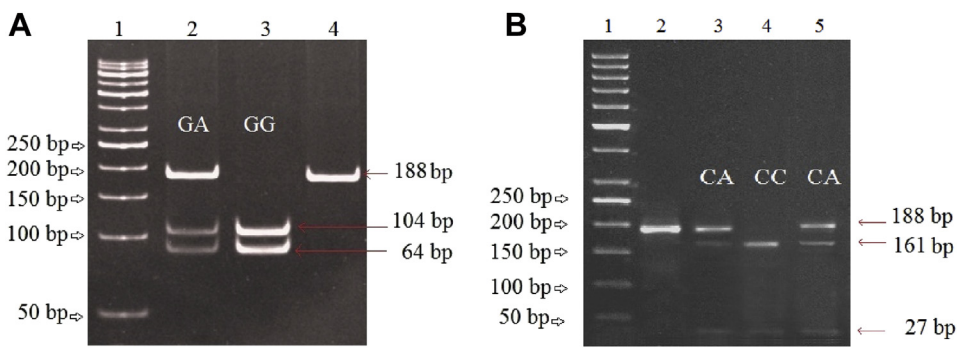

Fig. 2. (A) The electrophoresis patterns obtained after digestion with *AvaII* endonuclease for g.217G>A locus, lane 1: 50 bp marker, lane 2: GA genotype, lane 3: GG genotype, lane 4: undigested polymerase chain reaction (PCR) product (188 bp). (B) The electrophoresis patterns obtained after digestion with *SacII* endonuclease for g.291C>A locus, lane 1: 50 bp marker, lane 2: undigested PCR product (188 bp), lanes 3 and 5: CA genotype, lane 4: CC genotype.

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

Locus	Genotype	Overall litter size ± SE	Malabari (175)	Attappady Black (102)
g.217G>A	GG	1.59 ± 0.05[a]	1.83 ± 0.05[a]	1.37 ± 0.04
	GA	1.36 ± 0.08[b]	1.53 ± 0.10[b]	1.17 ± 0.03
	P value	0.02	0.03	0.07
g.291C>A	CC	1.56 ± 0.05	1.74 ± 0.09	1.42 ± 0.09
	CA	1.58 ± 0.07	1.83 ± 0.11	1.41 ± 0.09
	P value	0.42	0.38	0.87
Diplotypes with SNPs g.217G>A and g.291C>A	Diplotypes			
	AA	1.62 ± 0.07[a]	1.84 ± 0.06[a]	1.26 ± 0.06
	AB	1.35 ± 0.10[b]	1.50 ± 0.10[b]	1.11 ± 0.06
	AC	1.58 ± 0.06[a]	1.83 ± 0.09[a]	1.13 ± 0.08
	AD	1.67 ± 0.23[a]	[c]	[c]
	P value	0.04	0.04	0.29

Abbreviations: NGF, *Nerve Growth Factor*; SNPs, single-nucleotide polymorphisms.
[a,b]Values with different superscripts differ significantly ($P < 0.05$).
[c] Not included in analysis since number of observations were less than five.

expression of *NGF* was 4.65-fold higher ($P < 0.05$) in ovaries of goats with history of multiple births than singles.

4. Discussion

Marker-assisted selection based on proven genetic indicators would aid in creating a goat population with improved fecundity traits. The present study envisages the testing of *NGF* gene as a candidate marker for improved prolificacy in goats through PCR-SSCP and qRT-PCR assays. Initially, the exon 3 region covering the complete ORF of caprine *NGF* gene from Malabari and low-prolific Attappady Black goats was cloned and sequenced. Analysis of their nucleotide and amino acid sequences revealed a high degree of similarity between the two goat breeds except for a single nucleotide change in the ORF region resulting in the substitution of negatively charged aspartic acid by hydrophilic asparagine, p.Asp73Asn (AHC98008). The observed nonsynonymous mutation in the caprine *NGF* gene is the first report of its kind. Furthermore, to study the distribution of these mutations in the two breeds of goats, a set of primers were designed to amplify the region covering the identified mutation with an expected amplicon size of 188 bp. The genomic DNAs from both breeds were subjected to PCR to obtain amplicons of desired size, and representatives from both the breeds were sequenced and analyzed. It was observed that in addition to the previously detected g.217G>A nonsynonymous mutation, another synonymous mutation at g.291C>A was observed.

Furthermore, to unravel the influence of these SNPs at the exon 3 of the caprine *NGF* gene, association analysis was carried out with the data available in the farm records for both of these breeds of goats. Interestingly, it was observed that the SNP at the nonsynonymous mutation loci had a significant effect on litter size. In a previous study, An et al. [15] reported a synonymous mutation (g.705G>A) in the caprine *NGF* gene by PCR-RFLP and demonstrated its association with prolificacy. Although in the present study the mutation of g.291C>A did not significantly affect the reproductive production indices, it may not be regarded neglected as it was reported earlier that these changes could possibly influence the stability of the mRNA and can affect the mechanism of mRNA deadenylation and degradation [19].

Furthermore, the level of *NGF* mRNA expression in the ovarian tissues among goats with single and multiple births was quantified and compared using the qRT-PCR assay. Ovarian tissue was taken as the tissue sample based on the earlier report observing the highest expression for *NGF* gene in ovary and uterus [15]. Between multiple and monotocous goats in the follicular phase, a higher ovarian expression for *NGF* gene was observed in goats with a history of multiple birth, signifying its role in folliculogenesis and thereby influencing reproduction and prolificacy. The importance of *NGF* in ovarian angiogenesis, cell migration, proliferation, and ovulation [11,20] and as an intraovarian factor capable of promoting VEGF production by human ovarian follicular cells [13] was reported earlier. Also, the precise role of NGF as a follicular survival and maintenance factor was also reported earlier [21].

As the reproductive traits are complex quantitative traits, polygenic, involving multiple genes and loci, it is important to analyze the combined effect of multiple loci on litter size [22,23]. In this research, associations between SNPs of *NGF* gene and litter size were analyzed. The combined genotype/diplotype analysis of the two loci revealed that AB diplotype had lower litter size than other diplotypes, corresponding to the GA genotype in the g.217G>A locus. It was observed that both the SNPs were in linkage disequilibrium in low-prolific Attappady Black goats. It was reported earlier that the linkage disequilibrium with the causal mutation possibly affects the variation of reproductive traits in goats [24]. A significant departure from Hardy–Weinberg Equilibrium for the polymorphisms in Attappady Black population could be attributed to the nonrandom mating probably due to population stratification, selection, migration, or genetic drift as they are maintained by the tribal community in a geographically hilly isolated region of the Kerala State in South India. This might have influenced the nonsignificant association of GG genotype with litter size in goats, even though the GG genotype had higher values for litter size compared to GA genotypes in Attappady Black goats ($P = 0.07$).

PolyPhen-2 analysis revealed that the nonsynonymous mutation, g.217G>A, could probably be a damaging amino

acid substitution [16], i.e., the substitution of negatively charged aspartic acid (D) by polar asparagine (N) was predicted to have damaging effects on NGF protein structure and function with high confidence. This could be the probable reason for the absence of AA genotype in the whole goat population understudy for the locus g.217G>A and lower litter size for heterozygous GA genotypes. Thus, it is proved beyond doubt that these indices can be used as candidate markers for the genetic improvement of goat breeds under the study. Even though all the mechanisms of action of neurotrophins have not been elucidated yet, they are proven to provide a trophic support for the sympathetic innervations of ovaries and play a significant role in the development of reproductive tissues, early follicular development, its differentiation, and ovulation [12,25]. Earlier works also established the role of NGF in enhancing VEGF expression, ovarian angiogenesis, FSH receptor expression, and estradiol secretion before ovulation [13,20,26]. Thus, the biochemical and physiological roles of NGF, together with the results obtained in the present study, indicate that the SNPs associated with litter size in goats had potential applications in marker assisted selection for prolificacy in goat breeding. The novel PCR-RFLPs designed based on the identified SNPs would aid in the rapid screening of large number of animals so as to select the superior animals with desired genetic variants.

To conclude, the study reports the first identified non-synonymous mutation in the caprine *NGF* gene. The associations between the *NGF* genotypes with litter size and a significantly higher expression of ovarian *NGF* mRNA in goats with multiple births than monotocous goats emphasize the importance of *NGF* gene as a candidate target gene for improving the goat litter size based on marker-assisted selection.

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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.theriogenology.2016.07.011.

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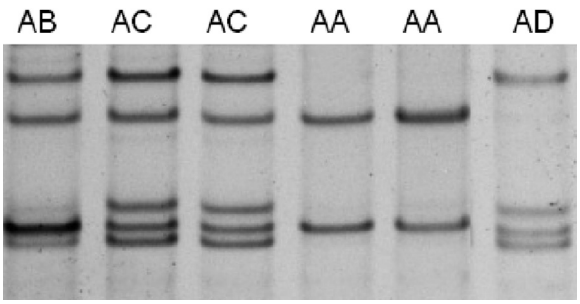
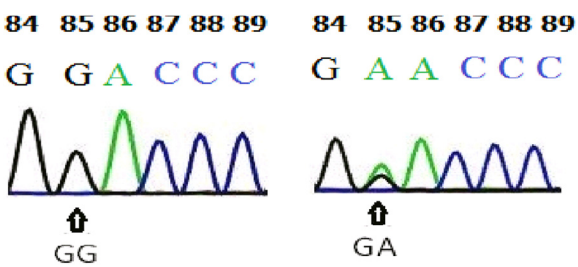
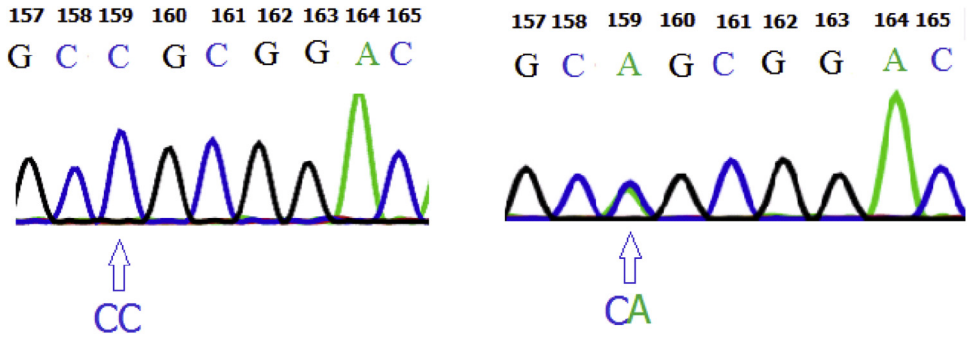


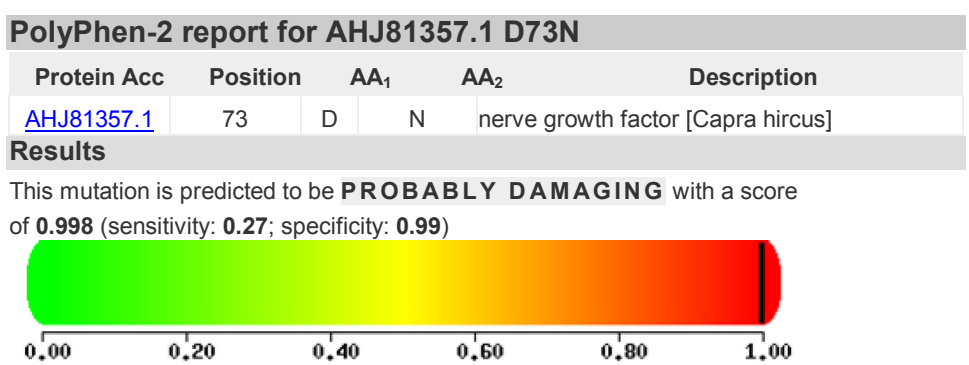
Supplementary Fig. 1. PCR-SSCP showing polymorphisms in the g.217G>A and g.291C>A loci of goat *Nerve Growth Factor* (NGF) gene resulting in four diplotypes AA, AB, AC, and AD.



Supplementary Fig. 2. Sequence maps of GG and GA genotypes with g.217G>A locus of goat *Nerve Growth Factor* (NGF) gene.



Supplementary Fig. 3. Sequence maps of CC and CA genotypes with g.291C>A locus of goat *Nerve Growth Factor* (NGF) gene.



Supplementary Fig. 4. Detailed results of the PolyPhen-2 analysis for single variant query (p.Asp72Asn). The top query section includes UniProtKB/Swiss-Prot description of query protein, if it was recognized as a known database entry. The large "heatmap" color bar with the black indicator mark dominates the display, illustrating the strength of the putative damaging effect for the variant.

Supplementary Table 1
Means with standard errors for litter size in Malabari and Attappady Black goats.

Factor	Litter size (mean ± SE)
Grand mean (277)	1.51 ± 0.07
Malabari (175)	1.75 ± 0.07[a]
Attappady Black (102)	1.25 ± 0.08[b]
	$P < 0.01$

Supplementary Table 2
Descriptive statistics of three candidate housekeeping genes based on their crossing point (CP) values.

Factor	GAPDH	β-actin	18S	BestKeeper (n = 3)	BestKeeper (n = 2)
N	12	12	12	12	12
GM [CP]	14.75	17.53	14.11	15.39	16.08
AM [CP]	14.76	17.55	14.18	15.41	16.09
min [CP]	14.00	16.33	13.05	14.75	15.43
max [CP]	15.56	18.39	18.20	17.15	16.65
SD [± CP]	0.41	0.68	**1.07**	0.51	0.40
CV [% CP]	2.76	3.90	7.54	3.33	2.51

In the two last columns, the BestKeeper index is computed together with the same descriptive parameters, either for three genes (*GAPDH*, *β-actin*, and *18S rRNA*) or for two genes after removal of 18S (*GAPDH* and *β-actin*). Since the SD of CP of *18S rRNA* gene was more than 1 (1.07), it was excluded (bold).
Abbreviations: AM [CP], the arithmetic mean of CP; CV [% CP], the coefficient of variance expressed as a percentage on the CP level; GM [CP], the geometric mean of CP; Min [CP] and Max [CP], the extreme values of CP; N, number of samples; SD [± CP], the standard deviation of the CP.