**Single nucleotide polymorphism of candidate genes in non-descript
local goats of Sri Lanka**

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Abstract

In the present study, genetic polymorphism in exon 4 of kappa casein (*k-CSN3*), exon 2-3 of alpha lactalbumin (*LALBA*) and exon 1 of gonadotropin releasing hormone receptor (*GnRHR*) genes were analyzed as candidate genes for milk production, milk quality and prolificacy aiming to provide information for future studies on genetic improvements in non-descriptive local goats in Sri Lanka. Altogether eleven, thirteen and three single nucleotide polymorphisms (SNPs) were identified in *k-CSN3*, *LALBA* and *GnRHR* gene fragments respectively utilizing the DNA sequencing technique in Sri Lanka. Seven polymorphic sites out of eleven in *k-CSN3* gene fragment and the recorded polymorphic site in exon 3 of *LALBA* gene fragment were homozygous while all three polymorphic sites in *GnRHR* gene fragment were heterozygous. Two of the SNPs recorded in the present study are found to unique for Sri Lankan non-descript goat population at G203T and A730G in *k-CSN3* and *GnRHR* genes respectively. The study records another two SNPs in *GnRHR* gene which are already known to be correlated with higher fecundity in goats (G757A and G891T). Results of the present study will be extremely important in future attempts to indicate markers to improve the milk production, composition of milk and litter size of non-descript local goats in Sri Lanka.

Key words; non-descript local goats, *k-CSN3*, *LALBA*, *GnRHR*, SNPs, DNA sequencing

Introduction

Sri Lankan non-descript local goats are highly localized, heterogeneous group which covers the majority of the goat population in the country (National Livestock Breeding Policy 2010) and are currently known to be under the threat of extinction due to intensive cross breeding with exotic breeds (Silva 2010). These non-descript goats are much more important at local circumstances as they hold invaluable genetic resources for high adaptability to local environmental conditions, disease resistance (Silva 2010) and high degree of prolificacy (National Livestock Breeding Policy 2010) though, they are poor in production parameters compared to exotic breeds (Silva 2010). Low performance in production of non-descript goats may be the result of poor management practices in rural areas and genetic makeup of animals (Azevedo et al. 1994). However, SNPs in production related genes affect significantly on the phenotypic characters though they arise at a lower rate in a population (Cargill et al. 1999) and production parameters can be greatly enhanced at a minor cost by promoting the inheritance of these beneficial mutations to next generation via selection. Consequently, modern researches are at an inspired

interest on association analysis of SNPs and related phenotypic characters such as milk, meat and wool production and prolificacy traits. *k-CSN3* and *LALBA* genes are milk trait related and *GnRHR* is a fecundity trait related gene which are collectively widely studied genes for genetic variability. Many researchers have reported the association between *k-CSN3* genetic variability and milk composition (Chiatti et al. 2005, Marletta et al. 2007) and milk production (Marletta et al. 2007). Further studies have revealed the association between *LALBA* gene polymorphism and nutrient content of milk (Lan 2007, An 2009, Zidi 2014) and relationship between genetic variability of *GnRHR* gene and prolificacy trait (Ming-Xing et al. 2009, Yang et al. 2011). The inclusive goal of this study was to identify SNPs in Caprine *k-CSN3*, *LALBA* and *GnRHR* genes aiming to genetically characterize non-descript local goats of Sri Lanka, thereby to offer genetic clues for future association analysis.

Material and methods

Collection of samples

A sample of 112 non-descript local goats from Eastern (49), Northern (15), Northwestern (37), and Southern (11) provinces was used for the study to cover different localities of Sri Lanka. Blood was collected from animals into sterile vacutainer tubes containing 100 µg/ml Ethylene diammine tetraacetic Acid (EDTA) using jugular puncture method.

Extraction, PCR amplification and Sequencing of DNA

DNA extraction from collected blood samples was done using salting-out protocol (Jawdat et al., 2011) and commercially available kits (Wizard Genomic DNA Purification Kit). Extracted genomic DNA was visualized on Ethidium bromide stained 1% agarose gels and quantified using a DNA spectrophotometer (BIOMATE 3). Exon 2-3 of Caprine *LALBA* (part of exon 2, intron 2 and part of exon 3) (667 bp), exon 4 of *k-CSN3* (458 bp) and exon 1 of *GnRHR* (746 bp) gene fragments were amplified using already published protocols by Jain et al., 2009; Kiplagat et al., 2010 and Yang et al., 2011 respectively. PCR was performed in a 25 µl reaction mixture containing 50 ng genomic DNA, 0.5 µM of each primer, 1× PCR reaction buffer, 1.5 mM of MgCl₂, 200 µM of dNTPs and 0.625 units of *Taq* DNA polymerase and amplifications were carried out in an ABI2720 (Applied Biosystems®) Thermal Cycler. Amplified PCR products were then visualized on 1% Agarose gel stained with Ethidium Bromide (1 µg/ml) and verified using a 100 bp ladder (Vivantis 6x). Following the visualization, PCR band was excised out of the agarose gel and PCR product was purified using PureLink PCR gel clean-up kit (Qiagen) and

quantified using Lambda markers. All 112 samples were sequenced for screening genetic variations using Bigdye terminator chemistry in an ABI 3500 genetic analyzer.

Sequence alignment

All DNA sequences were checked against Caprine genome sequences (BLAST+2.3.0) already published in Genbank and for presence of any insertions, deletions and for absence of stop codons using Sequencher software v 5.0.1 (Gene Codes Corp). Bases ambiguities were visualized and manually edited using CodonCode Aligner 5.1.5 (Codon Code Corporation, Dedham, MA, USA) and heterozygous bases were separated with the aid of PHASE 2.1 (Stephens et al., 2001). Poor allelic phase estimates (below Phred20 score) revealed by PHASE 2.1 were excluded from the analysis.

Polymorphic site mining

All the aligned sequences for all three gene fragments were realigned with corresponding gene sequences of other Caprine breeds already published in the GenBank database (accession no: L42937.1 for *GnRHR*; X60763 for *k-CSN3* and M63868 for *LALBA* genes) and polymorphic sites were visualized using Sequencher (v 5.3). Haplotypes list was identified using PHASE 2.1 and their frequencies were calculated using Hardy-Weinberg law with the aid of MS Excel 2010. DNA sequences were translated to amino acid sequences using ExPASy translate tool (Gasteiger et al., 2003).

Results and discussion

Three polymorphic sites were identified from *GnRHR* exon 1 region of non-descript local goats. All three polymorphic sites were heterozygous (Figure 1) and two of the sites were represented by G/A and the other was G/T alleles (Table 1). Heterozygous genotypes found at the polymorphic sites were represented by a minor population of goats while the majority of animals were represented by homozygous genotype. At both G757A and G891T loci G was the predominant allele whereas at locus A730G, A was predominant. The most common haplotype AA, GG, GG was represented by 17 goats out of 31 (57%). There were two haplotypes (AA, A/G, G/T and A/G, GG, GG) with similar frequency of occurrence and represented the rarest recombination (3%). Six possible allelic recombinations were predicted and AGG (74%) and AAT (1%) were among the highest and lowest expected haplotypic combinations respectively. From the six predicted haplotypes, five haplotypes (GenBank accession number: KX943532-KX943536) were carried by the goats analyzed in this study and only AAT combination was not

recorded. Commonly recorded haplotype was AGG (69%) whereas GGT (2%) was the rarest among non-descript goats studied (Table 1). Analysis of the deduced amino acid sequences in A730G and G757A sites showed presence of silent mutations and reported G891T variation is non-synonymous (Table 2). Studies by Yang et al., (2011) have reported the same two SNPs at G757A and G891T. To date, the reported synonymous mutation at A730G was not recorded from any goat breed of the world. Boer goats with GG and GT genotypes at G757A and G891T positions respectively showed a significantly positive correlation with the litter size ($p < 0.05$) (Yang et al., 2011). The present study was aligned with the findings of Yang et al., 2011 by demonstrating the genotype GG (at G891T and G757A). Even a silent mutation can have positive effects on prolificacy by changing the density of *GnRHR* on the gonadotropes as a result of increased or decreased concentrations of LH and FSH (Schubert et al., 2000).

Altogether 13 polymorphic sites were detected from exon 4 and partial intervening region of *k-CSN3* gene of the studied local goat population. Eleven out of thirteen polymorphic sites were located in exon 4 and two of them were located in intron 4 region (G656A and G657A) hence, are not responsible for amino acid variations of the corresponding peptide chain. Among all the polymorphic sites in exon 4, only 4 of the sites were heterozygous (Figure 2) while 7 other sites were homozygous (Table 2). At all four heterozygous sites the dominant genotype was homozygous and minority of the population was represented by the heterozygous genotype. At A274G and G309A polymorphic sites, all three genotypes (GG, AA, AG) were present and G was the dominant allele (0.525 and 0.9 respectively). At A471G and T591C sites only two genotypes were existed and allele A and T were prominent at 0.9 and 0.925 respectively where homozygous recessive genotypes were absent. Genotype GG at A471G and genotype CC at T591C were not recorded. At heterozygous polymorphic sites nine haplotypes were recorded. The most frequent haplotype at heterozygous polymorphic sites was GG/ GG/ AA/ TT (35%) and represented by 7 animals and was followed by AG/ GG/ AA/ TT (15%) and AA/ GG/ AA / TT (15%) haplotypes. Altogether those three genotypes cover 65% of the variability in the whole population and the remaining 35% split among other six haplotypes. Nine haplotypic combinations (GenBank accession number: KY003218- KY003226) out of ten predicted allelic haplotypic combinations were present in the studied population of goats. Among them GGAT was the dominant haplotype (23%) while GAAT (1%) and GGAC (1%) were the rarest and GAAC combination was not reported (Table 2). Analysis of the deduced amino acid sequences

showed point mutations at each polymorphic site and all of them were accountable as non-synonymous variations except for the site T245C and A284G (Table 3). However, to date the nucleotide substitution at 203 site (responsible for transition of G to T and Gln to His in nucleotide and amino acid sequences respectively) has not been reported from any goat breed of the world and might be another unique polymorphic site for Sri Lankan indigenous goat population (Table 3). Previous studies have revealed the presence of 16 polymorphic sites within complete exon 4 of *k-CSN3* gene of domestic goats (Prinzenberg et al., 2005). Thirteen out of 16 polymorphic sites were responsible for protein variants and 3 were synonymous mutations (Prinzenberg et al., 2005). From the recorded 11 sites in exon 4, 10 were previously reported by many researches (Angiolillo et al., 2002; Caroli et al., 2001; Jann et al., 2004; Prinzenberg et al., 2005; Yahyaoui, 2003 and Yahyaoui et al., 2001) except the novel mutation at G203T. Many researches (Caroli et al., 2001; Prinzenberg et al., 2005; Yahyaoui, 2003 and Yahyaoui et al., 2001) reported the silent mutation at 254 site. Synonymous mutation at 284 site was previously described in Spanish and French Saanen breeds (Yahyaoui et al., 2001) and in seven European breeds (Prinzenberg et al., 2005). Nucleotide A instead of G and G instead of A at 384 and 385 were recorded by Prinzenberg et al., 2005 and Angiolillo et al., 2002 in Montefalcone Italian breed. Further, the variabilities at 471, 550, 583 and 591 positions in comparison to accession number X60763 were formerly described in a single paper by Yahyaoui et al., 2001. However, none of the studies have taken attempts to evaluate the possible relationship between reported polymorphism and variations in milk traits of goats.

There were no polymorphic sites in partial exon 2 of *LALBA* gene studied in local indigenous goats. However, 12 polymorphic sites were there in intron region and were not responsible for amino acid variations. All the mutations found in the intervening region were A to G transitions (Table 4). Within the partial exon 3, a point mutation was recorded by nucleic acid exchange from T to C (Figure 3) which intern does not responsible for any transition of amino acid sequence. Eleven polymorphic sites out of thirteen were found as heterozygous sites and only two of the sites were recorded as homozygous (Table 4). The first homozygous site at G1541 was an insertion of nucleotide G compared to the reference sequence M63868 and was represented by all the tested local goats (allelic frequency of 100%). All the heterozygous sites were characterized only GG (dominant haplotype) and AG haplotypes. Further, G was the dominant allele for all the polymorphic sites investigated by this study except for C1897T site.

However, genotype AA was not recorded at any heterozygous site within intron 2 and exon 3 regions studied. Total of 35 haplotypic combinations (GenBank accession number: KY003183-KY003217) were investigated across exon 2-3 and only five of them were recorded more than once. The most common haplotype (G,G,G,G,G,G,G,G,G,G,G,G,C) was recorded from 22 local animals at a frequency of 36%.

The nucleotide exchange reported at exon 2 in this study was previously described (Cosenza et al., 2003 and Lan et al., 2007) and not known to be associated with milk trait. Non-synonymous mutations found in the current study can be used to suggest markers for marker assisted selection after confirming the link between the reported mutation and corresponding traits via association studies along with phenotypic data. Although the synonymous mutations are not directly change the amino acid sequences hence the corresponding trait, this type of mutations may be still important as effective genetic markers for association analysis (Thomas et al., 2011). However, markers based on recorded novel polymorphic sites will be reliable tool for genotypic characterization of local goats in Sri Lanka.

Since non-descript goats represent a highly localized population, it is apparent to observe an inbred population. However, exonic regions examined in current study revealed the presence of 5 combined haplotypes out of 6 predicted and 9 combined haplotypes out of 10 predicted haplotypes in *GnRHR* and *k-CSN3* respectively and their observed frequencies are not much deviated from the expected (Table 1 and 2). The reported intronic diversity in *LALBA* gene was also high and in turn this suggests that, they are currently not representing an inbred population. Further, the non-descript goats were previously noted as a phenotypically heterogeneous population while the significant genetic diversity reported in this study revealed their genetic heterogeneity as well. The genotypes and haplotypes which were not reported in the studied population may be attributed to low production frequency of those haplotypes where their expected haplotypic frequencies were also low and or low sample size. Although they were declared as a threatened population of goats owing to indiscriminate cross breeding, the newly recorded polymorphic sites in *GnRHR* and *k-CSN3* genes evident that they still possess preserved local genetic resources within the genome.

Conclusions

This study is a preliminary report on SNPs in *k-CSN3*, *LALBA* and *GnRHR* genes of non-descript local goats in Sri Lanka. Results of the current study reveal genetic variability of Sri Lankan

non-descript goats reporting; absolutely novel polymorphic sites (at G203T and A730G for *k-CSN3* and *GnRHR* genes respectively) and 9 out of 10 and 5 out of 6 combined haplotypes in above genes. These novel SNPs will be extremely important in genetic characterization of non-descript local goats and other mutations found in this study can be utilized to indicate molecular markers to be used in marker assisted selection and association analyses. Further studies would be essential to evaluate physiological role of different genetic haplotypes found in the current study in relation to production traits of non-descript goats in Sri Lanka.

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	730		757		891	
Hap1	ACTGTTTCAGCA	AATCAAC.....	CTAACACCC	RGGCAGCCTCCCCACC.....	AGGAAAGAGAAGAG	GAAAAAACT
Hap2	ACTGTTTCAGCA	AATCAAC.....	CTAACACCC	GGGCAGCCTCCCCACC.....	AGGAAAGAGAAGAG	GAAAAAACT
Hap3	ACTGTTTCAGC	RATCAAC.....	CTAACACCC	GGGCAGCCTCCCCACC.....	AGGAAAGAGAAGAK	GAAAAAACT
Hap3	ACTGTTTCAGCA	AATCAAC.....	CTAACACCC	RGGCAGCCTCCCCACC.....	AGGAAAGAGAAGAK	GAAAAAACT
Hap4	ACTGTTTCAGC	RATCAAC.....	CTAACACCC	GGGCAGCCTCCCCACC.....	AGGAAAGAGAAGAG	GAAAAAACT
L42937.1	ACTGTTTCAGCA	AATCAAC.....	CTAACACCC	RGGCAGCCTCCCCACC.....	AGGAAAGAGAAGAK	GAAAAAACT

Figure 1 Sequence alignment of *GnRHR* gene fragment compared to GenBank accession no. L42937.1

	274	309	471	591
Hap1	ATA G TCAATT.....AAAGCCA G TTGCAGTTAGGT.....ACAGAA A TCCC.....TGCGAGTGCA T CTGAGACCAA			
Hap2	ATA R TCAATT.....AAAGCCA G TTGCAGTTAGGT.....ACAGAA A TCCC.....TGCGAGTGCA T CTGAGACCAA			
Hap3	ATA A TCAATT.....AAAGCCA R TTGCAGTTAGGT.....ACAGAA A TCCC.....TGCGAGTGCA Y CTGAGACCAA			
Hap4	ATA A TCAATT.....AAAGCCA A TTGCAGTTAGGT.....ACAGAA A TCCC.....TGCGAGTGCA Y CTGAGACCAA			
Hap5	ATA A TCAATT.....AAAGCCA G TTGCAGTTAGGT.....ACAGAA R TCCC.....TGCGAGTGCA T CTGAGACCAA			
Hap6	ATA R TCAATT.....AAAGCCA G TTGCAGTTAGGT.....ACAGAA R TCCC.....TGCGAGTGCA T CTGAGACCAA			
Hap7	ATA G TCAATT.....AAAGCCA G TTGCAGTTAGGT.....ACAGAA R TCCC.....TGCGAGTGCA T CTGAGACCAA			
Hap8	ATA A TCAATT.....AAAGCCA G TTGCAGTTAGGT.....ACAGAA R TCCC.....TGCGAGTGCA T CTGAGACCAA			
Hap9	ATA R TCAATT.....AAAGCCA R TTGCAGTTAGGT.....ACAGAA A TCCC.....TGCGAGTGCA Y CTGAGACCAA			
X60-	ATA A TCAATT.....AAAGCCA G TTGCAGTTAGGT.....ACAGAA G TCCC.....TGCGAGTGCA T CTGAGACCAA			

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Figure 2 Sequence alignment of *k-CSN3* gene fragment compared to GenBank accession no. X60763

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Hap1	CCC.....TCCTGCCAGAGTTCTGGATGATGATCTTACTGATGACATTG
KF781121.1	CCC.....TCCTGCCAGAGTTCTGGATGATGATCTTACTGATGACATTG

Figure 3 Sequence alignment of *LALBA* gene fragment compared to GenBank accession no. KF781121.1

Table 1. The allelic and genotypic frequencies, recombined genotypic and haplotypic frequencies for the polymorphism in exon 1 of *GnRHR* gene in non-descriptive goats in Sri Lanka

Locus	Genotype	Genotype Frequency	Allele/Haplotype frequency (EF. $\pm$ SD.)
A730G	AA (26)	0.87	A 0.93
	AG (04)	0.13	G 0.07
G757A	GG (23)	0.77	G 0.88
	GA (07)	0.23	A 0.12
G891T	GG (24)	0.80	G 0.90
	GT (06)	0.20	T 0.10
Haplotypes	AA,A/G,GG (07)	0.24	AAG 0.15 (0.13 $\pm$ 0.014)
	AA,GG,GG (17)	0.57	AGG 0.69 (0.74 $\pm$ 0.035)
	A/G,GG,G/T (04)	0.13	AGT 0.02 (0.04 $\pm$ 0.014)
	AA,A/G,G/T (01)	0.03	GGG 0.12 (0.05 $\pm$ 0.049)
	A/T,GG,GG (01)	0.03	GGT 0.02 (0.03 $\pm$ 0.001)
			AAT NR (0.01)

EF; Expected frequency of combined haplotypes, SD; Standard deviation, NR; Not recorded

Table 2. The allelic and genotypic frequencies, recombined genotypic and haplotypic frequencies at the heterozygous polymorphism sites in exon 4 of *k-CSN3* gene in non-descriptive goats in Sri Lanka

Locus	Genotype	Genotype frequency	Allele/Haplotype frequency (EF. $\pm$ SD.)
A274G	GG (9)	0.45	G 0.525
	AA (8)	0.4	A 0.475
	A/G (3)	0.15	
G309A	GG (17)	0.85	G 0.9
	AA (1)	0.05	A 0.1
	A/G (2)	0.1	
A471G	AA (16)	0.8	A 0.9
	A/G(4)	0.2	G 0.1
T591C	TT (17)	0.85	T 0.925
	C/T (3)	0.15	C 0.075
Haplotype	GG/GG/AA/TT (7)	0.35	GGAT 0.23 (0.47 $\pm$ 0.168)
	AG/GG/AA/TT(3)	0.15	AGAT 0.19 (0.30 $\pm$ 0.082)
	AA/AG/AA/CT (1)	0.05	AAAC 0.18 (0.07 $\pm$ 0.078)
	AA/AA/AA/CT (1)	0.05	AGAC 0.13 (0.02 $\pm$ 0.078)
	AA/GG/AA/TT (3)	0.15	AAAT 0.13 (0.02 $\pm$ 0.078)
	AG/GG/AG/TT (1)	0.05	AGGT 0.06 (0.06 $\pm$ 0.001)
	GG/GG/AG/TT (1)	0.05	GGGT 0.06 (0.03 $\pm$ 0.015)
	AA/GG/AG/TT (2)	0.1	GAAC NR (0.01)
	AG/AG/AA/CT (1)	0.05	GAAT 0.01 (0.01 $\pm$ 0.002)
			GGAC 0.01 (0.01 $\pm$ 0.002)

EF; Expected frequency of combined haplotypes, SD; Standard deviation, NR; Not recorded

Table 3. *k-CSN3* (exon 4) and *GnRHR* (exon 1) genetic variability among locally adapted goats in Sri Lanka

GenBank accession no./ Reference	Nucleotide Position in <i>k-CSN3</i> gene											GenBank accession no./ Reference	Nucleotide Position in <i>GnRHR</i> gene		
	203	245	274	284	309	384	385	471	550	583	591		730	757	891
X60763	G(Q)	T(Y)	A(N)	G(L)	G(V)	G(D)	A(D)	G(V)	T(V)	C(A)	T(S)	Yang et al, 2011	-	A/G(P)	G/T(R/M)
AF485340								A(I)				This study	A(A)		G
AY090466		C(Y)					G(G)	A			C(P)	This study	A	G	G
AF521022			G(S)					A				This study	A/G(A)	G	
AY350425		C		A(L)	A(I)			A		T(V)		This study	A		
AY428577		C				A(N)		A	C(A)			This study	A/G	G	G
This study	T(H)		G					A							
This study	T		A/G					A							
This study	T				A/G			A			C/T				
This study	T				A			A			C				
This study	T							A							
This study	T		A/G					A/G							
This study	T		G					A/G							
This study	T							A/G							
This study	T		A/G		A/G			A			C/T				

Q; Gln, Y; Tyr, N; Asn, L; Leu, V; Val, D; Asp, A; Ala, S; Ser, H; His, I; Ile P; Pro, G; Gly, A; Ala, P; Pro, R; Arg and M; Met. *k-CSN3* nucleotide positions are compared to reference GenBank accession no X60763 and *GnRHR* nucleotides positions are compared to GenBank accession no L42937.1. Note; only the nucleotide and amino acid variations compared to reference are indicated in the table

Table 4 *LALBA* (intron 2 and part of exon 3) genetic variability among locally adapted goats in Sri Lanka

GenBank accession no.	Nucleotide position												
	1427	1541	1561	1598	1673	1719	1721	1724	1726	1807	1822	1892	1897
M63868	G	-	G	G	G	G	G	G	G	G	G	G	C(F)
KF781121.1	G	G	G	G	G	G	G	G	G	G	G	G	T(F)
This study	G A/G	G G	G A/G	G A/G	G A/G	G A/G	G A/G	G A/G	G A/G	G A/G	G A/G	G A/G	C(F)

F; Phe. Nucleotide positions are compared to GenBank accession no M63868. Note; only the nucleotide and amino acid variations compared to reference are indicated in the table