Indexing and Abstracting

CrossRef, Google Scholar, Global Impact Factor, Genamics, Index Copernicus, Directory of Open Access Journals, WorldCat, Electronic Journals Library (EZB), Universitätsbibliothek Leipzig, Hamburg University, UTS (University of Technology, Sydney): Library, International Society of Universal Research in Sciences (EyeSource), Journal Seeker, WZB, Socolar, BioRes, Indian Science, Jadoun Science, Jour-Informatics, Journal Directory, JournalTOCs, Academic Journals Database, Journal Quality Evaluation Report, PDOAJ, Science Central, Journal Impact Factor, NewJour, Open Science Directory, Directory of Research Journals Indexing, Open Access Library, International Impact Factor Services, SciSeek, Cabell's Directories, Scientific Indexing Services, CiteFactor, UniSA Library, InfoBase Index, Infomine, Getinfo, Open Academic Journals Index, HINARI, etc.

CODEN (Chemical Abstract Services, USA): IJASKD

Short Communication

DETECTION OF GENETIC DIVERSITY IN FOUR CANDIDATE GENES BY POLYMERASE CHAIN REACTION-SINGLE STRAND CONFORMATION POLYMORPHISM (PCR-SSCP) ANALYSIS OF GOATS IN SRI LANKA

HBPC Ariyarathne, LGS Lokugalappatti[*], HBS Ariyaratne and DMS Munasinghe

Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya 20400, Sri Lanka.

*Corresponding author's email: slokug@pdn.ac.lk

Abstract

Genetic diversity of Sri Lankan goats (*Capra hircus*) was evaluated using Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) method to detect polymophism in four candidate genes (LALBA, k-CSN3, GnRHR and BMP4) in a sample of 219 goats representing three phenotypic groups. All studied loci were polymorphic, having three morphs for BMP4, LALBA and two morphs for k-CSN3 and GnRHR in the study population. A significant difference between genotypic frequencies for BMP4 and GnRHR genes was found while PIC criterion revealed an intermediate polymorphism for all analyzed phenotypic groups except for k-CSN3 and GnRHR genes. Cross-bred animals for BMP4 gene and nondescript animals for LALBA gene were comparatively more polymorphic according to the effective allele number criterion. This study provides insight into the genetic diversity of Sri Lankan goats, which can be utilized to develop single nucleotide polymorphism markers to be used in association studies, and marker assisted selection.

Keywords: Goat; Polymorphism; LALBA; k-CSN3; GnRHR; BMP4

Introduction

The studies on genetic diversity allow not only the genetic characterization of desired breeds but also documentation of correlations between genotypic variations and related phenotypic traits. Alpha lactalbumin (LALBA), Kappa-Casein (k-CSN3), Gonadotropin Releasing Hormone Receptor (GnRHR) and Bone Morphogenetic protein 4 (BMP4) genes are such extensively studied potential candidate genes in goats (Supakorn, 2009). LALBA and k-CSN3 genes are known to associate with milk traits (Grosclaude, 1988; Pe´rez-Rodrı´guez *et al.,* 1998; Kumar *et al*., 2006; Lan *et al.,* 2007; An *et al.,* 2009; Jain *et al.,* 2009 and Dettori *et al.,* 2015) such as milk production, composition of milk and cheese making properties. GnRHR gene is a key regulator of the reproduction (MingXing *et al.,* 2009; Yang *et al.,* 2011 and Fu *et al.,* 2014) and BMP4 gene is known to significant in growth traits and reproduction (Dijke *et al.,* 2003; Fang *et al.,* 2009 and Sharma *et al.,* 2013).

The goat population in Sri Lanka consists mainly of a nondescript type local breed and their crosses with imported Jamnapari, Boer and Saanen animals. Among them, nondescript local goats are the highly adapted animals to local environmental conditions and are known to immune for disease and parasitic stresses compared to exotic breeds (Silva, 2010). At present only limited information is available on the genetic diversity of nondescript type local goats in Sri Lanka (Baker *et al.,* 2001; Silva 2010). In this paper, we present the genetic diversity of four candidate genes (LALBA, k-CSN3, GnRHR and BMP4) detected utilizing Single Strand Conformational Polymorphism (SSCP) in goats of Sri Lanka. This is a highly cost effective, simple and rapid method used to detect variations in DNA compared to DNA sequencing technique which is intensively used for detecting mutations within the target sequences. The SSCP protocol is capable of producing different banding patterns on polyacrylamide gels in the presence of single nucleotide variations in the target (Neibergs *et al.,* 1993; Sheffield *et al.,* 1993) allowing rapid identification of genetic diversity of organisms.

Materials and methods

Sample Collection

Altogether 219 goats representing phenotypically nondescript, cross-bred and pure Jamnapari animals were included in this study (Table 1). Genomic DNA was extracted from blood samples using either salting-out protocol (Jawdat *et al.,* 2011) or a commercially available kit (Wizard Genomic DNA Purification Kit, Promega). Extracted DNA was quantified using a DNA spectrophotometer (BIOMATE 3) and lambda marker.

DNA amplification by PCR

Four gene fragments representing four candidate genes (LALBA, k-CSN3, GnRHR and BMP4) of the *Capra hiruca* genome were amplified. The details of the gene fragments, primer pairs used and respective annealing temperatures are given in the Table 2. PCR amplification of LALBA, k-CSN3, BMP4 and GnRHR was done as described by Jain *et al.,* 2009; Kiplagat *et al.,* 2010; Fang *et al.,* 2009 and Yang *et al.,* 2011 respectively. PCR reactions were performed in a 25 µl reaction mixture containing 100-50 ng genomic DNA, 0.5 µM of each primer, 10× buffer 2.5 µl, 1.5 mM MgCl$_2$, 200 µM dNTPs, 0.625 units of *Taq* DNA polymerase and water for each gene. All PCR amplifications were carried out in ABI2720 (Applied Biosystems®) Thermal Cyclers. Amplified PCR products were visualized on 1% agarose gel stained with ethidium bromide (1 µg/ ml) and verified using a 100 bp ladder (VC DNA Ladder Mix, Vivantis).

Table 1: Details of the samples used in the study.

Sample site/ Province	No. of Samples collected			Predominant phenotype		
	Male	Female	Total	Nondescript	Cross bred	Jamnapari
Northern	15	39	54	44	10	-
Eastern	53	74	51	08	39	4
North Western	44	70	114	104	10	-

Table 2. Details of the four gene fragments amplified, primer sequences and the respective annealing temperatures used in PCR reactions.

Gene	Gene region	Fragment location within gene	Primer sequence	Fragment size (bp)	Annealing Temperature (°C)
LALBA	* Exon 2 to 3	1135 bp -1802bp	F'- 5'-CCAGTGGTTATGACACACAAGC-3' R'-5'-TCCAGAATCTTCTTGACACACA-3'	667	61
k-CSN3	Exon 4	60bp - 518bp	F'- 5'-TATGTGCTGAGTAGGTATCC-3' R'-5'-TTGTCCTCTTTGATGTCTCC-3'	458	54
GnRHR	Exon 1	458 bp- 1204bp	F'- 5'-CTCGTTCGCTTTAGCACCC-3' R'-5'-CTGTGGTCCAGCAAAGATG-3'	746	62
BMP4	Intron 2	1959bp- 2339bp	F'- 5'-CTGGGGAAATGTTTGGTA -3' R'-5'-GCTAAGAGTTGGGTGATGAG-3'	380	57

* Part of exon 2, intervening intron and part of exon 3

Single strand conformational polymorphism (SSCP) analysis

Number of samples included in SSCP analysis of each gene is presented in the table 3. A sample (4 µl) of each PCR product was mixed with a same amount of denaturation buffer (10 mM/L EDTA, pH = 8, 80 % (m/V) N, N-dimethyl formamide, 1mg/ ml Bromophenol blue, 1mg/ ml Xylene Cyanol) in PCR tubes, denatured at 95 °C in a thermo-cycler for 5 minutes, snap chilled on ice and resolved on polyacrylamide gels (12%). The electrophoresis was carried out in with a vertical unit (130 V, 5 W, 6 mA), in 1x TBE buffer at 4 °C for 18 hours. Gels were treated with (soaked in) silver staining to visualize the bands (Sun *et al.,* 2002). The above protocol was further modified for polyacrylamide gel concentration, volume of PCR product, volume and concentration of denaturation buffer components and gel running time for LALBA and k-CSN3 genes to get an optimum resolution (Bastos *et al.,* 2001). The gel phenotypes were identified and scored manually to obtain PCR-SSCP allele frequency after ascertained the reproducibility.

Statistical analysis

Genotypic and allelic frequencies for LALBA, k-CSN3, BMP4 and GnRHR loci within and between nondescript, cross-bred and pure Jamnapari populations were analyzed using Chi square test. Homozygous dominant, homozygous recessive and heterozygous genotypes were selected according to Hardy-Weinberg equilibrium equation. Gene heterozygosity, gene homozygosity, effective allele numbers and Polymorphism Information Content (PIC) were calculated by Nei methods (Nei and Roychoudhury, 1974; Nei and Li, 1979).

Results and discussion

All four gene fragments were polymorphic. BMP4 and LALBA genes showed three patterns whereas k-CSN3 and GnRHR gene fragments showed only two patterns (Fig. 1). In BMP4 gene both nondescript and cross-bred phenotypes

showed all three genotypic patterns whereas Jamnapari showed only two homozygous genotypes (Table 3). For BMP4 gene, AA genotype was predominant among both nondescript and cross-bred animals (66% and 72% respectively). Nondescript animals showed 40%, 38% and 22% genotypic distribution among AA, AB and BB genotypes for LALBA gene while cross-bred animals were represented by AA genotype (100%) only. The k-CSN3 gene had only two genotypes and AA was unique to nondescript ones (100%) and the BB was restricted to cross-bred animals (100%). GnRHR gene was limited for homozygous genotypes (AA and BB) where BB genotype

(85%) was the most shared among nondescript animals while AA genotype (75%) was shared amongst cross-bred animals (Table 3). A significant difference between genotypic frequencies for BMP4 and GnRHR genes was found ($\chi^2 = 12.29$, df = 4, P = 0.001 and $\chi^2 = 54$, df = 2, P = 0.00001 respectively). Our observed homozygosity values are higher than expected heterozygosity values for all the genes examined (Table 3) except for cross bred animals in BMP4 gene where both values were equal (H_o= 0.5, H_e=0.5). This could be due to subpopulation structure and inbreeding system of mating (Wahlund, 1928) taking place among the goats in Sri Lanka.
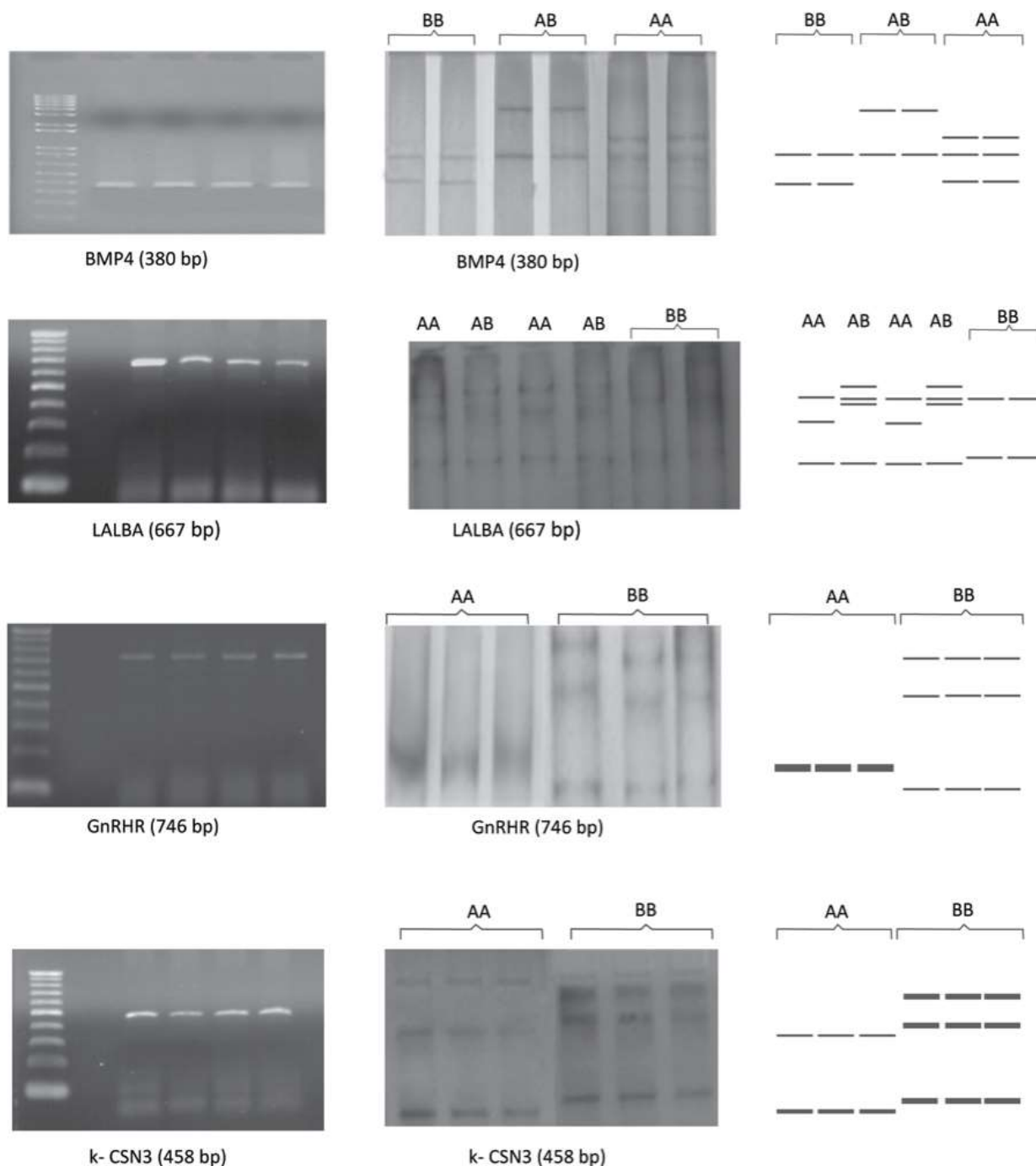
Fig. 1: The electropherogram of target PCR amplification of the four analyzed gene fragments (left) and the respective PCR-SSCP gel patterns (middle) with the schematic diagrams of each gel pattern (right).

Table 3: Genotypic, allelic frequencies and population genetic indices resulted for each of the four gene fragments.

Gene	Phenotypic group	Number of Samples	Genotypic Frequency			Allelic Frequency		χ^2 HWE	H_o	H_e	N_e	PIC
			AA	AB	BB	A	B					
BMP4	Nondescript	18	0.66 (12)	0.27 (05)	0.05 (01)	0.79	0.18		0.6867	0.3132	1.4562	0.2902
	Jamnapari	04	0.50 (02)	0(0)	0.50 (02)	0.50	0.50	P=0.001	0.6982	0.3018	1.4322	0.2802
	Cross-bred	54	0.72 (39)	0.18 (10)	0.09 (05)	0.81	0.19		0.5	0.5	2	0.5
LALBA	Nondescript	68	0.40 (27)	0.38 (26)	0.22 (15)	0.61	0.39	P=0.221	0.5217	0.4783	1.9168	0.4456
	Cross-bred	04	1.00 (04)	0(0)	0(0)	1.00	0.00		1	0	1	0
k-CSN3	Nondescript	42	1.00 (42)	0 (0)	0 (0)	1.00	0.00	P=1	1	0	1	0
	Cross-bred	10	0 (0)	0 (0)	1.00 (10)	0.00	1.00		1	0	1	0
GnRHR	Nondescript	26	0.15 (04)	0 (0)	0.85 (22)	0.15	0.85	P=0.00001	0.7396	0.2603	1.352	0.7560
	Cross-bred	28	0.75 (21)	0 (0)	0.25 (07)	0.75	0.25		0.625	0.375	1.6	0.3437

χ^2 (HWE) = Hardy-Weinberg equilibrium χ^2 value. Their P values were all above $\alpha = 0.05$. H_o: observed homozygosity, H_e : expected heterozygosity, N_e : effective allele numbers and PIC: polymorphism information content

According to the classification of PIC, PIC < 0.25 is considered as low polymorphism, 0.25 < PIC < 0.5 as intermediate polymorphism and PIC > 0.5 as high polymorphism (Ma *et al.,* 2010). However, as described by the PIC criterion all our analyzed breeds possessed intermediate polymorphism for all the genes tested except CSN3 and GnRHR genes. Both nondescript and cross-bred animals showed low polymorphism (PIC=0) for k-CSN3 whereas nondescripts were highly polymorphic (P=0.76) for GnRHR gene (Table 3).

Higher effective allele number (N_e) indicates higher polymorphism (Lan *et al.*, 2007) and it ranges from 1-2 in our studied goat population (Table 3). In this population the most polymorphic group was cross-bred animals for the BMP4 locus with the recorded N_e of 2 and the other two breeds (nondescript and Jamunapari) were equally diverse. For the LALBA gene, nondescript local goats showed the highest diversity (N_e= 1.9168) over the cross breds (N_e=1). Cross bred and non-descript animals for both k-CSN3 and GnRHR gene were also equally polymorphic.

Many authors have suggested that SSCP is a reliable, reproducible (Neibergs *et al.*, 1993, Sheffield *et al.,* 1993) and cost effective method compared to DNA sequencing (Orita *et al.,* 1989) for detecting structural variations in genome due to point mutations. According to studies by Yang *et al.,* (2011) and Kiplagat *et al.,* (2010) respectively on exon 1 of GnRHR gene and exon 4 of k-CSN3 gene indicated that these genes possess only two haplotypes and the results of our study confirm the above finding for Sri Lankan goats by showing only two haplotypes for both genes in Sri Lankan goats as well. Further, findings of this study reveal that both exon 2 of LALBA and intron 2 of BMP4 gene possess three haplotypes that were previously described by Cosenza *et al.*, (2003) and Xing-Tang *et al.*, (2010) respectively. The present study is a part of ongoing genetic characterization project aims at detection and development of genetic markers for marker-assisted selection, intended for improving the quantity and quality of goat production in Sri Lanka. DNA sequencing and association analysis with production traits are progressing for all four gene fragments at present.

Conclusion

All four gene regions analyzed are polymorphic in the sample of goats used in this study. A significant difference between genotypic frequencies for BMP4 and GnRHR genes was found while PIC criterion revealed an intermediate polymorphism for all analyzed phenotypic groups except for k-CSN3 and GnRHR genes. This study provides preliminary evidence for genetic polymorphism of Sri Lankan goats, which can be utilized to develop single nucleotide polymorphism markers to be used in association studies, and marker assisted selection. This will be critical for decision making on suitable management systems at farmers' level especially for low input systems in the country.

References

An XP, Han D, Hou JX, LiG, Wang JG, Yang MM, Song YX, Zhou GQ, Wang YN, Ling L, Yan QM, Cao BY (2009) GnRHR gene polymorphisms and their effects on reproductive performance in Chinese goats. *Small Ruminant Research*. **85**: 130-134. DOI: 10.1016/j.smallrumres.2009.09.001

Barker JSF, Ta SG, Moore SS, Mukherjee TK, Matheson JL, and Selvaraj OS (2001) Genetic variation within and relationships among populations of Asian goats (Capra hircus). *Journal of Animal Breeding and Genetics.* **118**: 213-233. DOI: 10.1046/j.1439-0388.2001.00296.x

Bastos E, Cravador A, Azevedo J and Guedes-Pinto H (2001) Single strand conformation polymorphism (SSCP) detection in six genes in Portuguese indigenous sheep breed "Churra da Terra Quente". *Biotechnology Agronomy Society and Environment.* **5** (1): 7–15.

Cosenza G, Gallo D, Illario R, Gregorio P, Senese C, Ferrara L, Ramunno L and MvaI A (2003) PCR-RFLP detecting a silent allele at the goat a-lactalbumin locus. *Journal of Dairy Research* .**70**: 355–357. DOI: 10.1017/S0022029903006265

Dettori ML, Pazzola M, Paschino P, Pira MG and Vacca GM (2015) Variability of the caprine whey protein genes and their association with milk yield, composition and renneting properties in the Sarda breed. 1. The LALBA gene. *Journal of Dairy Research* **82**(4):.434-441. DOI: 10.1017/S0022029915000461

Dijke P, Fu J, Schaap P and Roelen BAJ (2003) Signal Transduction of Bone Morphogenetic Proteins in Osteoblast Differentiation. *Bone and Joint Surgery.* **85**: 34-38.

Fang X, Xu H, Zhang C, Chen H, Hu X, Gao X, Gu C and Yue W (2009) Polymorphism in BMP4 gene and its association with growth traits in goat. *Molecular Biology Reports*. **36**:1339-1344. DOI: 10.1007/s11033-008-9317-1

Fu MZ, Li G and Zhou ZQ (2014) Polymorphisms of LHβ and GnRHR genes and their association with the number of embryos recovered in goats. *Animal Production Science*, *54*(8), pp.987-991. DOI: 10.1071/AN13076

Grosclaude F (1988) Genetic polymorphism of the main cow milk proteins. Relations with milk's quantity, composition

and cheese making attitude, INRA. *Productions Animales*. **1**:15–17.

Jain A, Gour DS, Bisen PS, Dubey PP, Sharma DK, Joshi BK and Kumar D (2009) Single nucleotide polymorphism (SNP) in alpha-lactalbumin gene of Indian Jamnapari breed of *Capra hircus*. *Small Ruminant Research* **82**(2):156-160. DOI: 10.1016/j.smallrumres.2009.02.013

Jawdat NG, Adnan F N and Akeel HA (2011) Simple salting–out method for genomic DNA extraction from whole blood. *Tikrit Journal of Pure Science* **16**: 9-11.

Kiplagat SK, Agaba M, Kosgey IS, Okeyo M, Indetie D, Hanotte O and Limo MK (2010) Genetic polymorphism of kappa-casein gene in indigenous Eastern Africa goat populations. *International Journal of Genetics and Molecular Biology* **2**: 1-5.

Kumar D, Gupta N, Ahlawat SPS, Satyanarayana R, Sunder S and Gupta SC (2006) Single strand confirmation polymorphism (SSCP) detection in exon I of the α - lactalbumin gene of Indian Jamunapri milk goats (Capra hircus). *Genetics and Molecular Biology* **29**: 271-274. DOI: 10.1590/S1415-47572006000200016

Lan XY, Pan CY, Chen H and Zhang CL (2007) An AluIPCR-RFLP detecting a silent allele at the goat *POU1F1* locus and its association with production traits. *Small Ruminant Research* **73**: 8-12. DOI: 10.1016/j.smallrumres.2006.10.009

Ma RN, Deng CJ, Zhang XM, Yue XP, Lan XY, Chen H and Lei CZ (2010) A novel SNP of α-Lactalbumin gene in Chinese dairy goats. *Molecular Biology* **44** (4):608-612. DOI: 10.1134/S0026893310040059

MingXing C, JieWen X, Ran D, XueWei L, Li F, YueHui M and Kui L (2009) Polymorphism of gonadotropin releasing hormaone receptor (GnRHR) gene and its relationship with prolificacy of lining grey goat. *Journal of Agricultural Biotechnology*, **17**(2): 218-223.

Nei M and Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science* **76**: 5269-5273. DOI: 10.1073/pnas.76.10.5269

Nei M and Roy Choudhury AK (1974) Sampling variances of heterozygosity and genetic distance. *Genetics.* **76**: 376-390.

Neibergs H, Dietz A and Womack J (1993) Single-Strand Conformation Polymorphisms (SSCPs) detected in five bovine genes. *Animal Genetics* **24**: 81-84. DOI: 10.1111/j.1365-2052.1993.tb00244.x

Orita M, Suzuki Y, Sekiya T and Hayashi K (1989) Rapid and sensitive detection of point mutation and DNA polymorphismsusing polymerase chain reaction. *Genomics* **5**: 874-879. DOI: 10.1016/0888-7543(89)90129-8

Pe´rez-Rodrı´guez ML, Martı´n-Alvarez PJ, Ramos M, Garcı´a-Muro E, Zaraga I and Amigo L (1998) Genetic polymorphism of bovine milk proteins in Holstein-Friesian and Fleckvieh breeds exploited in Spain. *Milchwissenschaft* **53**: 543–546.

Sharma R, Ahlawat S, Maitra, A, Roy M, Mandakmale S and Tantia MS (2013) Polymorphism of BMP4 gene in Indian goat breeds differing in prolificacy. *Gene* **532**(1): 140-145. DOI: 10.1016/j.gene.2013.08.086

Sheffield V, Beck J, Kwitek A, Sandstrom D and Stone E (1993) The sensitivity of single strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* **16**: 325-332. DOI: 10.1006/geno.1993.1193

Silva P (2010) Indigenous Animal Genetic Resources of Sri Lanka – Status, potential and opportunities. GEF-UNEP-ILRI –FAnGr Asia Project (ISBN 978-955-589-120-2).

Sun HS, Anderson LL, Yua TP, Kima KS, Klindt J and Tuggle CK (2002) Neonatal Meishan pigs show POU1F1 genotype effects on plasma GH and PRL concentration. *Animal Reproduction Science* **69**: 223–237. DOI: 10.1016/S0378-4320(01)00177-4

Supakorn C (2009) The Important Candidate Genes in Goats. *Walailak Journal of Science and Technology* **6**:17-36.

Wahlund S (1928) Zusammensetzung von Population und Korrelations erscheinung vom Standpunkt der Vererbungslehre aus betrachtet. *Hereditas* **11**: 65–106. DOI: 10.1111/j.1601-5223.1928.tb02483.x

Yang W, Tang K, Zhang C, Xu D, Wen Q and Yang L (2011) Polymorphism of the GnRHR gene and its association with litter size in Boer goats. *South African Journal of Animal Science* **41**: 398-402. DOI: 10.4314/sajas.v41i4.10