Meta Gene 2 (2014) 439–449

Contents lists available at ScienceDirect

Meta Gene

Designing, optimization and validation of tetra-primer ARMS PCR protocol for genotyping mutations in caprine *Fec* genes

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ARTICLE INFO

Article history:
Received 12 February 2014
Received in revised form 27 May 2014
Accepted 31 May 2014
Available online 27 June 2014

Keywords:
Tetra-primer ARMS-PCR
BMPR1B
GDF9
BMP15

ABSTRACT

New, quick, and inexpensive methods for genotyping novel caprine *Fec* gene polymorphisms through tetra-primer ARMS PCR were developed in the present investigation. Single nucleotide polymorphism (SNP) genotyping needs to be attempted to establish association between the identified mutations and traits of economic importance. In the current study, we have successfully genotyped three new SNPs identified in caprine fecundity genes viz. T(-242)C (*BMPR1B*), G1189A (*GDF9*) and G735A (*BMP15*). Tetra-primer ARMS PCR protocol was optimized and validated for these SNPs with short turn-around time and costs. The optimized techniques were tested on 158 random samples of Black Bengal goat breed. Samples with known genotypes for the described genes, previously tested in duplicate using the sequencing methods, were employed for validation of the assay. Upon validation, complete concordance was observed between the tetra-primer ARMS PCR assays and the sequencing results. These results highlight the ability of tetra-primer ARMS PCR in genotyping of mutations in *Fec* genes. Any associated SNP could be used to accelerate the improvement of goat reproductive traits by identifying high prolific animals at an early stage of life. Our results provide direct evidence that tetra-primer ARMS-PCR is a rapid, reliable, and cost-effective method for SNP genotyping of mutations in caprine *Fec* genes.

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http://dx.doi.org/10.1016/j.mgene.2014.05.004

Introduction

Growth factors synthesized from mammalian oocytes popularly known as oocyte secreted factors play numerous roles in ovarian folliculogenesis. In particular, a growing body of evidence in recent years has indicated that members of transforming growth factor-β superfamily genes and proteins are expressed in ovarian tissues (Ergin et al., 2008; Hatzirodos et al., 2011). Most notable members of TGFβ superfamily viz. Bone Morphogenetic Protein Receptor 1B (*BMPR1B*), Growth differentiation factor 9 (*GDF9*) and Bone Morphogenetic Protein 15 (*BMP15*) are intra-ovarian regulators of folliculogenesis in mammals. The identification of *BMPR1B*, *GDF9* and *BMP15* gene mutations (Table 1) as the causal mechanisms underlying either the highly prolific or infertile phenotypes of several sheep breeds in a dosage-sensitive manner highlighted the crucial role these genes play in ovarian function.

BMPR1B has been implicated as one of the type 1 receptors downstream of *BMP15* in sheep, mouse and rat granulosa cells. The Booroola sheep mutation *FecB* is a Q249R mutation in the kinase domain of *BMPR1B*, thereby increasing the fertility of females (Wilson et al., 2001). *BMPR1B* null mice are infertile and have cumulus expansion defects (Yi et al., 2001). *GDF9* and *BMP15* act synergistically to affect development of the cumulus–oocyte complexes in mice (Yan et al., 2001). *GDF9* plays critical roles in granulosa and theca cell growth as well as in differentiation and maturation of the oocyte (Hreinsson et al., 2002). *BMP15* has also been thought to be involved in oocyte maturation and follicular development alone or along with *GDF9*. Both the proteins are progressively expressed by the oocytes of growing follicles throughout folliculogenesis (Dube et al., 1998; Laitinen et al., 1998).

In an attempt to screen Indian goats for prolificacy associated mutations in sheep, we have previously reported that none of the mutations in the candidate genes associated with fecundity in sheep was detected in Indian goats. Two novel SNPs T(-242)C and G(-623)A in the promoter region of *BMPR1B* gene (Ahlawat et al., 2014), three non-synonymous SNPs (C818T, A959C and G1189A) in exon 2 of *GDF9* gene and two novel SNPs (G735A and C808G) in exon 2 of *BMP15* gene were detected (Ahlawat et al., 2013). These novel polymorphisms need to be associated with prolificacy trait for identification of molecular markers that can be used to identify high prolific animals at an early stage of life using robust and less costly genotyping techniques. A plethora of methods for genotyping of specific polymorphic loci are currently used and these include Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Single Strand Conformation Polymorphism (SSCP), direct DNA sequencing, and tetra-Primer Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR). In developing countries it is very difficult and costly to genotype large number of animals by methods involving PCR and post-PCR manipulations. To circumvent these problems, simple, fast and cost effective genotyping methods need to be developed.

Amplification refractory mutation system (ARMS)-PCR, also referred to as allele-specific oligonucleotide PCR is a technique that was originally designed by Newton et al. (1989) for the detection of known sequence polymorphisms. In ARMS-PCR, 2 pairs of primers in a single PCR tube, can simultaneously amplify both mutant and normal alleles as well as allow amplification of an internal DNA control. This technique has

Table 1
Genetic variants associated with prolificacy phenotype in sheep.

Gene	Mutation	Amino acid change	Founder breed	Reference
BMPR1B	*FecB*	Q249R	Booroola Merino, Garole and Javanese	Mulsant et al. (2001) Souza et al. (2001) Wilson et al. (2001)
BMP15	$FecX^{I}$	V299D	Romney	Galloway et al. (2000)
	$FecX^{H}$	Q291ter	Romney	Galloway et al. (2000)
	$FecX^{B}$	S367I	Belclare	Hanrahan et al. (2004)
	$FecX^{G}$	Q239ter	Belclare and Cambridge	Hanrahan et al. (2004)
	$FecX^{L}$	C321Y	Lacaune	Bodin et al. (2007)
	$FecX^{R}$	17 bp deletion	Rasa Aragonesa	Martinez-Royo et al. (2008) Monteagudo et al. (2009)
GDF9	$FecG^{H}$	S395F	Belclare and Cambridge	Hanrahan et al. (2004)
	$FecG^{T}$	S427R	Icelandic	Nicol et al. (2009)
	$FecG^{E}$	F345C	Santa Ineˆs	Silva et al. (2011)

been applied to study different mutations (Old et al., 1990; Vannucchi et al., 2006; Wanga et al., 2014). Ye et al. (1992) were the first to describe tetra-primer PCR in which allele-specific amplification is achieved in a single PCR reaction using two outer primers and two allele-specific inner primers. Ye et al. (2001) combined tetra-primer PCR with ARMS to form the tetra-primer ARMS-PCR or T-ARMS technique by introducing deliberate mismatches at position −2 from the 3′ end of inner primers to improve allele specificity. In a single step reaction, the outer primers amplify a large fragment of the target gene, irrespective of its genotype although each inner primer combines with a particular opposite outer primer to generate smaller allele-specific amplicons, which are of different sizes and can easily be discriminated on gel electrophoresis either as homozygous or heterozygous. In the present study, we developed rapid, efficient, cost effective and allele specific tetra-primer ARMS PCR for genotyping T(-242)C (*BMPR1B*), G1189A (*GDF9*) and G735A (*BMP15*) mutations so that these methods can be used to genotype large animals of animals in order to estimate association of these mutations with the prolificacy trait in goats.

Materials and methods

Sample collection

Blood samples (158) from Black Bengal goat breed from Kotulpur goat cum fodder farm, Bankura, West Bengal were collected from the jugular vein into EDTA containing vacutainer tubes. Genomic DNA was isolated and purified from the blood cells using the standard phenol–chloroform–isoamyl alcohol extraction followed by ethanol precipitation (Sambrook and Russell, 2001). The quality and quantity of isolated DNA were determined using agarose gel electrophoresis (0.8%) and NanoDrop spectrophotometer (GE Healthcare). Purified DNA ran as a single band on agarose gel and the OD 260/280 ratio for all the samples was between 1.8 and 2 indicating good quality of extracted DNA. The DNA samples were dissolved in TE buffer (pH 8.0) and stored at −20 °C until use.

Primer designing

Tetra-primer ARMS PCR was developed for genotyping T(-242)C, G1189A and G735A mutations in *BMPR1B*, *GDF9* and *BMP15* genes respectively. Primers were designed by the original software on the website: http://cedar.genetics.soton.ac.uk. The mutation points were positioned asymmetrically with respect to the common (outer) primers so that allele specific amplicons with different product lengths could be easily separated by standard agarose gel electrophoresis. 'BLAST' program at http://www.ncbi.nlm.nih.gov/blast was used to check for the specificity of the primers. The primers used in this study are listed in Table 2.

Table 2
Sequence of primers used, product size and annealing temperature for T-ARMS PCR.

Gene	Primer sequence	Product size	Annealing temp. °C
BMPR1B	Forward inner primer: GTCAAATACAACTATTATAGCTTAAGGT	Control fragment: 238 bp	54
	Reverse inner primer: TAAACCTTTCAGAGTAGTAGCTTATG	T allele: 168 bp	
	Forward outer primer: ATAATATTTACTCACAGTATTACATGCA	C allele: 124 bp	
	Reverse outer primer: AAATAGTAAGGATTTGTAACACTTAAAG		
GDF9	Forward inner primer: TGCTTTTGTATCTGAACGACACAAGGGT	Control fragment: 323 bp	68
	Reverse inner primer: GGAATGCCACCTGTGAAAAGCCTTAG	T allele: 207 bp	
	Forward outer primer: CCTCCCACAAGAGGAATATTCACATGTCT	C allele: 170 bp	
	Reverse outer primer: AACCAGAGGCTTCTTCAATTCAGAGCTG		
BMP15	Forward inner primer: TTTCAATGACACTCAGAGTGTTCAGCAA	Control fragment: 425 bp	67
	Reverse inner primer:TCAGGCCTTTAGGGAGAGGTTTGTTC	A allele: 257 bp	
	Forward outer primer: CTTGGACAGAGATGGATATCATGGAACA	G allele: 216 bp	
	Reverse outer primer: GTAGTTTGGGGTATAGAGATGGGGAGCA		

PCR method

Gradient PCR was carried out on BIORAD iCycler to determine the best annealing temperature for each primer set. The optimal annealing temperatures for primers specific for −T242C (*BMPR1B*), C818T (*GDF9*) and G735A (*BMP15*) loci were 54, 68 and 67 °C respectively. For all sets of outer–inner primer pair, optimum concentration of $MgCl_2$ producing the highest yield of specific PCR products was 2 mM. Outer–inner primer ratio for tetra-primer ARMS PCR was 1:1 for −T(242)C (*BMPR1B*) and 1:10 for C818T (*GDF9*) and G735A (*BMP15*) loci. Finally, optimized PCR was performed in a single tube containing 25 μl of reaction volume made up of the following components: 10 μl master mix, 50–100 ng of genomic DNA and optimized concentrations of each primer. PCR cycling was performed at 95 °C for 4 min followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at specific temperature for 45 s, extension at 72 °C for 45 s and final extension at 72 °C for 8 min. The PCR products were separated by running on 3% agarose gel with DNA size marker of 50–1000 bp followed by staining with ethidium bromide and visualized and semi-automatically analyzed by the gel documentation system.

Validation of the assay

To evaluate the efficiency and accuracy of the assay, selected PCR-amplified DNA samples (n = 3, respectively, for each genotype) were examined by DNA sequencing and the results obtained by T-ARMS PCR were compared with those determined by sequencing.

Results

Three point mutations in important candidate genes for fecundity, one each in *BMPR1B*, *GDF9* and *BMP15* were genotyped by tetra-primer ARMS PCR based methodology. PCR fragments were generated as per expectations for all the loci. All the PCR products were well resolved and sized by agarose gel electrophoresis, allowing easy identification of different genotypes. Heterozygotes and homozygotes were unambiguously assigned from the gel profile. The size of DNA fragments amplified with these four primers for −T242C of *BMPR1B* (239 bp control fragment, 168 bp T allele, 125 bp C allele), C818T of *GDF9* (323 bp control fragment, 207 bp T allele, 170 bp C allele) and G735A of *BMP15* (425 bp control fragment, 222 bp G allele, 257 bp A allele) was suitable for separation on 3% agarose gels (Figs. 1, 2, 3). Several factors, including primer concentration and PCR cycling conditions, which can affect PCR specificity and efficiency were optimized. For all sets of outer–inner primer pairs we tested Mg^{2+} concentrations from 1.5 mM to 4 mM and best results were obtained at Mg^{2+} concentration of 2 mM for all the primer sets. Gradient PCR was carried out on the BIORAD iCycler to determine the best annealing temperature for each primer set (change per reaction was

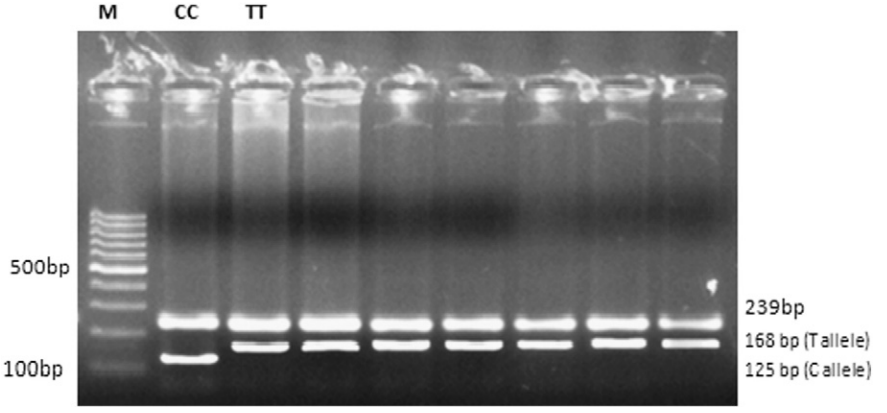

Fig. 1. Agarose gel electrophoresis (3%) of polymerase chain reaction (PCR) product of tetra-primer ARMS-PCR for T(−242)C locus of *BMPR1B* gene.

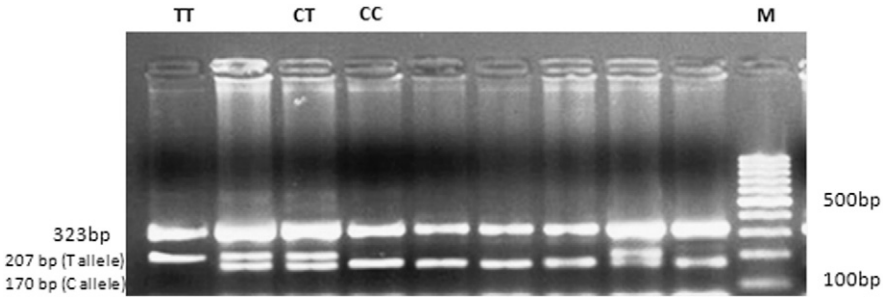

Fig. 2. Agarose gel electrophoresis (3%) of polymerase chain reaction (PCR) product of tetra-primer ARMS PCR for C818T locus of *GDF9* gene.

1 °C) and optimum annealing temperatures were finalized to be 54, 68 and 67 °C for −T242C (*BMPR1B*), C818T (*GDF9*) and G735A (*BMP15*) loci.

To improve the amplification efficiency of the shorter and allele-specific products, titration of primer concentrations is of paramount importance in tetra-primer ARMS PCR (Etlik et al., 2008; Okayama et al., 2004; Ye et al., 2001). In our study also, optimization of primer concentrations was the trickiest one, since the usually suggested outer–inner ratio (1:10) for tetra-primer ARMS PCR was not useful for one of the primer sets (−T242C, *BMPR1B*). Thus, we performed PCR with three more ratios (1:5, 1:2, 1:1) of outer–inner primers and 1:1 was found to give best results for −T(242)C (*BMPR1B*) and 1:10 for the other two loci. Validation of the developed methodology was done by direct sequencing of representative samples from each set using the outer primers for each SNP. We observed complete concordance between the methods. The genotypes scored from the assay were in 100% accordance with direct sequencing (Figs. 4, 5, 6).

Allele and genotype frequencies for the three SNPs in 158 animals of Black Bengal goats are given in Table 3. All the three possible genotypes were expressed for the three investigated loci. The predominant alleles were T, C and G for −T242C (BMPR1B), C818T (GDF9) and G735A (BMP15) mutations, respectively. Black Bengal goat population deviated from Hardy–Weinberg equilibrium ($P < 0.05$) with respect to all the three loci.

Discussion

Single nucleotide polymorphism (SNP), a novel molecular marker technology, refers to a sequence polymorphism caused by a single nucleotide mutation at a specific locus in the DNA sequence. This sort of polymorphism includes single base transitions, transversions, insertions and deletions (Lander, 1996), and the minor allele frequency should be 1% or greater (Vignal et al., 2002). Of all the SNP mutation types, transitions are the most common (approx. 66.6%) (Zhao and Boerwinkle, 2002). Currently, SNP markers are

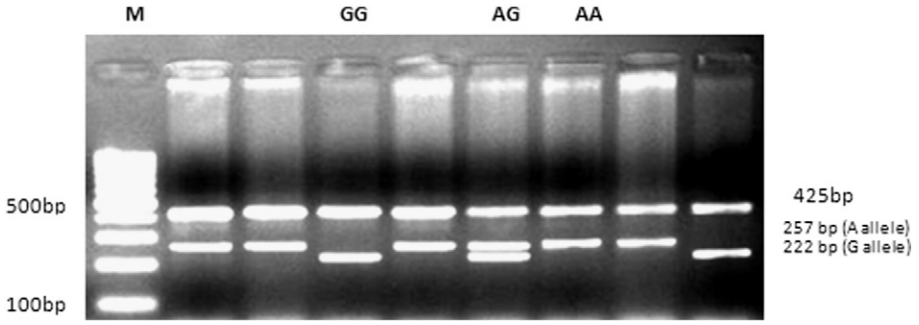

Fig. 3. Agarose gel electrophoresis (3%) of polymerase chain reaction (PCR) product of tetra-primer ARMS PCR for G735A locus of *BMP15* gene.

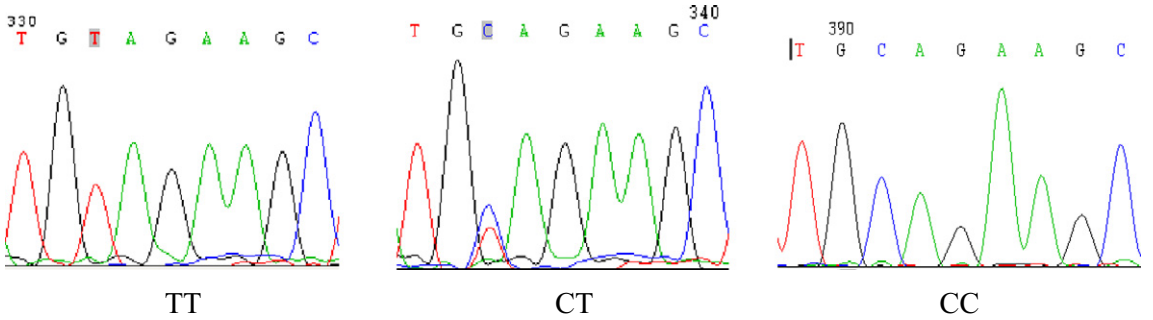

Fig. 4. Genotypes for T(−242)C mutation of *BMPR1B* gene.

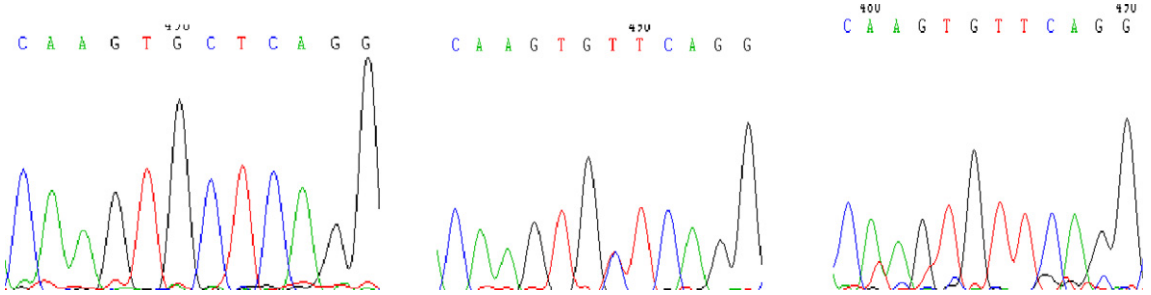

Fig. 5. Genotypes for C818T mutation of *GDF9* gene.

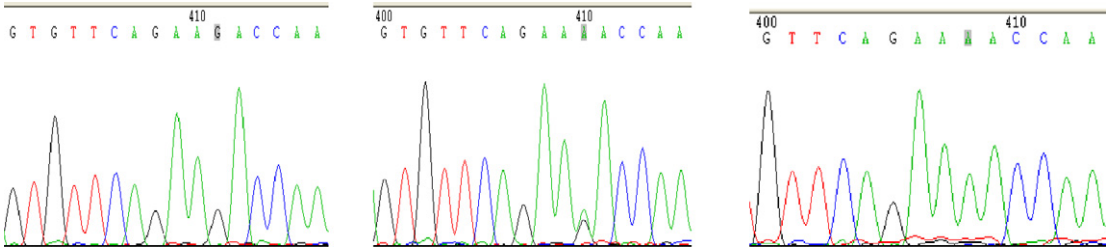

Fig. 6. Genotypes for G735A mutation of *BMP15* gene.

Table 3
Allele and genotype frequencies for the SNPs in three *Fec* genes in Black Bengal goats (158).

Gene	Mutation	Genotypes	Genotype frequency	Allele frequency	H-W test
BMPR1B	T(−242)C	TT-139	TT − 0.88	T − 0.905	$\chi^2 = 78.61^*$
		TC-8	TC − 0.05	C − 0.095	P value = 0.0000
		CC-11	CC − 0.06		
GDF9	C818T	CC-94	CC − 0.60	C − 0.75	$\chi^2 = 9.29^*$
		CT-46	CT − 0.30	T − 0.25	P value = 0.0023
		TT-18	TT − 0.10		
BMP15	G735A	GG-70	GG − 0.44	G − 0.63	$\chi^2 = 5.28^*$
		GA-60	GA − 0.38	A − 0.37	P value = 0.0216
		AA-28	AA − 0.18		

* Allele frequency differs significantly at $P < 0.05$.

one of the preferred genotyping approaches, because they are abundant in the genome, genetically stable and amenable to high throughput automated analysis.

Variation in candidate genes for traits of economic importance in livestock species may be useful in guiding genetic selection for desirable traits and thus designing a genotyping protocol for the differentiation of polymorphic nucleotides is required for investigation of an animal population for which phenotypic data is available. The workers in the past have used techniques like Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Single Strand Conformation Polymorphism (SSCP), direct DNA sequencing, and tetra-primer Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) for genotyping of specific polymorphic nucleotide loci. Among these the tetra-primer ARMS-PCR could be a useful tool for genotyping, since SSCP may not be repeatable some times, direct DNA sequencing is a cumbersome, time consuming, technically demanding and costly procedure and the possibility of getting a restriction site for an enzyme could be rare for genotyping by RFLP. In our study also PCR-RFLP protocol could be developed and standardized for SNP G(−623)A in the promoter region of *BMPR1B* gene, two SNPs (A959C and G1189A) in exon 2 of *GDF9* gene and one SNP (C808G) in exon 2 of *BMP15* gene but no restriction enzyme site was identified for the three loci dealt with in the present study viz. T(−242)C of *BMPR1B*, C818T of *GDF9* and G735A of *BMP15*. For genetic analysis, fast and economical assays that can be performed with standard PCR instruments are highly desirable. Compared to other genotyping techniques, tetra-primer ARMS-PCR has been reported to be a rapid, reliable, simple and economical assay for SNP genotyping (Little, 2001; Okayama et al., 2004; Ye et al., 2001). Etlik et al., 2011 compared tetra-primer ARMS assay with routinely used methods such as typical PCR-RFLP analysis, real time PCR assay and DNA sequencing. They concluded that although real time PCR and DNA sequencing are sensitive and accurate techniques, tetra-primer ARMS PCR assay could be beneficial in terms of total time, cost and applicability in a typical laboratory.

Hence tetra-primer ARMS-PCR based methodology was developed for genotyping three mutations in Black Bengal goats in the present study. The assay described here is more convenient than the traditional PCR-RFLP since it eliminates the need for incubation with restriction enzymes. This not only avoids any consequent errors and artifacts from such procedures but also reduces the amount of DNA required for the digestion step in PCR-RFLP. No special equipment and only a small amount of standard PCR reagents are needed in tetra-primer ARMS-PCR.

Black Bengal goat is a prolific and major meat producing animal in eastern states of India (Zeshmarani et al., 2007). They kid twice a year or more commonly thrice in two years and the number of kids at one time varies from single to quadruplet (Zeshmarani et al., 2007). Twinning is more frequent (56.32%) and quadruplet is least frequent (2.11%) litter size (Hassan et al., 2007). The allele and genotype frequencies for the three SNPs showed significant differences among the population studied. If phenotypic data (litter size) is available for the investigated animals, an association between genotype and prolificacy can be conveniently established. SNPs are the most widely tested markers in association studies. These SNPs were tested for Hardy–Weinberg equilibrium (HWE) by comparing the observed genotype counts in Black Bengal goats with those expected under HWE. Since all the three loci deviated significantly from HWE it may indicate non-random mating and possibly population stratification, non-random genotyping error or missing genotype data so that one allele or genotype is more often misclassified or missing than the other. However, this may be due to the small sample size taken for the standardization of the technique. Thus

there is a need to increase the population size in actual association studies to fulfill the assumptions for HWE, that there is random mating (with respect to that locus), no selection, no mutation, no gene flow and a population large enough to avoid the random effects of genetic drift.

The tetra-primer ARMS-PCR methods described above are the first reported methods allowing one to genotype three novel SNPs in goat *Fec* genes with no post-PCR treatment other than electrophoresis. These methods are rapid, simple, reliable, easy to perform, economical and require minimum level of expertise that can be used for both large- and small-scale genotyping studies.

Conclusion

With the rapid advances in molecular techniques, various methods for genotyping single-nucleotide polymorphisms (SNPs) are available. Still, the search for easy, robust, and less costly techniques continues. We wished to develop a tetra-primer Amplification Refractory Mutation System-Polymerase Chain Reaction based technique for genotyping the SNPs in *Fec* genes so that these methodologies could be used to establish association between the studied mutations and prolificacy trait, provided that the phenotypic data is available. Any such associated SNP could be used to accelerate the improvement of goat reproductive traits by identifying high prolific animals at an early stage of life.

Acknowledgment

We acknowledge the financial support provided by Network Project on Animal Genetic Resources (ICAR) for carrying out the work.

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