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Design of a tetra-primer amplification refractory mutation system technique for cost effective genotyping of single nucleotide polymorphism of gonadotropin releasing hormone receptor gene in goats

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Abstract
The single nucleotide polymorphisms (SNP) in gonadotropin releasing hormone receptor (GNRHR) gene contributes substantially to the litter size trait in goats. In the present study, analytical validity of tetra primer amplification refractory mutation system polymerase chain reaction (tetra primer ARMS PCR) assay and PCR restriction fragment length polymorphism (PCR-RFLP) were compared based on sequencing method results for genotyping of GNRHR promoter SNP (rs642351318; A/G) in goats. The sensitivity and specificity were 100 per cent for tetra primer PCR method, sensitivity and specificity were 100 and 97.43 percent respectively for PCR-RFLP. The results indicate that tetra-primer ARMS PCR provides an easy and cost-effective technique for GNRHR SNP genotyping.

Keywords: Tetra-primer ARMS-PCR, genotyping, GNRHR, goat

Introduction
Reproductive traits are of utmost importance in goat production. The gonadotropin releasing hormone receptor (GNRHR) gene plays a pivotal role in the development and maintenance of reproduction in mammals [1]. The single nucleotide polymorphisms (SNPs) in GNRHR were reported to be associated with litter size trait in Chinese goats and West African Dwarf goats [2, 3]. Screening of goat population to explore GNRHR region for more SNPs was presumed to be relevant for enhancing litter size trait in goats. Hence, simple and streamlined genotyping methods are needed for the cost effective detection of SNPs in GNRHR.
Tetra primer Amplification Refractory Mutation System Polymerase Chain Reaction (tetra-primer ARMS PCR) is regarded as a rapid, efficient and economic genotyping assay that did not require the preparation of high-quality DNA suitable for restriction enzyme digestion [4, 5]. The tetra primer ARMS PCR utilises two primer pairs, the outer and inner primers. The outer primers are used to isolate and amplify DNA sequence of interest; while the inner primers are employed to identify allelic variation. The reaction of tetra primer ARMS PCR is run in a single tube and in one PCR step, and genotype variation from SNPs of interest could be visualized directly using agarose gel electrophoresis [6]. The tetra primer ARMS PCR does not involve post-PCR modification, making the assay efficient, even though the optimisation phase can be time-consuming [7, 8].
It is essential that the molecular tests are properly evaluated before widespread dissemination in practice. The analytical validity of a molecular test is defined as its ability to measure accurately and reliably the genotype of interest [9]. In the present study, a tetra primer ARMS PCR assay and PCR- RFLP method were performed to genotype GNRHR SNP in native goats in order to investigate and compare the analytical validity of the two assays. The analytical validity of assays was evaluated as sensitivity and specificity.

Materials and Methods
Study goat population and DNA samples The study goat population comprised of 53 goats randomly sampled from farmers herd and government farms in Kerala, India. Blood samples were collected from study goats and genomic DNA was extracted using standard phenol chloroform method [10]. The DNA integrity was evaluated using 0.8 per cent agarose gel electrophoresis.

The concentration and purity of DNA was quantified in Nanodrop TM 1000 spectrophotometer (Thermo Scientific, USA) and adjusted to 50 ng/µl by adding TE buffer.

DNA pooling sequencing assay

Target region comprised of promoter region (6:84163028 to 84162608) of goat GNRHR. The goat GNRHR reference sequence (NC_030813.1) was retrieved from NCBI genome browser (https://www.ncbi.nlm.nih.gov/). The SNPs in the target region were identified using DNA pooling sequencing assay [11]. The PCR amplification of target region was performed using primers including forward: 5'TTCATTCACCTGCCAAGAGAGTAT3' and reverse: 5'GCACGTTATTTCATAGGGTAGCTTG3' The PCR was performed in a final reaction volume of 20 µl, containing 1µl genomic DNA (50 ng/µl) as template, 10µl of 2XPCR smart mix (Origin labs), 0.6µl of each of forward and reverse primer (10 pM/µl) and nuclease free water which was added to make up a final reaction volume. The PCR cycling profile involved an initial denaturation step at 94 ºC for 5 minutes followed by 35 amplification cycles consisting of denaturation at 94 ºC for 30 s, annealing for 30 s at 61.4 ˚C and an extension at 72 ºC for 30 sec. A final extension at 72 ºC for 5 minutes completed PCR reactions. Amplification was performed in Bio-Rad thermal cycler (Bio- Rad, USA). The DNA pooling sequencing assay revealed a single SNP (rs642351318; A/G) in the promoter region of goat GNRHR in native goats.

All the DNA samples in the study goat population were sequenced individually and genotypes frequencies were recorded. DNA sequencing was set as the gold standard for the analytical validity of tetra primer PCR and PCR RFLP.

PCR-RFLP method

The SNP (rs642351318) was also genotyped in the whole study population by PCR-RFLP. For converting the SNP into PCR-RFLP marker, information regarding type II restriction enzyme was retrieved from data base, REBASE using online programme NEBcutter version 2.0 (www.labtools.us/nebcutter-v2-0). The restriction enzyme DdeI having a recognition sequence of 'CTNAG' was used for PCR- RFLP assay (Table 1.). The PCR-RFLP was performed in a reaction volume of 10 µl containing 5µl of PCR product, 1 µl of 10x reaction buffer, 0.08 µl of restriction enzyme (10 U/ µl) and nuclease free water which was added up to make final reaction volume.

Tetra primer ARMS-PCR method

For carrying out tetra primer ARMS- PCR, primer pairs (Table 2) was designed using Primer 1 software (http://primer1.soton.ac.uk/primer1.html). The expected PCR product lengths of control, wild and variant were 746 bp, 470 bp and 330 bp, respectively.

Table 1: The PCR-RFLP marker information

SNP	Restriction enzyme	Recognition sequence and variant			Incubation conditions		Expected product size (bp)	
					o	Period (h)	Allele A	Allele G
rs6423513 18	DdeI	C TNA C TNA	G A	/	37	2	217,129,75	129,109,108,75

Ti: incubation temperature

Table 2: Primer information for tetra-primer ARMS PCR

Primer	Sequence (5'-3')
Forward inner	AACTACAAGAATGGGCCATCTCAG
Reverse inner	AATGTTAAAAGTAAGGGTGGTTATTTTCAT
Forward inner	GACACTGAAGAGGTACGTAAGGTAAGTC
Reverse outer	CGTTATTTCATAGGGTAGCTTGGTATTA

Two important aspects in the optimisation of tetra primer ARMS PCR were the standardisation of annealing temperature and determining the ratio of outer and inner primers. The annealing temperature was optimised by using gradient PCR system. Reactions involving different outer and inner primer ratios were carried out during the optimizing process. The tetra-primer ARMS PCR was carried out in a final volume of 20 µL, containing 1 µL of the template DNA, 10µl of 2XPCR smart mix (Origin), 0.6 µL of each outer primer, 1.8 µL of the forward inner primer, 1.2 µL of the reverse inner primer and nuclease free water which was added to make up a final reaction volume of 20µl. The tetra primer ARMS PCR cycling profile involved an initial denaturation at 94 ºC for 5 minutes followed by 35 amplification cycles consisting of denaturation at 94 ºC for 30 s, annealing for 30 s at 61.4 ºC, an extension at 72 ºC for 45 s and a final extension at 72 ºC for 5 minutes. The PCR products were analysed using 2 per cent agarose gel electrophoresis.

Statistical analysis

The allele and genotype frequencies were computed based on the observations in each assay. The analytical validity of tetra primer ARMS and PCR-RFLP were estimated as per Kroese *et al*. (2004). The analytical sensitivity was estimated based on the probability that the test detects the specific genotype of interest that the test was intended to detect; the analytical specificity was calculated as the probability that the test did not detect specific genotype of interest that were not present. The genotype of interest considered was AG genotype with G mutant allele.

Results and Discussion

The DNA pooling sequencing assay revealed a single SNP (rs642351318; A/G) in the promoter region of GNRHR in native goats. The SNPs in promoter region of GNRHR were reported to be associated with litter size trait in West African Dwarf goats [3]. The SNPs in the promoter region could modify transcription factor binding sites and considered crucial in the regulation of gene expression [12]. Therefore development of an easy and inexpensive method benefitting from high specificity and accuracy for screening of SNPs in GNRHR is important.

In the present study, PCR sequencing assay was considered as the gold standard for the evaluation of analytical validity of PCR- RFLP and tetra primer ARMS PCR assays. Though, the sequencing assay is the most reliable and precise technique for screening SNPs, the high cost limits its wider application. The sequencing assay revealed three genotypes in the study goat population. The chromatograms corresponding to each genotype is given in Figure 1. The allele and genotype frequencies observed in PCR sequencing assay are given in Table 3. There were only two samples with GG genotypes in the study population.

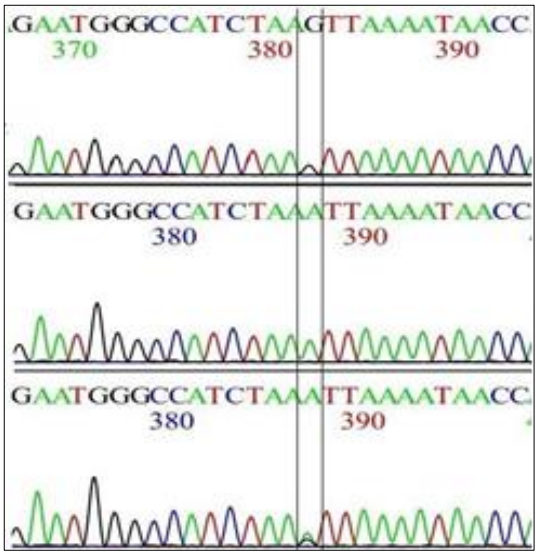

Fig 1: Sequence chromatogram showing three genotypes (GG, AA and AG) of SNP (rs642351318) in GNRHR

The genotyping of SNP (rs642351318) in native goats by PCR-RFLP revealed three RFLP banding patterns (Figure 2) corresponding to three genotypes; AA (217 bp,129 bp, and 75 bp fragments) and AG (217 bp, 129 bp, 109 bp, 108 bp and 75 bp fragments) and GG (129 bp,109 bp,108 bp and 75 bp fragments). The RFLP is advantageous in being accurate, fast and simple molecular tool for the profiling of population [13]. Among the two samples with GG genotype, only one GG sample was correctly genotyped in PCR-RFLP. The other was wrongly genotyped as AG in PCR-RFLP. Nevertheless, the incomplete and/or partial digestion is a major drawback of RFLP method resulting in false negative or false positive outcomes. The other technical limitation of RFLP method is the high price and non-availability of restriction enzymes [14].
Tetra primer ARMS-PCR revealed three different genotypes for the SNP (rs642351318) of GNRHR *viz*., AA, AG and GG in the whole study goat population. The Tetra-primer ARMS PCR banding patterns of the three genotypes are shown in Figure 3. Compared to PCR-RFLP, the tetra primer ARMS PCR is easy as it does not involve post-PCR modification [7, 8].

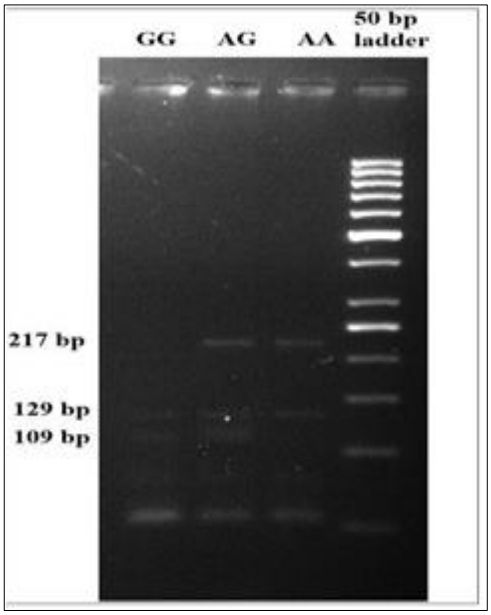

Fig 2: The PCR-RFLP banding pattern showing three genotypes (GG, AG and AA) of SNP (rs642351318) in GNRHR.

Nevertheless, standardisation of the tetra primer ARMS PCR assay was time consuming compared to PCR-RFLP. The success of the genotyping method by tetra primer ARMS-PCR relies on many factors, the major one being the type of polymorphism present in the loci. A transition from A to G produces a strong destabilising strength at the 3' end of the inner primers and the subsequent weak mismatch (A to C) deliberately produced at penultimate position of polymorphism in inner primers facilitated more specificity of the reaction [15]. There are reports of unsuitability of the method for genotyping polymorphisms present in CG rich regions due to unspecific amplification of primer [16]. The low CG percentage 37.34 per cent of the GNRHR promoter region harbouring the polymorphism facilitated easy optimisation of the reaction. In the present study, all the primer pairs were amplified equally in a wide range of temperature from 60 ˚C to 66 ˚C that made the standardisation less time consuming. Minor variations in the concentration of primers brought about differences in the intensity of the bands produced. The higher concentration of the forward inner primer (G allele) compared to reverse inner primer (A allele) produced better intensity of amplicon with G allele following Medrano and de Oliveira [7].
The tetra primer ARMS PCR and PCR-RLP results were compared with sequencing assay and specificity and sensitivity were statistically measured and the results are presented in Table 4. The sensitivity and specificity were 100 per cent for tetra primer PCR method, sensitivity and specificity were 100 and 97.43 percent, respectively for PCR-RFLP. The results suggest the poorer specificity of PCR-RFLP technique in discriminating the homozygotes from heterozygotes. Incomplete and/or partial digestion was reported as one disadvantage of PCR-RFLP method resulting in false negative or false positive outcomes [14]. Therefore, it is suggested that cost effective tetra primer ARMS-PCR assay could replace the PCR- RFLP technique for screening the SNP in the GNRHR promoter region of goats.

Table 3: Genotype and allele frequencies of SNP (rs642351318; A/G) in GNRHR

Genotype/allele	AA	AG	GG	A	G
Frequency	38	13	2		
Relative Frequency	0.717	0.245	0.038	0.847	0.153

Table 4: Comparison of genotyping results obtained by sequencing, PCR-RFLP and tetra-primer ARMS PCR

Genotype	Sequencing	PCR-RFLP	Tetra-primer ARMS PCR
GG	2	1	2
AG	13	14	13
AA	38	38	38
Sensitivity		100	100
Specificity		97.43	100

Conclusion

In conclusion, analytical validity of tetra primer ARMS assay was compared with that of PCR-RFLP method based on sequencing method. The results of the current study revealed higher specificity and accuracy of tetra primer ARMS assay compared to PCR-RFLP specifically for discriminating the heterozygotes from homozygotes for genotyping the SNP in the GNRHR promoter region of goats.

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