Polymorphism of receptor genes of Follicle Stimulating Hormone (FSHR), Luteinizing Hormone (LHR) and Estrogen receptor alfa (erα) in Marathwadi buffaloes

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Abstract

Marathwadi buffaloes are found in Marathwada region of Central India and are primarily reared by marginal farmers and landless laborers for its modest milk production. In buffaloes, inadequate reproduction is a limiting factor for rapid genetic development. FSH and LH receptors are transmembrane receptors that are required for hormonal activity during reproduction. Similarly, ERα playes a critical role in the development of anatomical, functional, and behavioural qualities required for species reproduction. Present study was carried out to investigate polymorphism in 100 Marathwadi buffaloes for certain locus of FSHR (exon 10), LHR (exon 11) and ERα (exon 1) gene. Three loci of each gene fragments were studied to demonstrate that three out of nine loci studied were polymorphic and others were monomorphic.

Key words: allele frequency, FSHR, genotypic frequency, Marathwadi buffalo

Introduction

Indian subcontinent is an excellent reservoir of livestock biodiversity in the form of species, breeds and strains esp buffaloes (Gautam et al, 2021). India ranks first in the world for buffalo population and accounts for nearly 57 % of world population (Hegde, 2019). Marathwadi buffalo is found in Marathwada region of Maharashtra state with an average milk production of about 950±2.50 lits. Genetic improvement of buffalo has been dependent on concepts of quantitative genetics of various traits of economic importance to promote more efficient and relatively easy selection of Indian buffaloes. The use of polymorphism in candidate genes for marker assisted selection may increase the genetic gain achieved by selection as a result of more accurately predicted breeding values. The economics of any dairy farm largely depends on the reproductive traits of animals. In cows with the CC genotype at the FSHR locus, the hormone concentration during ovulation was 1.1–2.5 ng/ml, which is within the physiological range for healthy reproduction (Sarybayev et al., 2023). Follicle Stimulating Hormone (FSH) is secreted by anterior pituitary gland and is essential for follicle growth, development, differentiation, triggering the maturation and ovulation of ovarian follicles. It is mainly responsible for follicular development in the ovary. Follicle-Stimulating Hormone (FSH) starts and maintains follicular development by binding to its specific receptor (FSHR) on the surface of the granulosa cells in the ovary (Palta *et. al.,* 1998). Luteinizing hormone (LH) is a glycoprotein hormone of pituitary origin that regulates gonadal function, including steroidogenesis and gametogenesis. LHR gene is located on chromosome number 11 (GeneID: 281900) and consists of 11 exons and 10 introns in Bovines. LHR responses include the regulation of male sex differentiation and male and female fertility. In females, production of androgens by the ovarian theca cells that surround the growing follicles, are dependent on LHR (Rahe *et al.,* 1980). Estrogen hormone is also one of the important hormones found to be linked with reproductive traits and affect the growth, differentiation and function of reproductive tissues like mammary gland, ovary and uterus. Therefore, the present investigation was carried out to study molecular profiling of this invaluable buffalo breed for polymorphism of genes related to the reproduction traits.

Materials and methods

The present research was carried out in the Department of Animal Genetics & Breeding, College of Veterinary and Animal Sciences, Parbhani (Maharashtra Animal & Fishery Sciences University, Nagpur). The data were collected from 100 Marathwadi buffalo reared at MAFSU Sub-centre Marathwadi buffalo farm, Udgir, Dist-Latur and Marathwadi buffalo breeding tract *i.e.* from Marathwada region of Maharashtra state. About 4 ml blood sample was collected from jugular vein of each animal in aseptic conditions in vaccuteiner containing EDTA as an anticoagulant. The samples were collected then carried to the laboratory on ice and were stored at -20^{o}C till DNA isolation was performed. Genomic DNA was isolated using DNA isolation kit (*Qiagen* DNA isolation kit) according to manufacturer's instruction. Quality of DNA was checked by nanodrop 100 (eppendorf) and in 0.8% agarose gel electrophoresis. DNA samples showing OD value (260/280) between 1.7 - 1.9 were used for the further study. The primers were obtained from Bangalore GeneiPvt. Ltd, Bangalore. The PCR reaction was carried out in 25 µl volume. The PCR reaction conditions were standardized and accordingly PCR amplification was performed using initial denaturation (94^{o}c for 3 mins), denaturation (94^{o}c for 30 Sec), annealing (60^{o}c for 30 Sec), extension (72^{o}c for 30 Sec), final extension (72^{o}c for 5 Mins) and PCR products were checked in 1 per cent agarose gel for specific amplification.

A PCR cocktail was prepared consisting of 1.0 µM upper and lower primers specific for each tested gene (table 1), 0.2 mMdNTPs, 10 mMTris (pH 9), 50 mMKCl and 1.5 mM MgCl2, 0.01 % gelatin (w/v), 0.1 % Triton X-100 and 1.25 units of *Taq polymerase*. The cocktail was aliquot into PCR tubes with 100 ng of buffalo DNA. The reaction was cycled for 1 min. at 94°C, 1 min at an optimized annealing temperature that would be determined for each primer and elongation for 1 min. at 72°C for 30 cycles. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide to test the amplification success. The gel was examined under UV light transilluminator and photographed for documentation.

The reaction mixture for restriction digestion was prepared in 0.2 ml tubes. After proper mixing, it was distributed to all the tubes (30 µl to each tube). 15 µl of PCR product was added in each tube. All these steps were carried out on ice. The 30 µl reaction mixture was kept for digestion in incubator at 37°C overnight. The RE digested products were electrophoresed in 1.5% agarose gel.

Genotypic frequencies of each variant genotype were calculated using the formula:

$$\text{Genotypic frequency} = \frac{\text{Number of animals with specific (AA, AB or BB) genotype}}{\text{Total number of animals}}$$

Table 1: List of primers used for amplifications of FSHR, LHR and ERα genes along with Restriction enzyme used in Marathwadi buffaloes

Genes	Sequences	PCR Product Size	Restriction enzyme and Expected cutting site	Region
FSHR	F: 5'-CTG CCT CCC TCA AGG TGC CCC TC - 3' R: 5'-AGT TCT TGG CTA AAT GTC TTA GGG GG-3'	306 bp	*Alu* I cuts at 243-244 nt	Exon 10
	F:5'-AGA GAT TCCC TCC GAC CTCC- 3' R:5'- TTC AAA ACT TAG CCC CAT GAAAGA - 3'	400 bp	*Hae*III cuts at 200nt *Hind* III cuts at 56 nt	Exon 10
	F:5'-TGG GGC TAA GTT TTG AAA GTA TGAC-3' R:5'- TGC CAT CTT TCC AGC GTGAT-3'	910bp	*Hind* III cuts at 264 nt	Exon 10
LHR	F: 5'-CAA ACT GAC AGT CCC CCG CTT T-3' R: 5'- CCT CCG AGC ATG ACT GGA ATG GC-3'	303bp	*Hha*I cuts at 155-156nt	Exon 11
	F:5'-AGT CGA TGC CCA GAC CAAAG-3' R: 5'- ACG GAT TGG CGC ATG AATTG-3'	611 bp	*Hinf* I cuts at 344 nt	Exon 11
	F:5'-TGC CTT TGA CAA CCT CCT CAAT - 3' R:5'-CTT TGG TCT GGG CAT CGACT-3'	995 bp	*Hpall* cuts at 379 nt	Exon 11
ERα	F: 5' TTT GGT TAA CGA GGT GGA G -3' R: 5' TGT GAC ACA GGT GGT TTT TC-3'	248 bp	*Bgl*I cuts at 171-172	Exon 1
	F: 5'-ACC GCC CGC AGC TCAAG-3' R: 5'-TGT CGC CTT CCT GCA CCCGC- 3'	294 bp	*Bgl*I cuts at 232 - 526 nt	Exon 1
	F 5'- TAC ATG GAC AGC AGC AAGCC-3' R 5'- AGG TAA TAG GGC ACC TGTTGG-3'	272 bp	*Bgl*I cuts at 101 Nt	Exon 1

Allelic frequencies were calculated as follows:

A = AA+ ½ AB

B = BB+ ½ AB

Where, AA, BB = genotypic frequency of homozygote, AB= genotypic frequency of heterozygote

A, B = Allelic Frequencies

The chi square ($\chi 2$) test ($P \leq 0.05$) was performed to test whether the distribution of the genotype frequencies was in the Hardy-Weinberg equilibrium (Snedecor and Cochran, 1989).

Results & Discussion

The Agarose gel electrophoresis (1% agarose gel) was used to assess the quality of extracted genomic DNA. All of the examined samples were showed a single intact band with no smearing around the well, as confirmed by the UV-trans illuminator system (Fig. 1).

PCR amplification of exon 10 of FSHR gene was carried out by using sequence specific primers (table 1). The amplified fragments of the FSHR gene revealed 306 bp product (Fig. 2) by performing 1% agarose gel electrophoresis and it was documented using UV-Trans illuminator system. PCR amplification of FSHR gene (fragment 2) was carried out by using primers (table 1) as per protocol. The amplified fragments of the FSHR gene of all samples revealed 400bp product by performing 1% agarose gel electrophoresis and it was documented using UV-Trans illuminator system. Similarly, the third fragment of exon 10 of FSHR gene was amplified by using forward primer FSHR3f and reverse primer FSHR3r revealing a PCR product of 910bp.

Exon 11 of LHR gene was also amplified in three fragments using forward and reverse primers (table 1), producing amplicons of 303bp, 611bp and 995bp (Fig 3). PCR amplification of exon 1 of ERα gene was also carried out by using primers denoted in table 1 to reveal PCR product of 248bp, 294bp and 272bp when visualised on 1% agarose gel electrophoresis (Fig 4).

PCR-RFLP assay of FSHR, LHR and ERα gene

All the PCR products were subjected to restriction digestion using different restriction enzymes (table 1) for fragments of FSHR, LHR and ERα genes revealing different banding patters. The FSHR/Alu I RE PCR-RFLP assay revealed 243 and 63 bp (BB genotype) and heterozygous pattern having 306, 243 and 63 bp bands (AB genotype) for fragment 1 (Fig 5). The FSHR/HaeIII RE revealed 400bp (AA genotype) band for fragment 2. Similarly, FSHR/Hind III RE assay revealed 910bp (AA genotype) only for fragment 3 of exon 10 of FSHR gene. The LHR/HhaIRE PCR-RFLP assay revealed 303bp (TT genotype) for fragment 1 (Fig 6). The LHR/HinfI RE revealed 344bp and 267bp bands (TC genotype) for fragment 2 of LHR exon 11 gene and

LHR/HpaIIRE PCR-RFLP assay revealed 995bp (TT genotype) for fragment 3 of LHR exon 11. The ERα/BglI RE revealed different banding patters in all the three fragments of exon 1 of ERα gene. Genotype GC (248bp,171bp and 77bp) and CC (171bp and 77 bp) were observed for fragment 1. For fragment 2 of ERα gene, 294bp, 232bp and 62bp were seen for genotype GC and 232bp and 62bp were seen for genotype CC. All the samples cleaved to demonstrate 171bp and 101bp bands for genotype CC of fragment 3 of exon 1 of ERα gene. (Fig.7).

Genotypic frequencies for fragment 1, 2 and 3 for exon 10 of FSHR gene are represented in table 2. Fragment 1 of the FSHR gene revealed two genotypes AB and BB with genotypic frequencies 6 (AB) and 94 (BB), allelic frequencies were 0.03 and 0.97 for allele B and allele A respectively. Fragment 2 and 3 for exon 10 of FSHR gene were monomorphic in the studied population of Marathwadi buffalo. These findings are contrary to the findings in B.indicus (Hernadez et. al., 2009), European-Zebu (Marson et. al., 2005) or in Indian cattle breeds like Sahiwal, Rathi and Kankrej (Amitosh et. al., 2017). The effective number of allele for fragment 1 of FSHR exon 10 ranged from 1-2 and the value of PIC was around 0.1. The decreased genetic variation in the FSHR gene indicated a lesser adaptation potential of the population. All the three fragment for exon 11 of LHR gene were monomorphic (table 3) in the studied population which was also reflected in a study by Othman and

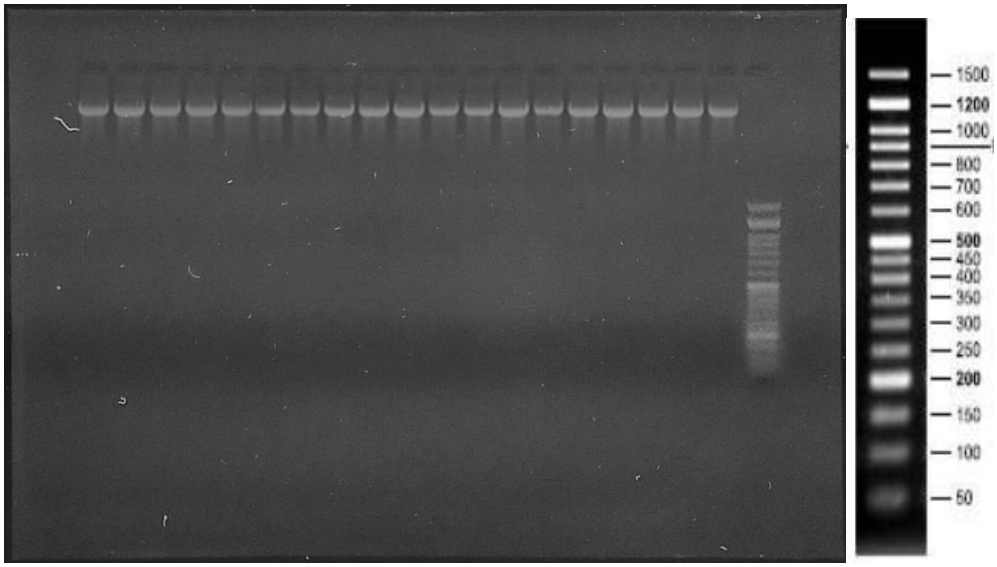

Fig.1 Genomic DNA of Marathwadi buffalo isolated from blood samples on 1% Agarose gel & Reference Himedia 50 bp DNA marker; Lane 20: 50 bp DNA marker; Lane 1-19: Blood Genomic DNA of Marathwadi buffalo sample 1-19

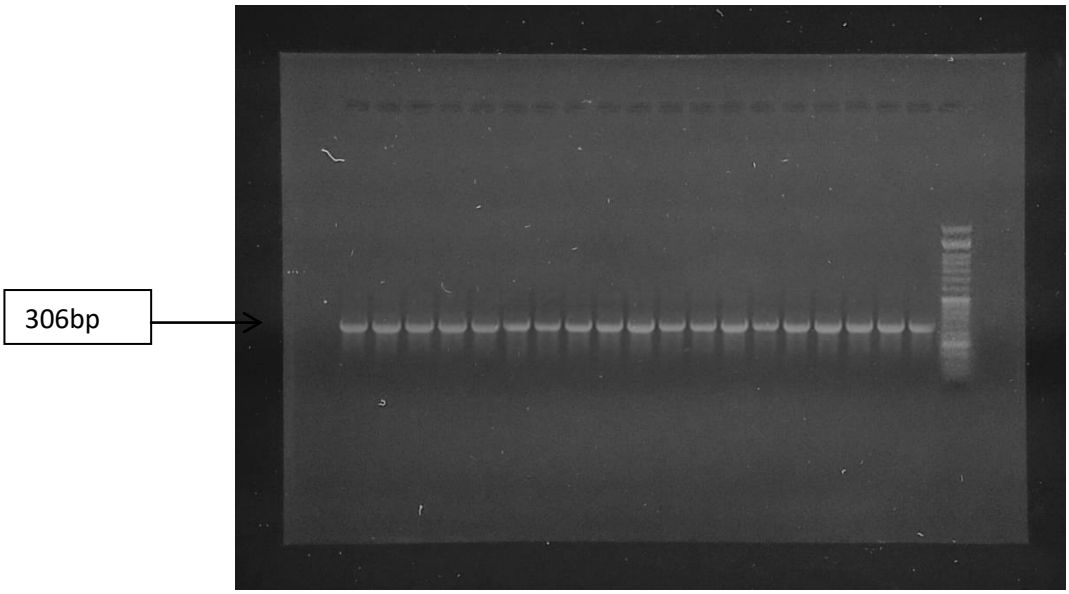

Fig.2 PCR Amplification of FSHR gene (primer set 1for samples 1-19 on 1% Agarose gel)
Lane 20: 50 bp DNA marker Lane 1-19: PCR Product of FSHR gene (306 bp) sample 1-19 for primer set 1

Abdel-Samad, 2013. However different allele frequencies have been reported for the alleles of these fragments. (Marson et. al., 2005; Omar et. al., 2016). This study revealed presence of only one allele in the indigenous breed, indicating good level of genetic purity in analyzed cattle population at considered locus.Genotype CC and GC were observed for fragment 1 and 2 of exon 1 of ERα gene with frequencies 0.74 (CC genotype) and 0.26 (GC genotype) for fragment 1 and 0.94 (CC genotype) and 0.06 (GC genotype) for fragment 2. The locus in fragment three was monomorphic contrary to the previous findings (Hernadez et. al., 2009; Amitosh et. al., 2017). Allele frequency was abundant for allele C in both fragments with frequency 0.87 and 0.97 for fragment 1and 2 respectively (table 4).

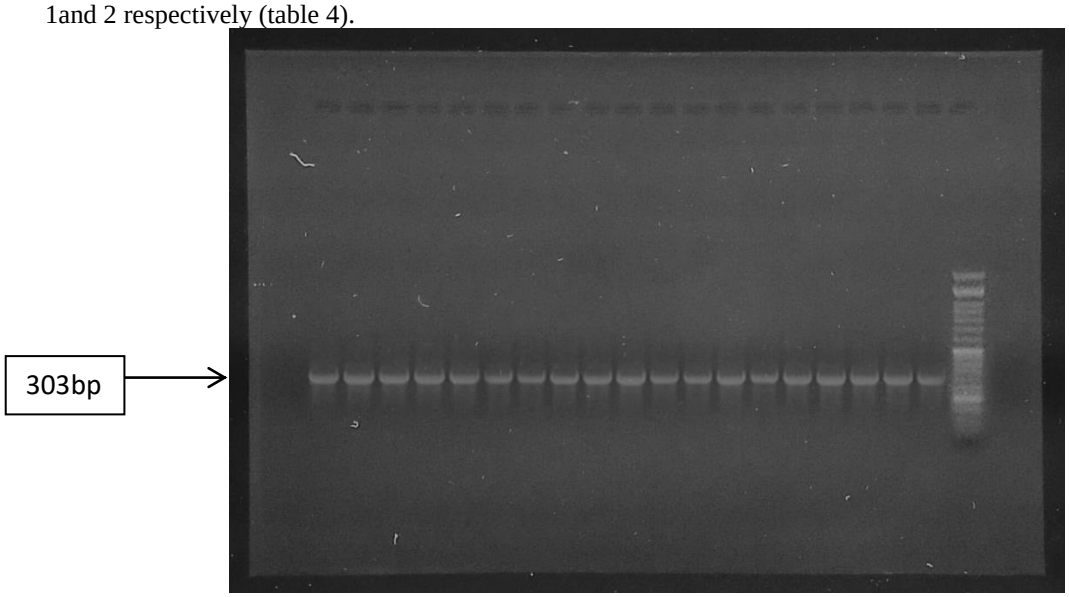

Fig.3 PCR Amplification of LHR gene (primer set 1for samples 1-19 on 1% Agarose gel)
Lane 20: 50 bp DNA marker, Lane 1-19: PCR Product of LHR gene (303 bp) sample 1-19 for primer set 1

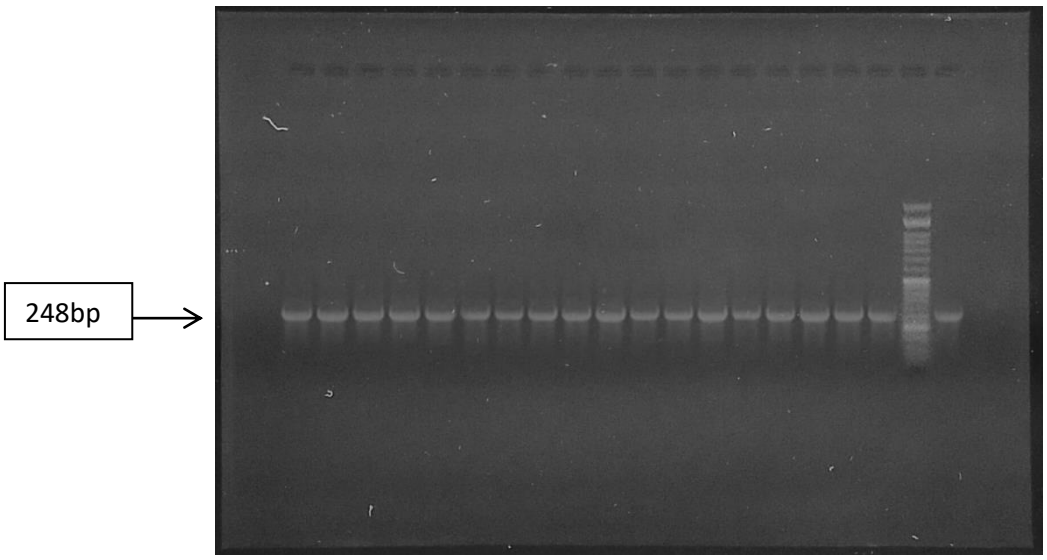

Fig.4 PCR Amplification of ERα gene (primer set 1for samples 1-19 on 1% Agarose gel)
Lane 19: 50 bp DNA marker; Lane 1-18, 20: PCR Product of ERα gene (248 bp) sample 1-19 for primer set 1

Table 2: Gene and Genotypic frequencies of FSHR gene

Primer set	Genotype frequency (%)			Allelic Frequency		$\chi 2$	P	S/ NS
	AA	AB	BB	A	B			
FSHR set 1 (N=100)	0	6 (n=6)	94 (n=94)	0.03	0.97	0.5642	0.7541	NS
FSHR set 2	100	0	0	1	0	-	-	-
FSHR set 3	100	0	0	1	0	-	-	-

Where; N= Sample size, n= Number of animals in particular genotype

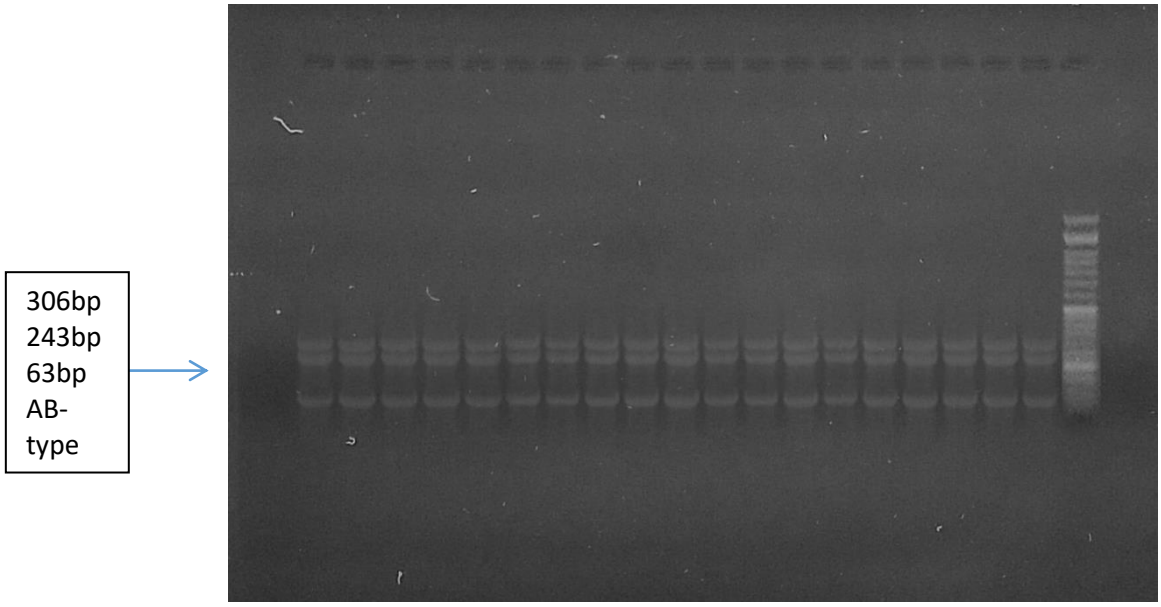

Fig 5: PCR-RFLP Pattern of FSHR gene (digested with *Alu*I for primer set 1 on 1% agarose gel samples 1-19); Lane 20: 50 bp DNA marker; Lane 1-19: Genotypic patterns of FSHR gene sample 1-19 for primer set 1

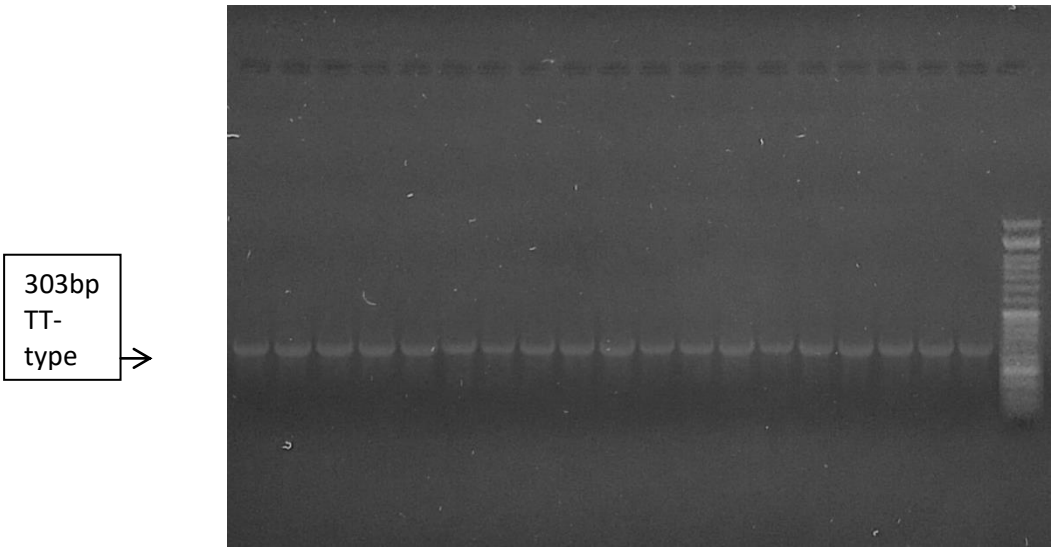

Fig. 6: PCR-RFLP Pattern of LHR gene (digesed with *Hha I* for primer set 1 on 1% agarose gel samples 1-19); Lane 20: 50 bp DNA marker; Lane 1-19: Genotypic patterns of LHR gene sample 1-19 for primer set 1

Table 3 Gene and Genotypic frequencies of LHR gene

Primer set	Genotype frequency (%)			Allelic Frequency		χ^2	P	S
	TT	TC	CC	T	C			
LHR set 1 (N=100)	100 (n=100)	0	0	1	0	-	-	-
LHR set 2	0	0	100	0	1	-	-	-
LHR set 3	100	0	0	1	0	-	-	-

Table 4 Gene and Genotypic frequencies of ERα gene

Primer set	Genotype frequency (%)			Allelic Frequency		χ^2	P	S
	GG	GC	CC	G	C			
ERα set 1 (N=100)	0	26 (n=26)	74 (n=74)	0.13	0.87	1.18	0.55	NS
ERα set 2 (N=100)	0	6 (n=6)	94 (n=94)	0.03	0.97	0.5642	0.7541	NS
ERα set 3 (N=100)	0	0	100	0	1	-	-	-

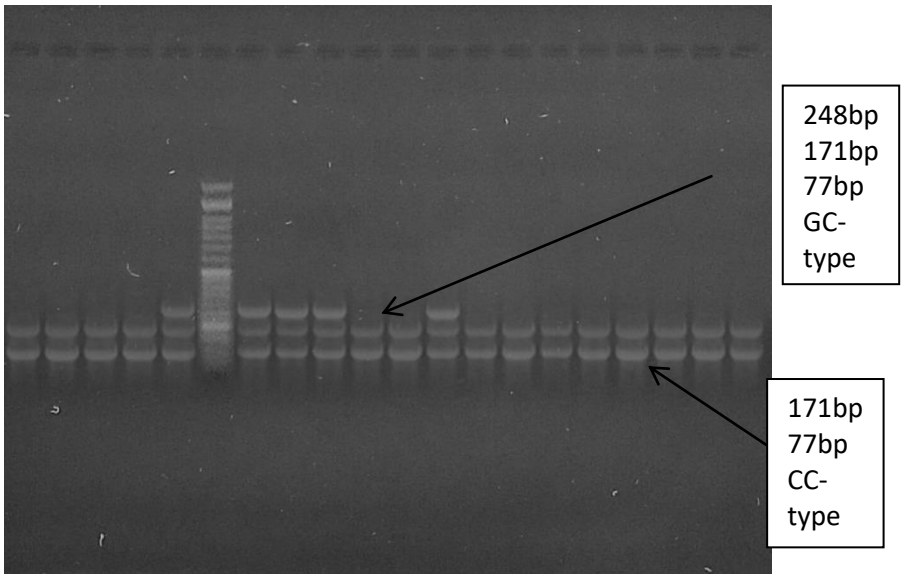

Fig. 7. PCR-RFLP Pattern of ER α gene (digesed with *Bgl*I for primer set 1 on 1% agarose gel samples 1-19); Lane 6: 50 bp DNA marker; Lane 1-5, 7-20: Genotypic patterns of ER α gene sample 1-19 for primer set 1

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Conclusions

The FSHR/ *HaeIII* RE assay and FSHR/*Hind III* RE assay for fragment 2 and 3 respectively revealed monomorphic pattern and allelic frequency of FSHR/*Alu I* RE assay was 0.03 and 0.97 for allel B and A respectively. The LHR/*HhaI* RE assay, LHR/*HinfI* RE assay and LHR/*HpaII* RE assay for fragment 1,2 and 3 revealed monomorphic pattern at the locus. The ER*α/BglI* RE assay for fragment 1 and 2 demonstrated allelic frequencies of 0.13, 0.87 and 0.03, 0.97 for alleles G and C respectively.

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