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Molecular characterization and association studies of CYP19 (aromatase) allelic variants with reproduction and production traits in Sahiwal and Hariana cattle

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ABSTRACT

Anestrus is a major reproductive problem in bovines which occurs due to less secretion of estrogen from the ovarian follicles. Enzyme cytochrome P450 aromatase which helps in estrogen biosynthesis is encoded by *CYP19* gene. In the current study, characterization of exon 9 and 10 region and polymorphic study in 5' UTR region of *CYP19* gene was undertaken in Indian Sahiwal and Hariana cattle. Studied Indian cattle breeds showed 100% homology to Nelore and crossbred cattle, and 99.8% to *Bos taurus* at nucleotide level while at amino acid level, they showed 100% homology to Nelore and crossbred cattle, and 99.4% to *Bos taurus*. CYP19/*Pvu*II PCR-RFLP assay conducted in 100 Sahiwal and 100 Hariana cattle revealed three types of genotype namely AA (405 bp), AB (405 bp, 327 bp and 78 bp) and BB (327 bp and 78 bp). The AA genotype was more frequent (58.5%) followed by AB (37.0%) and BB (4.5%) genotypes and Chi square analysis revealed the populations was in Hardy-Weinberg equilibrium. The allelic frequency of A and B allele was observed as 0.77 and 0.23, respectively. Association study of CYP19/*Pvu*II genotypes with reproduction and production traits revealed no significant difference.

Keywords: *CYP19* gene, *Pvu*II/PCR-RFLP, Indian cattle, Association study

Anoestrus is a broad term which indicates lack of estrous sign (Peter *et al*. 2009) and one of the major causes of infertility among dairy cattle. Not only it increase postpartum interval, but also decrease farmers' income by selling milk due to overall reduction in number of pregnancies (Mwaanga and Janowski 2000). Periodic cyclicity of estrus and manifestation of estrus signs is mainly regulated by collective action of hypothalamus-pituitary and ovarian hormone. One of the major ovarian hormones is estrogen secreted by theca interna of follicles. It promotes sexual behaviour, development of secondry sexual characters and has anabolic effect to increase body weight gain and growth (Hafez and Hafez 2000). The enzyme involved in estrogen biosynthesis is Cytochrome P450 which is encoded by *CYP19* gene (Simpson *et al*. 1994) by aromatization of androgens. This gene has been mapped to band q2.6 of chromosome 10 in cattle (Goldammer *et al*. 1994) and consists of 10 exons. Coding region includes exon 2 to 10 with translation start site in exon 2. The gene expression is regulated by tissue-specific alternative promotor regions (P1.1, P1.2, P1.3, P1.4, P1.5 and P2) which correspond to the 5' UTR transcripts, but the coding region is identical for all tissues (Simpson and Davis 2001). These characteristics make this gene a suitable candidate for marker assisted selection (MAS). Furbass *et al*. (1997) proposed that the gene variants in *CYP19* might influence the fertility of cattle. *Pvu*II/PCR-RFLP has been employed in 5' UTR region to found out allelic variants of *CYP19* gene of Sahiwal and Hariana cattle. Reproductive potential and productivity of indigenous breeds can be increased by selection and breeding strategies. Molecular markers are efficient tools than traditional breeding to bring genetic change in short period of time. Sequence analysis and detection of single nucleotide polymorphism (SNP) will be helpful in tracking certain mutations which may cause aromatase deficiency that will result in absence of estrous sign consequently infertility. In the present study, we cloned and characterized the exon 9 and exon 10 of *CYP19* gene in Sahiwal and Hariana cattle. Moreover, *Pvu*II/PCR-RFLP has also been conducted in 5' UTR region of this gene along with association study of genotypes with reproduction and milk production traits.

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MATERIALS AND METHODS

The venous blood was collected from three adult females from each breeds (Sahiwal and Hariana) maintained at Livestock farm complex (LFC), DUVASU, Mathura for characterization study. For PCR-RFLP assay, blood samples

Table 1. Primer sequences used for amplification of exon 9 and 10 and 5' UTR region of *CYP19* gene

Gene	Region	Sequence	Amplicon size
CYP19	Exon 9	F 5′-CATTCTACGGAACAAGCACAGG-3′ R 5′-TTGATGCTTTCAAGGGTGGC-3′	463 bp
CYP19	Exon 10	F 5′-TCAAACGGAGACGCATGACT-3′ R 5′-AAGCTCCCAATTCAGTGTAG-3′	790 bp

were taken randomly from 200 animals (100 Sahiwal and 100 Hariana). Genomic DNA was isolated using standard phenol-chloroform DNA isolation protocol (Sambrook and Russell 2001).

Exon 9 and 10 of *CYP19* gene were amplified using specific primer pair (Table 1) designed using VNTI software and commercially synthesized (Imperial Life Sciences, Gurugram, Haryana). PCR reactions were carried out in 25 µl reaction mixture containing 1× PCR buffer (NEB, USA), 2 mM $MgCl_2$, 2.5 mM of dNTPs, 5 pmole of each primer and one unit of *Taq* DNA polymerase (NEB, USA). Cycle conditions used were: an initial denaturation of one cycle at 94°C for 5 min; 35 cycles of exon specific amplification denaturation at 94°C for 30 sec; annealing at 60°C for 30 sec; extension at 72°C for 30 sec followed by final extension of 72°C for 8 min. The amplified product was run on agarose (1.0%) gel electrophoresis. The amplified products were cloned into pTZ57R/T cloning vector using Quick clone PCR-cloning kit (PUREGENE). The positive recombinant clones were identified from the transformed bacterial colonies of *E. coli* (DH5α strain) using blue and white colony selection. The positive clones were sequenced commercially (Eurofins genomics India Pvt. Ltd, Bengaluru, Karnataka) by automated sequencer using standard cycle conditions by Sanger's dideoxy chain termination method.

A deduced partial coding sequence (CDS) obtained from exon 9 and 10 sequences of *Cyp19* gene. The nucleotides as well as deduced amino acid sequences of partial CDS for exon 9 and 10 of *CYP19* gene of Sahiwal and Hariana cattle breeds were aligned with homologous sequence of Nellore cattle (XM_019968827), (Angus × Brahman F_1) crossbreed cattle (XM_027554016), *Bos taurus* (U18447), buffalo (NM_001290963), American bison (XM_010839491), wild yak (XM_005900467), goat (NM_001285747), sheep (NM-_001123000) and pig (U92245) available in the GenBank database using ClustalW method of MegAlign program of Lasergene software (DNASTAR, USA). Phylogenetic tree was constructed by using MEGA version 4.0 (Tamura *et al*. 2007).

For PCR-RFLP assay, primers used for amplification of 405 bp fragment comprising 5′ UTR of *CYP19* gene was F: 5′-CTCTCGATGAGACAGGCTCC-3′ and R: 5′-ACAATGCTGGGTTCTGGACT-3′ (Vanselow *et al*. 1999). PCR reaction mixture components were described earlier. Cycle conditions used were: an initial denaturation of one cycle at 94°C for 5 min; 35 cycles of exon specific amplification, denaturation at 94°C for 30 sec; annealing at 60°C for 30 sec; extension at 72°C for 30 sec followed by final extension of 72°C for 8 min. The restriction digestion was carried out at 37°C for overnight in 15 µl volume of containing 10 µl of PCR product, 1.5 µl of 10× RE buffer, 10 units of 0.5 µl *Pvu*II enzyme and 3 µl nuclease free water. The digested products were electrophoresed in 2.5% agarose gel in 1× TBE buffer.

The data were generated by estimating the frequency of different RFLP genotypes. The allelic and genotypic frequencies of *Pvu*II/PCR-RFLP genotypes were estimated by standard procedure (Falconer and Mackay 1996). The association study of *Pvu*II genotypes was carried out with milk production and reproduction traits, viz. age at first calving (AFC), lactation period (LP), total milk yield (TMY), service period (SP), calving interval (CI). One way ANOVA was performed using SPSS statistical software (version 16.0).

RESULTS AND DISCUSSION

Characterization of exon 9 and 10 of CYP19 gene, partial CDS: Agarose gel electrophoresis (1%) revealed amplified product of 463 bp of exon 9 and 790 bp for exon 10 (Fig. 1) in Sahiwal and Hariana cattle breeds. Partial CDS encoding exon 9 and 10 sequence of Sahiwal and Hariana were submitted in GenBank database with accession no. MT876567 and MT876566, respectively.

Multiple sequence alignment of deduced amino acid sequence of *CYP19* gene encoding exon 9 and 10 of Sahiwal and Hariana cattle breeds with exotic cattle breeds, buffalo and other species is presented in Fig. 2. The per cent identity of studied Indian cattle breeds in comparison to homologous

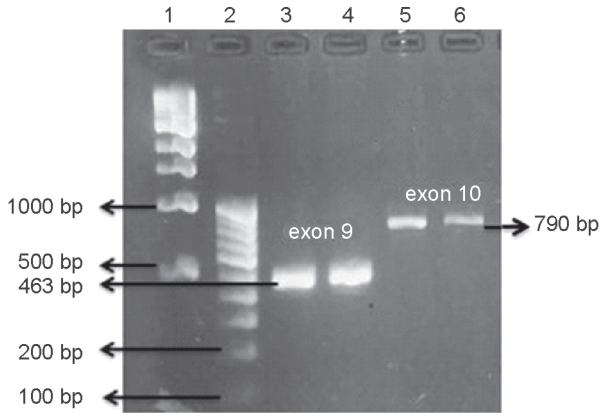

Fig. 1. Amplification of PCR products for exon 9 and 10 of *CYP19* gene, partial CDS of Sahiwal and Hariana cattle. Lane 1–2 (Marker), Lane 3–4 (463 bp product for exon 9) and Lane 5–6 (790 bp product for exon 10).

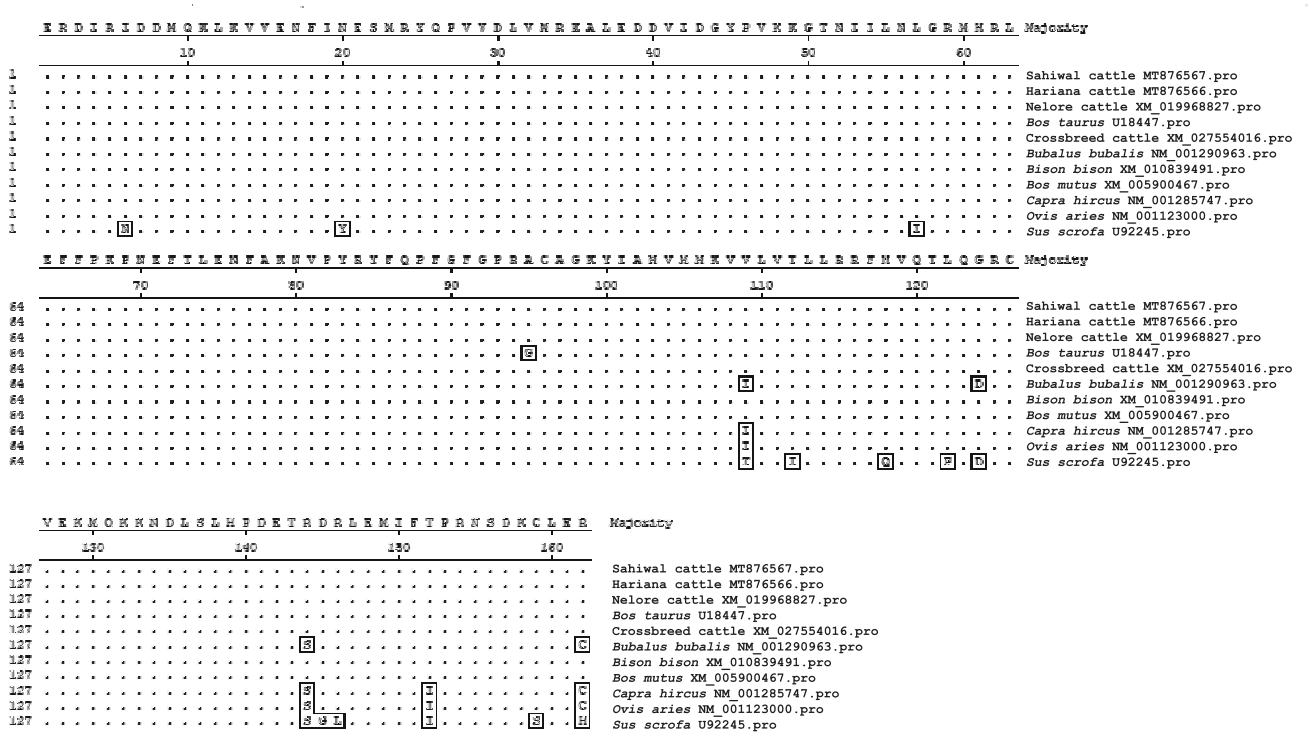

Fig. 2. Multiple sequence alignment (Clustal W) of *CYP19* amino acid sequence encoding exon 9 and 10 of Indian cattle breeds with other exotic cattle breeds, buffalo and other species.

sequence of other breeds and species at nucleotide as well as amino acid level is presented in Table 2.The present study reported the partial CDS of Sahiwal and Hariana cattle comprises exon 9 and 10 encoding 162 amino acids. Hinshelwood *et al*. (1993) reported partial CDS from exon 3 to 10 of bovine *CYP19* gene encoding 503 amino acids proteins. However, Vanselow and Furbass (1995) reported complete CDS of bovine *CYP19* gene of encoding 563 amino acid proteins.

Table 2. Per cent identity of exon 9 and 10 of *CYP19* gene of Indian cattle breeds compared with other exotic cattle breeds, buffalo and other species at nucleotide and amino acid level

Species and accession no.	Nucleotide (%)		Amino acid (%)	
	Sahiwal	Hariana	Sahiwal	Hariana
Sahiwal MT876567	**	100	**	100
Hariana MT876566	100	**	100	**
Nellore cattle (XM_019968827)	100	100	100	100
Crossbreed cattle (XM_027554016)	100	100	100	100
Bos taurus (U18447) (NM_001290963)	99.8	99.8	99.4	99.4
Bubalus bubalis	98.8	98.8	97.5	97.5
Bison bison (XM_010839491)	99.4	99.4	100	100
Bos mutus (XM_005900467)	99.8	99.8	100	100
Capra hircus (NM_001285747)	97.6	97.6	97.5	97.5
Ovis aries (NM_001123000)	97.6	97.6	97.5	97.5
Sus scrofa (U92245)	91.0	91.0	91.4	91.4

In the present study, studied Indian cattle breeds showed 98.8% and 97.5% homology with *Bubalus bubalis* in nucleotide and amino acid sequence of *CYP19* gene, respectively. Kumar *et al*. (2009) cloned and characterized 206 bp 5' UTR region in buffalo and observed 78.65% and 97.6% homology with cattle and sheep nucleotide sequence. Aboelenin *et al*. (2017) studied 409 bp of 5' UTR region in buffalo and 97% homology was observed with cattle sequence, whereas, in present study, partial CDS of Sahiwal and Hariana for exon 9 and 10 of *CYP19* nucleotide sequence showed 98.8% homology with *Bubalus bubalis* respective nucleotide sequence. Bobes *et al*. (2004) studied complete CDS of goat *CYP19* and reported 95% to 97.6% similarity with cattle nucleotide and amino acid sequence, whereas, partial CDS of Sahiwal and Hariana for exon 9 and 10 of *CYP19* nucleotide and amino acid sequence showed 97.6% and 97.5% homology with goat.

Hinshelwood *et al*. (1993) and Vanselow and Furbass (1995) reported one non-synonymous nucleotide substitution at 1489 (C→G) in exon 10 in bovine, causes amino acid change 'Ala' to 'Gly' at 436 similar to present study wherein a non-synonymous nucleotide substitution found in *Bos taurus* at position 286 (C→G) caused amino acid change 'Ala' to 'Gly' at position 95.

Bobes *et al*. (2004) studied evolutionary relationship where goats and sheep diverged from each other very recently and also suggested that the cow diverged from the

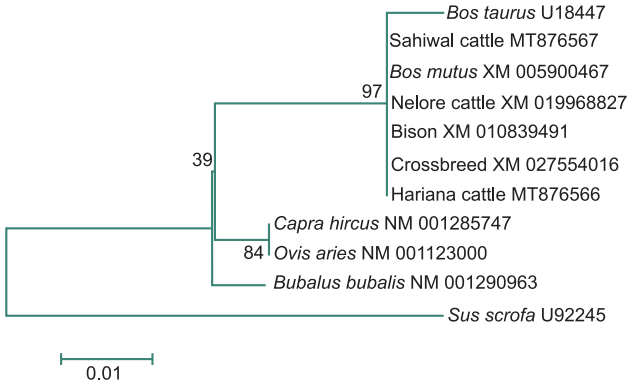

Fig. 3. Phylogenetic tree based on deduced amino acid sequences of *CYP19* gene for exon 9 and 10 for Indian cattle breeds, buffalo and other related species.

sheep and goat more recently than the human diverged from the monkeys. Whereas, caprinae was more closely related to cattle breeds than *Bubalus bubalis* and suidae was farthest from cattle breeds in present study as shown in Fig. 3.

PvuII/PCR-RFLP assay: As expected, 405 bp PCR product of *CYP19* gene was amplified in 5' UTR region. CYP19/*Pvu*II PCR-RFLP assay revealed three types of genotype namely AA (405 bp), AB (405, 327 and 78 bp) and BB (327 and 78 bp) which confirmed the polymorphic pattern of 5' UTR region of this gene in both the cattle breeds (Fig. 4). The allelic and genotypic frequencies were calculated and presented in Table 3. The genotype AA (58.5%) and allele A (0.77) was more frequent in all the screened animals followed by AB (37.0%) and BB (4.5%) genotypes. Chi square (χ^2) analysis at 1 degree of freedom showed that population was in Hardy-Weinberg equilibrium. Kumar *et al*. (2017a) and Kumar *et al*. (2017b) also reported higher frequency of AA genotype and allele A in Sahiwal and Rathi cattle. Genetic polymorphism of *CYP19* gene has also been studied in many exotic cattle breeds by various authors in polish Holstein Friesian by Szatkowska *et al*. (2011), Black and white cattle by Jedrzrijczak *et al*. (2011), Slovak Simmental cattle by Trakovicka *et al*. (2015) and Holstein Friesian by Keskin *et al.* (2015). They found higher frequency of AA genotype

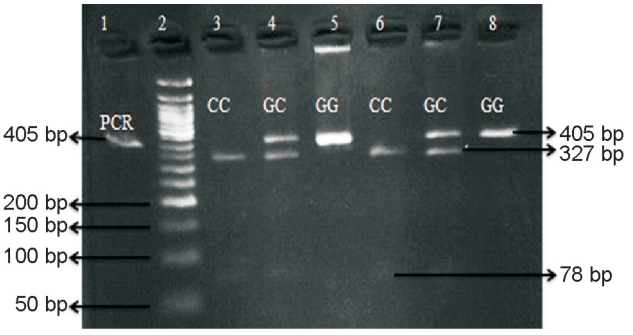

Fig. 4. CYP19/*Pvu*II PCR-RFLP pattern for 5' UTR region of Sahiwal and Hariana cattle on 2.5% agarose gel electrophoresis. Lane 1 (405 bp PCR product), Lane 2 (50 bp Marker), Lane 3 and 6 (CC genotype), Lane 4 and 7 (GG genotype), 5 and 8 (GC genotype).

in respective cattle breeds but value of AA genotype was in range of 74% to 98% which was not in accordance to our observation. This may be due to different native tract and population size. The frequency of BB genotype was lowest in all the exotic cattle breeds except in Holstein Friesian where BB genotype was absent as observed by Keskin *et al*. (2015). Likewise higher frequency of A allele was observed by all the authors in Indian and exotic cattle breeds.

Association analysis: The overall mean±SEM of AFC, TMY, LP, SP and CI in first and second lactation is given in Table 4. No significant difference was observed of

Table 4. Genotypic and allelic frequencies of CYP19/*Pvu*II gene in Sahiwal and Hariana cattle breeds

Breed	Genotypic frequency (%)			Allelic Frequency		Chi square (df = 1)
	AA	AB	BB	A	B	
Sahiwal (n=100)	55.0 (n=55)	39.0 (n=39)	6.0 (n=6)	0.745	0.255	0.068 (*P<0.05*)
Hariana (n=100)	62.0 (n=62)	35.0 (n=35)	3.0 (n=3)	0.795	0.205	0.5438 (*P<0.05*)
Total (N=200)	58.5 (n=117)	37.0 (n=74)	4.5 (n=9)	0.77	0.23	0.199 (*P<0.05*)

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

genotypes with milk production and reproduction traits which is in accordance with the findings of Jedrzejczak *et al*. (2006), Jedrzejczak *et al*. (2011) and Kowalewska-Luczak *et al*. (2013) where they also found no significant difference of CYP19/*Pvu*II genotypes with milk production traits of investigated cows. But the present investigation was not in accordance with reports of Kowalewska- Luczak (2010) who found statistically significant (*P≥0.05*) associations between *CYP19* genotypes and different milk production traits. They found AA genotype showed highest value of all analysed milk performance traits including milk yield, milk fat, milk protein in all the three lactations and BB genotype showed least value for milk traits. Szatkowska *et al*. (2011) analyzed the significant association of calving to conception intervals and the average calving intervals in Polish Holstein-Friesian cows. They found BB genotype in the first and second lactation were characterized by the shortest calving-to-conception interval and calving interval turned out to be significantly longer in third lactation in cows of AA genotype compared to AB genotype.

In the present study, we reported the cloning and characterization of partial CDS of exon 9 and exon 10 of *CYP19* gene in Indian cattle breeds. Moreover, nucleotide and amino acid sequence of Sahiwal and Hariana cattle breeds was compared with homologous sequence of other species. We identified a non-synonymous nucleotide subsitutions found in *Bos taurus* at 286 (C→G) caused amino acid change 'Ala' to 'Gly' at position 95. Furthermore, *Pvu*II/PCR-RFLP in 5' UTR was also conducted and we obtained polymorphic pattern of this gene

Table 4. Association study for 5' UTR region of CYP19/*Pvu*II with milk production and reproduction traits in Sahiwal and Hariana cattle

Lact	Breed	Geno	n	AFC (days)	TMY (ltr)	LP (days)	SP (days)	CI (days)
I	Sahiwal (N=70)	AA	39	1840.0±74.6	1880.0±138.0	388.0±20.2	221.0±19.2	499.0±16.9
		AB	28	1640.0±50.0	1740.0±96.5	335.0±18.5	221.0±21.2	503.0±22.2
		BB	3	1620.0±273.0	1550.0±116.0	353.0±33.3	294.0±102.0	509.0±161.0
	Hariana (N=57)	AA	34	2010.0±68.0	1710.0±102.0	330.0±16.3	163.0±18.7	438.6±19.0
		AB	21	2030.0±84.9	1570.0±139.0	288.0±15.4	162.7±22.4	438.4±22.4
		BB	2	1590.0±287.0	1400.0±247.0	271.0±6.0	160.0±18	407.0±65.0
	Total (N=127)	AA	73	1919.1±71.5	1800.8±121.0	360.9±18.4	193.9±18.9	470.8±17.8
		AB	49	1807.0±64.9	1667.0±114.7	314.8±17.1	196.0±21.7	475.3±22.3
		BB	5	1608.0±278.6	1490.0±168.4	320.0±22.4	240.0±68.4	468.2±122.6
II	Sahiwal (N=70)	AA	39	–	1980.0±99.6	355.0±12.9	210.0±20.5	490.0±18.0
		AB	28	–	1940.0±98.9	370.0±20.2	208.0±32.0	488.0±25.0
		BB	3	–	1970.0±265.0	423.0±22.8	282.0±87.0	510.0±75.5
	Hariana (N=57)	AA	34	–	1730.0±99.4	309.0±14.8	168.0±19.5	444.0±25.0
		AB	21	–	1480.0±71.8	311.0±14.3	172.0±25.5	465.0±30.0
		BB	2	–	1340.0±217.0	237.0±11.5	162.0±30.0	425.0±55.0
	Total (N=127)	AA	73	–	1863.5±99.5	333.5±13.7	190.4±20.0	468.5±21.2
		AB	49	–	1742.8±87.2	344.7±17.7	192.5±29.2	478.1±27.2
		BB	5	–	1718.0±245.8	348.6±18.3	234.0±64.2	476.0±67.3

Lact, Lactation; Geno, genotype; AFC, age at first calving; TMY, total milk yield; CI, calving interval; LP, lactation period; SP, service period.

in Sahiwal and Hariana cattle breeds. Association study revealed no significant difference among genotypes with milk production and reproduction traits. Further, investigation needs to be done to found out good performing genotypes which may be helpful in MAS.

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