Original Article

Buffalo Bulletin (October-December 2022) Vol.41 No.4
https://doi.org/10.56825/bufbu.2022.4143569

ASSOCIATION OF MELATONIN RECEPTOR 1A (MTNR1A) GENE POLYMORPHISM WITH SEASONAL SUPPRESSION OF FERTILITY IN BUFFALOES

Muddarangaiah Madyavenkatapura Rangashamaiah[1], Sarvpreet Singh Ghuman[2,*] and Ranjna Cheema[3]

Received: 02 May 2020
Accepted: 24 December 2022

ABSTRACT

The polymorphism in Melatonin receptor 1A (MTNR1A) gene and its association with reproductive cyclicity (cyclic, n=51; non-cyclic, n=50) during summer season and age at first calving (<3.5 years, n=38; >3.5 years, n=60) was investigated in buffaloes subjected to blood sampling. Polymerase chain reaction (PCR) of genomic DNA was carried out using three primers *viz*. 824 bp (primer pair 1), 856 bp (primer pair 2) and 268 bp (primer pair 3) corresponding to exon-II of MTNR1A gene. Restriction fragment length polymorphism was performed using PCR products of primer pair 1 and 2. The digestion of PCR product of primer pair 1 with restriction enzymes *viz*. MnII, RsaI or Eco3II generated total restriction sites respectively as 14, 4 and 5. Based upon differential restriction sites, six genotypes revealed in buffaloes using MnII were MM-1 to MM-6, four genotypes using RsaI were RR-1 to RR-4, and four genotypes using Eco3II were EE-1 to EE-4. The digestion of PCR product of primer pair 2 with MnII, RsaI or Eco3II generated restriction sites respectively as 13, 5 and 6. Based upon differential restriction sites, six genotypes revealed using MnII were mm-1 to mm-6, five genotypes using RsaI were rr-1to rr-5, and three genotypes using Eco3II were ee-1 to ee-3. For primer pair 3, single stranded conformational polymorphism analysis exhibited 5 genotypes *viz*. AA, BB, AB, CC and DD. The results revealed that the buffaloes during summer had polymorphic genotypes for MTNR1A gene as buffaloes showing reproductive cyclicity had MM-3, MM-4, RR-2, EE-3, mm-2, mm-4, rr-3, ee-3, CC and DD genotypes, whereas their non-cyclic counterparts had MM-2, MM-6, EE-4, mm-3, mm-6, AA, BB and AB genotypes. However, no such differences were recorded with respect to age at first calving. In conclusion, polymorphic genotypes of MTNR1A gene may be considered as genetic markers to identify buffaloes with better reproductive potential during summer season.

Keywords: *Bubalus bubalis*, buffaloes, genotypes, MTNR1A gene, polymorphism, summer

[1]Veterinary Dispensary, Karnataka, India

[2]Department of Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India,*E-mail: ghuman_s@yahoo.co.in

[3]Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

INTRODUCTION

The buffaloes exhibit a seasonal trend in pattern of reproductive activity with compromised activity in summer, and regular reproductive cyclicity and conception during autumn and winter season (Das and Khan, 2010). Understanding the cause of this reproductive limitation in buffaloes by unravelling their reproductive endocrinology remains an unsolved puzzle (Warriach *et al*., 2015). The associated environmental factors with reproductive seasonality in buffalo are photoperiod, temperature, relative humidity and rainfall (Ribeiro *et al*., 2003). In fact, the period of day length is negatively correlated with number of buffaloes expressing estrus, and the short periods of artificial darkness reduced the problem of anestrus during long day length (Singh *et al*., 2000).

Various ovine studies have revealed photoperiod and melatonin as the modulators of reproductive axis at hypothalamic level (Karsch *et al*., 2013). Melatonin hormone is produced by pineal gland in direct proportion to the period of darkness (Malpaux *et al*., 2001). The Mediterranean buffaloes showing seasonal reproductive trend had highest night-time plasma melatonin concentration in winter and lowest in summer (Parmeggiani *et al*., 1994). During summer anestrus, subcutaneous insertion of sustained-release melatonin implants in buffaloes mimics the effect of short days by lengthening the daily presence of melatonin and has been widely tested for the activation of reproductive axis (Ghuman *et al*., 2010; Ramadan *et al*., 2016; Kavita *et al*., 2018).

Melatonin regulates GnRH secretion from hypothalamus through binding with its receptors *viz*. melatonin receptor 1A gene (MTNR1A) and melatonin receptor 1B gene (MTNRIB), the G-protein couple receptors, but only the former is involved in reproductive seasonality (Dubocovich *et al*., 2000). Precisely, MTNR1A consists of two exons divided by ~8,000 base pair (bp) long intron. The first exon (Exon I) codes for the first intracellular loop, whereas second exon (Exon II) codes for the rest of the receptor (Carcangiu *et al*., 2009a).

In sheep, polymorphism in exon II of MTNRIA gene was observed at MnII and RsaI restriction enzyme cut at position 604 and 605, respectively (Chu *et al*., 2006). Others found two polymorphic restriction sites for MnII and the absence of restriction sites for RsaI (Ibrahim *et al*., 2011). The polymorphic cleavage sites at position 612 for ewe (Carcangiu *et al*., 2009b) and at position 52 for goat were associated with the decrease in seasonal reproductive activity (Chu *et al*., 2007; Carcangiu *et al*., 2009a). In other studies, in ewes, polymorphism in MTNR1A gene exhibited an association with a greater percentage of estrus (Martinez *et al*., 2012) and age at first conception (Luridiana *et al*., 2016). In Mediterranean Italian buffalo, MTNR1A gene polymorphism was associated with mating period and calving (Carcangiu *et al*., 2011), but was not associated with age at first conception (Luridiana *et al*., 2012). However, in water buffaloes, a genotype of MTNR1A gene was shown to regulate the conception rate following melatonin treatment (Pandey *et al*., 2019). Thus, the investigations in polymorphism in MTNR1A gene are important for buffaloes.

The hypothesis of present study is based upon the fact that a proportion of non-pregnant buffalo during summer season do not experience summer-stress induced cessation of ovarian activity and continue to breed in spite of summer stress as well as seasonal change in daylight and fodder availability. This warrants investigations

into genotype of these two populations (which continue cycling *vis-à-vis* which stop cycling during summer) to identify the possible genetic markers for selection of buffalo. Thus, the present study was designed to determine the association of MTNR1A gene polymorphism with fertility parameters in buffaloes particularly reproductive cyclicity during summer season and the age at first calving.

MATERIALS AND METHODS

Animals and collection of blood samples

The history of buffaloes maintained at different dairy farms around Ludhiana, Punjab was collected, and those exhibiting uninterrupted (cyclic, n=51) or disrupted (non-cyclic, n=50) reproductive activity during summer season were selected for the present study. Furthermore, the buffaloes calving by the age of 3.5 or >3.5 years were grouped as early (n=38) or late calvers (n=60), respectively. From all the buffaloes, about 5 ml blood was collected through jugular venepuncture in the vials containing 500 µl of 0.5M EDTA.

Genomic DNA preparation

Genomic DNA from buffy coat was extracted by Cetyl trimethyl ammonium bromide method. The quantity of DNA was measured at A260/280 absorbance in a biospectophotometer and the purity of DNA was confirmed by 0.8% (w/v) agarose electrophoresis. The template DNA ranging from 400 to 500 ng was used for Polymerase chain reaction (PCR) using the already described primers (Messer *et al*., 1997; Lai *et al*., 2013), and the primer sequences (Table 1). The PCR products of each sample were separated by electrophoresis on 1% agarose gel in parallel with a 100 bp DNA marker.

Restriction Fragment Length Polymorphism (RFLP) of MTNR1A gene

About 10 µl PCR product of primer pair 1 and 2 was digested separately with each restriction enzyme (MnlI, RsaI and Eco3II) at 37°C for 3 to 5 minutes in a water bath. The digestion reaction was carried out in 30 µl volume containing 10 µl PCR product, 2 µl Fast digest green buffer (10x), 1 µl Fast digest enzyme and 17 µl nuclease free water. The resulting fragments were separated by electrophoresis on 3% (w/v) agarose gel in parallel with 50 bp DNA marker.

Single-stranded conformational polymorphism (SSCP)

The SSCP analysis of PCR product of primer pair 3 was carried out using 12% (w/v) non-denaturing polyacrylamide gel electrophoresis (PAGE) in a vertical gel electrophoresis unit. The gel mix was prepared by adding 300 µl of 10% ammonium persulphate and 15 µl of N,N,N',N'-tetramethylenediamine to the PAGE solution (30 ml). Gel mix was filled from upper side of gel sandwich using 5.0 ml micropipette, and clamps were immediately applied over the comb to ensure sharp wells. The gel was kept undisturbed at least 45 minutes. After polymerization, the comb was removed, and wells were flushed with 0.5X buffer. The gel sandwich was placed in electrophoresis tank with notched plate facing towards the buffer reservoir. The reservoir of the electrophoresis tank was filled with 0.5X TBE and the gel was given pre-run at 200 volts at 4°C for 45 minutes. At same time, about 3 µl PCR product and 12 µl of a formamide dye was prepared in PCR tube and denatured at 95°C for 10 minutes in PCR machine. After denaturation the samples were immediately

kept in ice-chilled box and kept at -20°C for 10 minutes. Subsequent to the completion of pre-run, the wells were flushed again using buffer. Thereafter, the samples were loaded with gel loading tip and immediately electrophoresis was performed in 0.5X Tris borate (pH 8.3)-EDTA buffer at 9-10 volts/cm for 9 to 14 h at 4°C. After the completion of electrophoresis, glass plates were removed from the assembly. Gel was detached from the plates carefully and was subjected to silver staining or ethidium bromide (ETBR) staining to visualize SSCP bands under UV light in Synegene Gel doc.

Statistical analysis

The data generated from RFLP analysis was compared on the basis of reproductive cyclicity (cyclic or non-cyclic) and age at first calving (early or late calving age), whereas the results of SSCP analysis were compared only on the basis of reproductive cyclicity. The genotypic frequency was calculated as number of buffaloes in a genotype divided by total number of buffaloes tested.

RESULTS AND DISCUSSIONS

In buffaloes of present study, the yield of pure DNA at the ratio 260/280 ranged between 1.7 to 1.8 and the concentration of DNA was 342 to 472 ng/µl blood. Further, the bright band of DNA on agarose gel electrophoresis confirmed the purity of DNA (Figure 1). The PCR of genomic DNA using primer pairs 1, 2 and 3 resulted in successful amplification of 824, 856 and 268 bp that correspond to main part of exon II of MTNRIA gene (Figure 1).

Assessing the relationship of MTNR1A gene polymorphism with suppression of reproductive cyclicity of buffaloes during summer season and age at first calving through RFLP analysis of MTNR1A gene

Primer pair 1

MnlI: Using RFLP, the digestion of PCR product of primer pair 1 with MnlI restriction enzyme resulted in 14 restriction sites (824, 600, 375, 350, 325, 300, 265, 240, 225, 200, 170, 140, 125 and 60 bp). As these restriction sites were not similar in all the tested buffaloes, therefore, these animals were assigned six genotypes based upon restriction sites *viz*., MM-1, MM-2, MM-3, MM-4, MM-5 and MM-6, which had respective overall frequency in all the tested buffaloes as 0.16, 0.07, 0.05, 0.12, 0.47 and 0.13 (Table 2, Figure 2). This suggested the presence of polymorphic restriction sites at different positions in MTNR1A gene. A similar study in Murrah buffaloes revealed that the digestion of 824 bp PCR product with MnlI resulted in one polymorphic cleavage site at position 50 bp with genotypic frequency as 0.62 (Cheema *et al*., 2016). In another study on Mediterranean Italian buffaloes, the digestion of 824 bp product with HpaI restriction enzyme resulted in one polymorphic site at position 82 bp (Luridiana *et al*., 2012). In a goat breed, the polymorphism was observed at position 612 bp, resulting in three genotypes with frequency as 0.68, 0.19 and 0.13 (Carcangiu *et al*., 2009a). On the contrary, the digestion of 824 bp product with MnlI did not exhibit MTNR1A gene polymorphism in other six goat breeds (Chu *et al*., 2007). With respect to reproductive cyclicity, MM-1 and MM-5 genotypes were detected in similar proportion of cyclic and non-cyclic buffaloes, MM-3 and MM-4 were present only in cyclic, and MM-2 and MM-6 were present only in non-cyclic buffaloes (Table 2). With respect to age at first calving, MM-2 was only

in late calvers, MM-1 and MM-3 were in similar proportion of early or late calvers, and with respect to their counterparts, MM-4 was higher (by 11%) in early calvers, and MM-5 and MM-6 was higher (by 33% and 4%, respectively) in late calvers (Table 2).

Primer pair 1 - RsaI

The PCR product of primer pair 1 digested with RsaI resulted in four restriction sites (404, 325, 265 and 125 bp). The buffaloes were assigned four genotypes based upon restriction sites *viz*., RR-1, RR-2, RR-3 and RR-4, which had respective frequency as 0.12, 0.02, 0.10 and 0.76. (Table 2, Figure 2). In a study on Murrah buffalo, the digestion of 824 bp PCR product with RsaI restriction enzyme resulted in three restriction sites and two genotypes of frequency as 0.95 and 0.33 (Cheema *et al*., 2016). Out of four genotypes of RsaI, RR-2 was present only in cyclic buffaloes. Furthermore, RR-1 and RR-3 were higher (by 6% each) in cyclic, and RR-4 was higher (by 12%) in non-cyclic buffaloes with respect to their counterparts (Table 2). In early and late calving buffaloes, all the genotypes of RsaI were present in almost similar proportion of buffaloes of both the groups, except RR-4 genotype, which was present in higher proportion (by 21%) of late calving buffaloes (Table 2).

Primer pair 1 - Eco3II

The PCR product of primer pair 1 digested with Eco3II resulted in five restriction sites (824, 600, 404, 317 and 240 bp). Thereby, the buffaloes were assigned four genotypes *viz*., EE-1, EE-2, EE-3 and EE-4, which had respective genotypic frequency as 0.77, 0.11, 0.09 and 0.03 (Table 2, Figure 2). Out of four genotypes of Eco3II, the proportion of non-cyclic buffaloes with EE-1 was higher (by 7%) compared to their cyclic counter parts, EE-2 was in similar proportion of both the group of buffaloes, whereas, EE-3 was only in cyclic, and EE-4 only in non-cyclic group (Table 2). With respect to age at first calving, the proportion of late calving buffaloes with EE-1 genotype was higher (by 40%) with regard to their counterparts, whereas the remaining Eco311 genotypes were in similar proportion in both early or late calving buffaloes (Table 2).

Primer pair 2 - MnII

The PCR product of primer pair 2 digested with MnII resulted in 13 restriction sites (856, 600, 350, 300, 265, 240, 225, 200, 170, 140, 125, 70 and 60 bp). Due to differential restriction sites in individual buffaloes, six genotypes were assigned *viz*., mm-1, mm-2, mm-3, mm-4, mm-5 and mm-6, which had respective overall frequency as 0.05, 0.07, 0.15, 0.10, 0.48 and 0.15 (Table 3, Figure 3). In another study on Murrah buffaloes, the digestion of 856 bp PCR product with MnII resulted in five restriction sites (236, 218, 150, 75 and 50 bp) and four genotypes with frequency as 0.04, 0.76, 0.96 and 0.24 (Cheema *et al*., 2016). Out of six genotypes of MnII, only mm-1 and mm-5 were detected in almost similar proportions of both cyclic and non-cyclic buffaloes, whereas mm-2 and mm-4 were detected only in cyclic, and mm-3 and mm-6 only in non-cyclic buffaloes (Table 3). Furthermore, out of MnII genotypes, mm-1 was only in late calvers, whereas the remaining all genotypes were present in early as well as late calvers (Table 3). The proportion of mm-5 and mm-6 genotypes in late calving buffaloes was higher (by 25% and 7%, respectively) compared to their counterparts (Table 3).

Primer pair 2 - RsaI

The PCR product of primer pair 2 digested with RsaI resulted in five restriction sites (700, 404, 325, 265 and 125 bp) in buffaloes, and these animals were assigned five genotypes *viz*., rr-1, rr-2, rr-3, rr-4 and rr-5, with respective genotypic frequency as 0.08, 0.28, 0.04, 0.55 and 0.06 (Table 3, Figure 3). In a previous study on Murrah buffaloes, the digestion of 856 bp product with RsaI also resulted in five restriction sites (411, 320, 267, 110, 50 bp) and four genotypes with frequency as 0.10, 0.05, 0.90 and 0.95 (Cheema *et al*., 2016). The assessment of genotypic difference based upon reproductive cyclicity revealed that out of five genotypes of RsaI, rr-3 was present only in cyclic buffaloes, whereas, rr-1, rr-2 and rr-5 were in similar proportion of cyclic and non-cyclic buffaloes, and rr-4 was higher by 8% in non-cyclic buffaloes compared to their counter parts (Table 3). Based upon the age at first calving, out of five genotypes of RsaI, rr-5 was present only in late calvers, whereas rr-1 and rr-3 were in similar proportion of early and late calvers, and rr-2 and rr-4 were higher by 14 and 8%, respectively in late calvers compared to their counter parts (Table 3).

Primer pair 2 - Eco3II

The PCR product of primer pair 2 digested with Eco3II resulted in six restriction sites (856, 800, 700, 600, 404 and 265 bp). Subsequent analysis revealed the presence of three genotypes *viz*., ee-1, ee-2 and ee-3, and their respective frequency in buffaloes as 0.67, 0.26 and 0.08. (Table 3, Figure 3). In various goat breeds, the RFLP analysis of 856 PCR product digested with Eco3II restriction enzyme resulted in three restriction sites and three genotypes (Lai *et al*., 2013). Out of three genotypes of Eco3II, ee-3 was only in cyclic buffaloes, and the frequency of ee-1 and ee-2 genotype was higher (by 14% and 6%, respectively) in non-cyclic and cyclic buffaloes, respectively as compared to their counter parts (Table 3). All the buffaloes calving at early or late age had all the three genotypes of Eco3II with the frequency of ee-1and ee-2 higher (by 13% and 7%, respectively) in late calvers compared to their counterparts (Table 3).

Assessing the relationship of MTNR1A gene polymorphism with suppression of reproductive cyclicity of buffaloes during summer season through SSCP analysis of MTNR1A gene

Using primer pair 3, the SSCP analysis resulted in five genotypes *viz*., AA, BB, AB, CC

Table 1. Primer sequences synthesized.

Primer	Primer sequence (5'-3')	Product size (bp)
P1	F1:TGT GTT TGT GGT GAG CCT GG R1: ATG GAG AGG GTT TGC GTT TA	824
P2	F2: GCC TGG CAG TTG CAG ACC TG R2: CAT TTT TAA ACG GAG TCC ACC	856
P3	F3: AGCTCAGCCTACACGATCGC R3: CCAGCAAATGGCAAAGAGGAC	268

Table 2. Genotypic frequency of primer pair 1 digested with three restriction enzymes in buffaloes (n) and frequency distribution with respect to reproductive cyclicity (C, cyclic; NC, non-cyclic) and age at first calving (EC: calving at early age <3.5 y; LC: calving at late age >3.5 y).

Restriction enzyme	n	Genotypes					
		MM-1, RR-1, EE-1	MM-2, RR-2, EE-2	MM-3, RR-3, EE-3	MM-4, RR-4, EE-4	MM-5	MM-6
Genotype frequency, Overall							
MnlI (MM)	91	0.16	0.07	0.05	0.12	0.47	0.13
RsaI (RR)	101	0.12	0.02	0.10	0.76	-	-
Eco3II (EE)	101	0.77	0.11	0.09	0.03	-	-
Genotype frequency with respect to reproductive cyclicity							
MnlI	C, 44	0.08	0.00	0.05	0.12	0.23	0.00
	NC, 47	0.08	0.07	0.00	0.00	0.24	0.13
RsaI	C, 51	0.08	0.02	0.08	0.32	-	-
	NC, 50	0.04	0.00	0.02	0.44	-	-
Eco3II	C, 51	0.35	0.06	0.09	0.00	-	-
	NC, 50	0.42	0.05	0.00	0.03	-	-
Genotype frequency with respect to age at first calving							
MnlI	EC, 29	0.07	0.00	0.02	0.14	0.02	0.08
	LC, 57	0.07	0.06	0.03	0.03	0.35	0.12
RsaI	EC, 38	0.04	0.06	0.04	0.24	-	-
	LC, 60	0.04	0.04	0.08	0.45	-	-
Eco3II	EC, 29	0.17	0.05	0.05	0.02	-	-
	LC, 69	0.57	0.06	0.05	0.02	-	-

Table 3. Genotypic frequency of primer pair 2 digested with three restriction enzymes in buffaloes (n) and frequency distribution with respect to reproductive cyclicity (C: cyclic; NC: non-cyclic) and age at first calving (EC: calving at early age <3.5 y; LC: calving at late age >3.5 y).

Restriction enzyme	**n**	**Genotypes**					
		mm-1, rr-1, ee-1	**mm-2, rr-2, ee-2**	**mm-3, rr-3, ee-3**	**mm-4, rr-4**	**mm-5, rr-5**	**mm-6**
Genotype frequency, Overall							
Mn1I (mm)	86	0.05	0.07	0.15	0.10	0.48	0.15
RsaI (rr)	101	0.08	0.28	0.04	0.55	0.06	-
Eco3II (ee)	101	0.67	0.26	0.08	-	-	-
Genotype frequency with respect to reproductive cyclicity							
MnII	C, 38	0.02	0.07	0.00	0.11	0.25	0.00
	NC, 45	0.02	0.00	0.13	0.00	0.23	0.16
RsaI	C, 51	0.05	0.15	0.04	0.21	0.05	-
	NC, 50	0.03	0.13	0.00	0.34	0.01	-
Eco3II	C, 51	0.26	0.16	0.08	-	-	-
	NC, 50	0.40	0.10	0.00	-	-	-
Genotype frequency with respect to age at first calving							
MnII	EC, 24	0.00	0.02	0.05	0.03	0.13	0.05
	LC, 62	0.01	0.05	0.08	0.07	0.38	0.12
RsaI	EC, 30	0.01	0.06	0.06	0.17	0.00	-
	LC, 68	0.02	0.20	0.10	0.35	0.02	-
Eco3II	EC, 38	0.29	0.04	0.04	-	-	-
	LC, 60	0.42	0.11	0.04	-	-	-

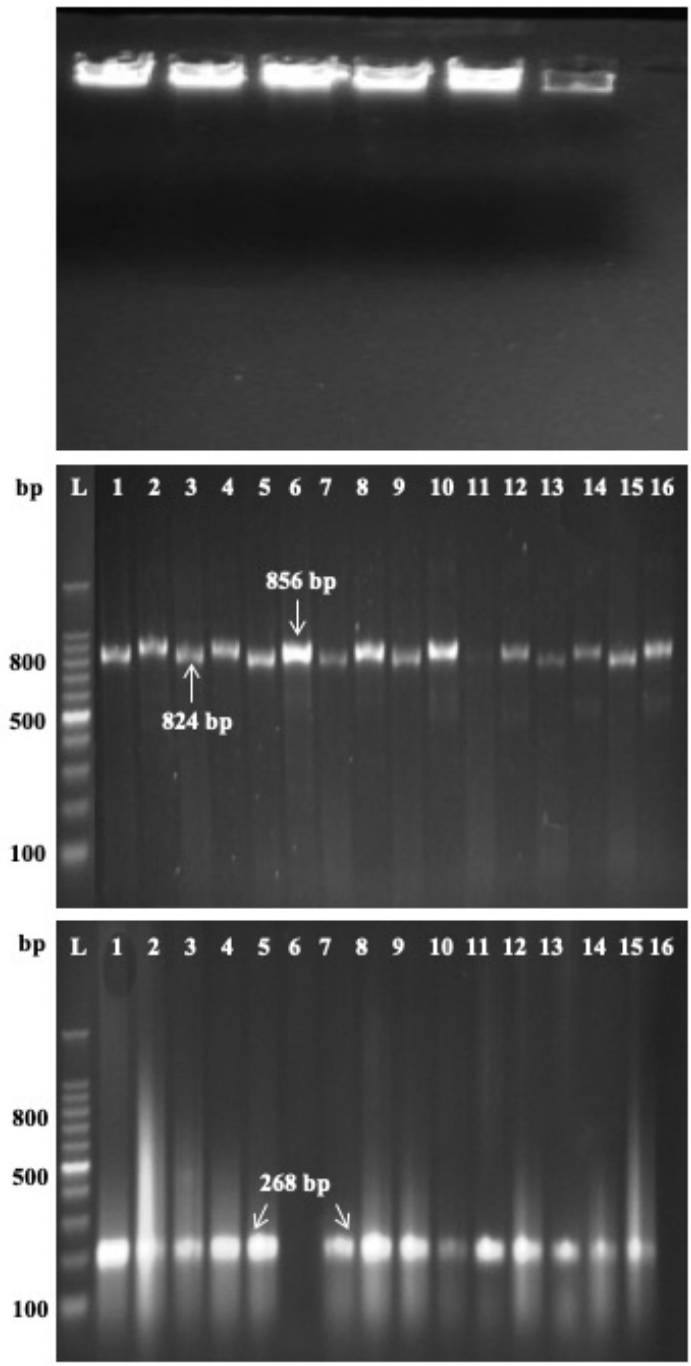

Figure 1. Upper panel - Genomic DNA; Middle panel - Amplified 824 bp (Lane 1, 3, 5, 7, 9, 11, 13, 15) and Amplified 856 bp (Lane 2, 4, 6, 8, 10, 12, 14, 16) PCR product; and Lower panel - 268 bp PCR product on 1% agarose gel.

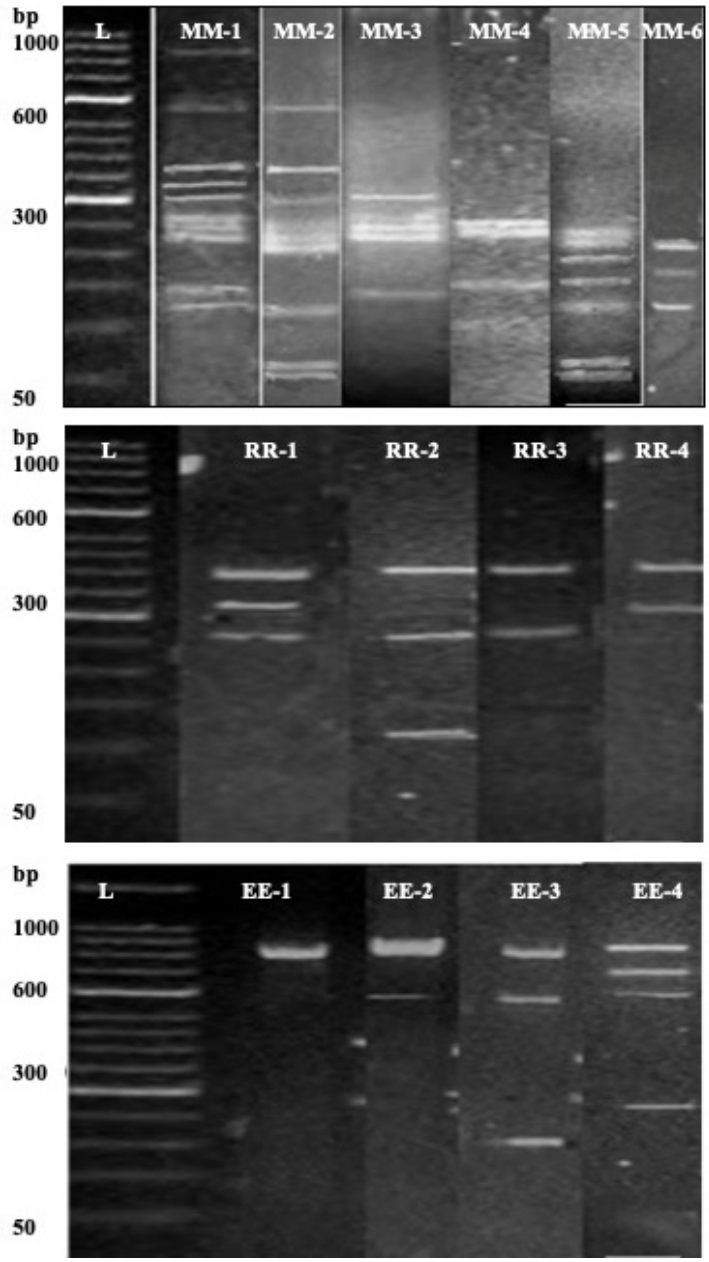

Figure 2.RFLP analysis of MTNR1A gene in buffaloes showing digestion of PCR product of primer pair 1 with MnII, RsaI or Eco3II which generated total restriction sites respectively as 14, 4 and 5. Based upon differential restriction sites, the genotypes assigned to these animals using MnII were MM-1 (824, 600, 375, 325, 300, 265, 240, 225, 140, 125 bp), MM-2 (600, 350, 300, 240, 225, 200, 140, 125, 70, 60 bp), MM-3 (300, 265, 240, 225, 140 bp), MM-4 (265, 240, 140 bp), MM-5 (240, 225, 200, 140, 125, 70, 60 bp) and MM-6 (225, 140, 125 bp), genotypes using RsaI were RR-1 (404, 325, 265 bp), RR-2 (404, 265, 125 bp), RR-3 (404, 325 bp) and RR-4 (404, 265 bp), and genotypes using Eco3II were EE-1 (824 bp), EE-2 (824, 600 bp), EE-3 (824, 600, 240 bp) and EE-4 (824, 600, 404, 317 bp).

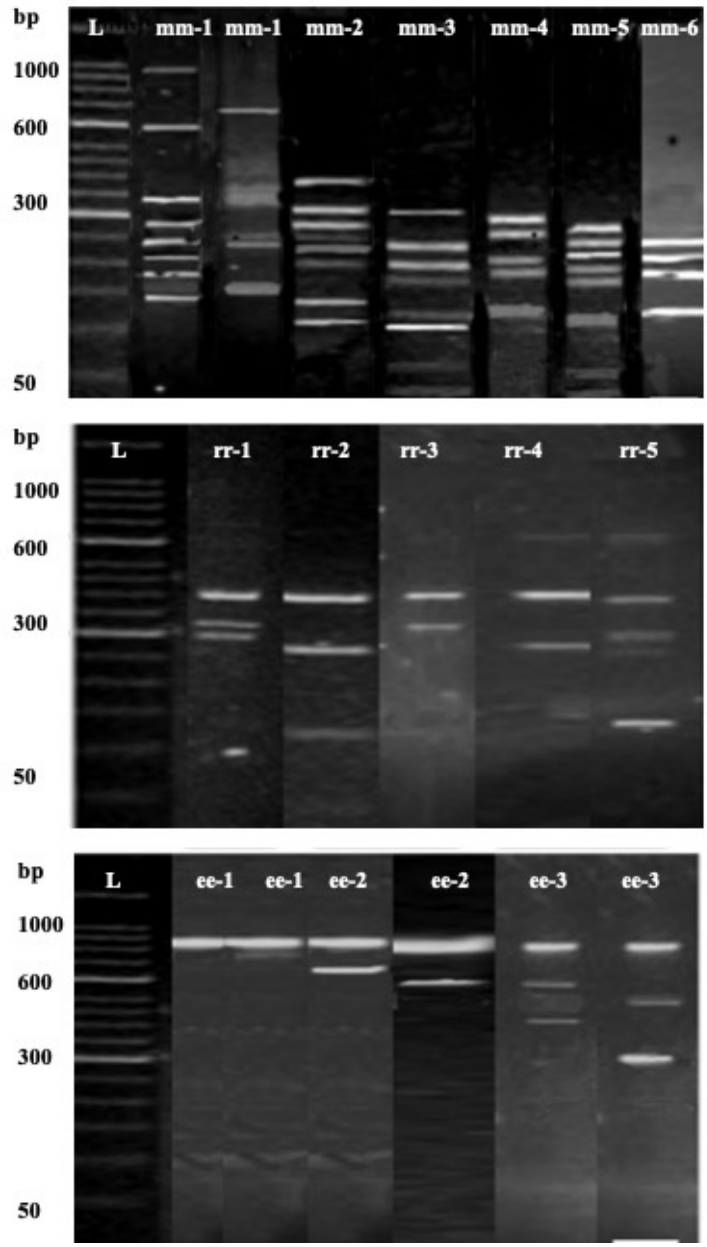

Figure 3. RFLP analysis of MTNR1A gene in buffaloes showing digestion of PCR product of primer pair 2 with MnII, RsaI or Eco3II which generated total restriction sites respectively as 13, 5 and 6. Based upon differential restriction sites, the genotypes assigned to these animals using MnII were mm-1 (856, 600, 300, 265, 240, 170, 140 bp), mm-2 (350, 300, 240, 225, 140 bp), mm-3 (300, 240, 225, 200, 170, 140, 125 bp), mm-4 (265, 240, 200, 170, 140 bp), mm-5 (240, 225, 200, 170, 140, 125, 70, 60 bp) and mm-6 (225, 200, 170, 125 bp), genotypes using RsaI were rr-1 (404, 325, 265 bp), rr-2 (404, 265, 125 bp), rr-3 (404, 325 bp), rr-4 (404, 265 bp) and rr-5 (700, 404, 325, 265, 125 bp), and genotypes using Eco3II were ee-1 (856 bp), ee-2 (856, 800/700/600 bp) and ee-3 (856, 600, 404/265 bp).

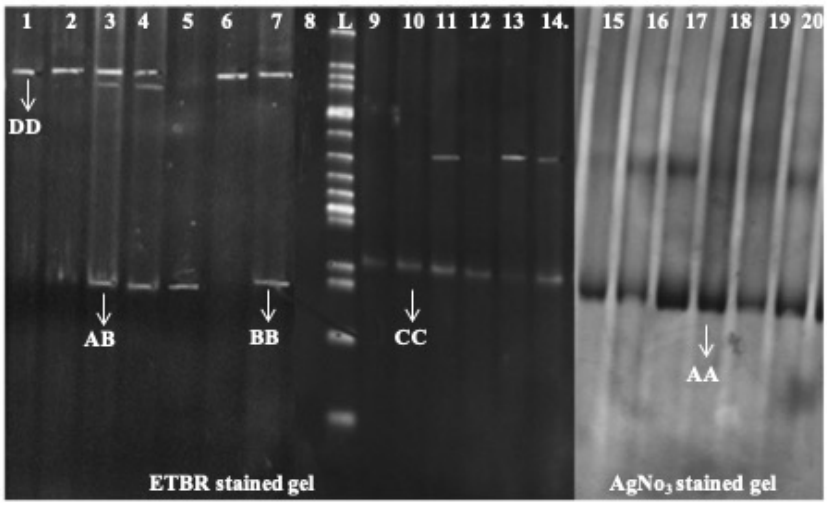

Figure 4. SSCP analysis of MTNR1A gene in buffaloes showing PCR product of primer pair 3. Non-cyclic buffaloes revealed AA (200/475bp; Lane 11, L13, L14-20), BB (200/800bp; L7) and AB (200/700/800bp; L3, L4) genotypes, whereas, the cyclic buffaloes revealed CC (200bp; L5, L9-10) and DD (800bp; L1, L2, L6) genotypes.

and DD (Figure 4) with their overall respective frequency in buffaloes as 0.30, 0.5, 0.2, 0.32 and 0.68. With respect to reproductive cyclicity, the genotype AA, BB and AB were present only in non-cyclic, whereas CC and DD genotypes were present only in cyclic buffaloes (Figure 4). In the present study, SSCP analysis of MTNR1A gene revealed mutation at position 475 bp and 700 bp in non-cyclic buffaloes. In goat, the SSCP analysis of 268 bp PCR product resulted in three genotypes with frequency as 58.5, 36.7 and 9.8%, respectively (Lai *et al*., 2013).

Thus, the present study through RFLP and SSCP analysis of MTNR1A gene revealed the existence of relationship between MTNR1A gene polymorphism and suppression of reproductive cyclicity in buffaloes during summer season. In brief, the buffaloes subjected to blood sampling during summer period had polymorphic genotypes for MTNR1A gene as the buffaloes showing reproductive cyclicity had MM-3, MM-4, RR-2, EE-3, mm-2, mm-4, rr-3, ee-3, CC and DD genotypes, whereas their non-cyclic counterparts had MM-2, MM-6, EE-4, mm-3, mm-6, AA, BB and AB genotypes. Others also reported the existence of association of polymorphism in MTNR1A gene with reproductive activity during days with long photoperiod in buffalo (Carcangiu *et al*., 2011; Luridiana *et al*., 2014). Moreover, in several breeds of goat, the polymorphism in MTNR1A gene was also associated with seasonal reproductive cyclicity (Chu *et al*., 2007).

However, RFLP analysis of MTNR1A gene revealed similar genotypes between buffalo calving at early or late age, which suggested that there is no association of MTNR1A with age at first calving. In fact, only MM-2 and rr-5 genotypes were differentially detected in later calvers

compared to early calves, rest all the genotypes were present in buffaloes calving at early as well as late age. Another study in buffalo also exhibited the absence of association of MTNR1A gene with age at first calving and calving interval (Luridiana *et al*., 2014). The absence of association between MTNR1A gene polymorphism and age at first calving could be due to the impact of season of birth, time of puberty and season of conception or calving (Penchev *et al*., 2014; Sharma *et al*., 2016).

In conclusion, the polymorphic genotypes of MTNR1A gene could be the potential candidate genetic markers to identify buffaloes with the ability to reproduce better during summer season.

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