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Association of Aquaporin 7 gene variants with semen quality in Murrah buffaloes

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ABSTRACT

The Aquaporin 7 (AQP7) gene, a member of the aqua-glyceroporins, was characterised using Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) analysis, in order to investigate the association of genetic variants of the AQP7 gene with semen quality in Murrah bulls. In this study, genomic DNA was extracted from the blood of Murrah bulls (n = 69) using the high salt method. Sequence data were analyzed using Bio Edit software (version 7.0.5) for detecting single nucleotide polymorphisms (SNPs). Statistical analysis was carried out using repeated GLM procedure measures (SAS 9.3). Analysis revealed 17 different SSCP variants in the AQP7 gene in Murrah buffaloes. Three unique SSCP band patterns were observed in exons 1 and 4. Exons 5, 7 and 8 showed five, four and two unique SSCP band patterns, respectively. Sequence analysis revealed a total of 20 SNPs (8 in exonic and 12 in intronic region). PCR-SSCP variants of exon 5 were significantly associated ($P<0.01$) with sperm concentration. SSCP variants of exons 5 and 8 were associated ($P<0.05$) with sperm viability and post thaw motility (PTM), respectively. The study revealed a high degree of genetic variability in the AQP7 gene in Murrah bulls. The SSCP variants observed are associated with semen parameters which suggests the possibility of using the AQP7 gene as a candidate gene for identification of markers for semen quality traits in buffaloes.

Key words: AQP7 gene; Murrah buffalo; PCR-SSCP; SNP

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Introduction

Buffaloes (*Bubalus bubalis*) contribute significantly towards milk, meat and draught power in the Indian subcontinent. India has the largest buffalo population in the world, with 108.70 million head (DAHD, 2014), which constitutes 56.7% of world's buffalo population. Murrah buffaloes are well-known for their milk production as well as their adaptability to tropical conditions. They are used for upgrading local buffaloes in many parts of Asia and other parts of the world, which necessitates the selection of highly fertile buffalo bulls. Since semen quality is a direct indicator of bull fertility, candidate genes with a major effect on semen quality traits could be used as a marker for early selection of males for fertility. However, very few reports are available on candidate marker genes for fertility traits in buffaloes.

Aquaporins (AQP) are integral membrane proteins from a family of major intrinsic proteins (MIP) that form pores in the membrane of a biological cell (AGRE, 2006) and are located on a wide variety of cells, that is: the testes, kidneys, lungs, pancreas, brain, gastrointestinal tract, eyes, ears, immune system, skin, adipose, muscles and uterus (BEITZ et al.,1999; LI et al., 1994). There are 13 members in the AQP family which are subdivided into: orthodox AQPs, aquaglyceroporins and superaquaporins (AGRE et al., 2002; ZARDOYA, 2005). The Aquaporin 7 (AQP7) gene, a member of the aquaglyceroporins, is located on chromosome BTA8 and has 7 coding and 1 non-coding exons. It consists of 16.25 kilobase pairs and encodes the AQP7 protein of 269 amino acids. The AQP7 gene is expressed in the tail of spermatids, spermatozoa and the anterior tail portion of ejaculated sperm (SUZUKI-TOYOTA et al., 1999). Immuno-histochemical analysis demonstrated that the AQP7 protein exists in elongated spermatids, testicular spermatozoa, and residual bodies (CALAMITA et al., 2001). The differential expression pattern of the AQP7 in infertile and fertile ejaculated human sperm, as well as the different motility rates between AQP7 positive or negative sperms, proves its role in male fertility and the transient expression of mRNA in the late phase of spermatogenesis (SUZUKI-TOYOTA et al., 1999; SAITO et al., 2004). In addition, it also contributes to generating the seminiferous tubule fluid (RUSSELL et al., 1989). In epididymal spermatozoa AQP7 play an important role in the maintenance of sperm quality through sperm maturation, by transporting glycerol, urea and other small non-electrolytes from the epididymal fluid (CALAMITA et al., 2001). On the other hand, AQP7 helps in the cryopreservation of sperm because it has the permeable properties to water, as well as urea and glycerol. MA et al., 2011 reported that SNPs in the exon 2 and 3 of the AQP7 gene are associated with semen quality parameters in cattle. However, no information is available on the association of genetic variants of the AQP7 gene with semen quality traits in buffaloes. Therefore, the present study was undertaken to characterize the aquaporin 7 gene and to investigate the association of its genetic variants with semen quality in Murrah bulls.

Materials and methods

Location and experimental animals. The experimental study was carried out in Murrah bulls (n = 69) maintained at various semen stations, that is the Livestock Training Centre, Dharwad, Karnataka, and Nandini Sperm Station, Hessarghatta, Karnataka, India. The Livestock Training Centre, Dharwad is situated at an altitude of 768 meters above mean sea level, at 15.49 ºN latitude and 74.98 ºE longitude. The Nandini semen station and the state semen collection centre, Hessarghatta, are situated at an altitude of 874 meters above mean sea level at 13.15 °N latitude and 77.49 °E longitude. The bulls were kept in herds at the livestock training centre under natural conditions, according to the animal welfare rules. The semen parameters, that is: sperm concentration (SCON), individual motility (MOT), semen volume per ejaculate (VOL) and post thaw motility (PTM) were recorded from April, 2014 to March, 2015 for each bull from the records maintained in the semen stations (SMILE software, IMV technologies, France). Sperm viability and hypo osmotic swelling (HOS) reaction in both fresh and frozen semen were estimated in three different seasons. For determination of live and dead spermatozoa, the eosin-nigrosine staining technique was used (BLOOM, 1950; HANCOCK, 1951). The functional integrity of the plasma membranes of the spermatozoa, was checked by HOS reaction, as described by CORREA and ZAVOS (1994).

DNA extraction. Blood samples (8-10 mL) were collected from healthy bulls using lithium coated vacutainer tubes (BD Biosciences) containing heparin as the anticoagulant by jugular vein puncture under sterile conditions, and were promptly transported to the laboratory under refrigeration. Genomic DNA was isolated from the blood samples by the High Salt Method as described by MILLER et al. (1988). The quality of DNA was checked by 0.8% agarose gel electrophoresis. The quality and quantity of DNA were also estimated using a Biospec-nano spectrophotometer. The ratio between OD260 and OD280 was observed for each sample. A DNA sample with a ratio of 1.8 was considered good and taken for further analysis. The genomic DNA was diluted to a final concentration of 100 ng/µL and stored at -20 °C for subsequent analysis.PCR primers and amplifications

Eight sets of primers were designed for amplifying the entire coding region using Primer 3 (Version 0.4.0) online software based on the publishing sequence of *Bos taurus* cattle (Ensemble Ref Seq: ENSBTAG00000020105) located on BTA-8. The primers were procured from Amnion Biosciences Pvt. Ltd., Bengaluru, India. The sequence of primers, their respective nucleotide numbers, and amplicon size are given in (Table 1). The PCR amplification was carried out in programmed thermo cycler (Genetix, India) containing a final volume of 25 µL of reaction mixture. The PCR reaction mixture comprising of 20 pM containing 0.5 µL of each primer, 200 µM of each dNTPs (2.5 µL), 2.5 µL of 10× PCR assay buffer containing 1.5 mM $MgCl_2$, 1 µL of 1 U/µL *Taq* DNA polymerase, 1 µL of 100 ng/µL genomic DNA and final dilution was made by adding nuclease free

water. The thermo cycler conditions included an initial denaturation at 94 °C for 5 min for exons 1 and 8, and 95 °C for 10 min for rest of the exons, followed by 35 cycles with denaturation at 94 °C for 1 min with varying annealing temperatures based on primer set, extension at 72 °C for 1 min followed by a final extension at 72 °C for 5 min for exons 1 and 8, and 10 min for the other exons. The PCR products, along with 100bp DNA markers, were electrophoresed at 150 V for 1 hour in 1.5% agarose gel using 1X TBE buffer containing 0.5 µg/mlethidium bromide. The gels were visualized and photographed using the Gel documentation system (Gel Doc 1000, Bio-Rad, USA).

Table 1. Details of primer sequences (5' to 3' sequences) used for PCR amplification

Exon	Sequence	Location of exon on gene (AQP7)	Product size (bp)	Ta (°C)
1	F-GAAGGGGTGCTATTTTGGGC R- AGGCAGCAACTCAGGACTAA	601-841	459	58
2	F- AATGAGGGGCCAAGTTCTGT R- AGGGAGTCTTGGTGCATCAG	2008-2056	227	60
3	F- CAAGAGCAGGCATGTGTGG R- AAGGGACAGTGTGATCCAGG	6869-6986	365	61
4	F- AAGGGCAATGCAGGGAGAC R- CTTGTGAAGTGTCTGGGCAG	14763-14886	384	59
5	F- ATCATCGCTGCTGTCATTGC R- AGGACACACTCAGATCTGCC	15252-15389	287	56
6	F- GCAGGTTCACACTCAGTCTG R- TCCAGGGTTCTTGTCCACTC	15597-15715	249	58
7	F- GAGTGGACAAGAACCCTGGA R- CTTTTCCACCCTCCACATGC	15923-16140	413	58
8	F- CATCTTGGCCTGGGGTAGAC R- AACCTGGGAGCTTCTGAGAG	16529-16854	478	56

F = forward, R = reverse, Ta = annealing temperature, bp = base pair

PCR-SSCP analysis. The PCR products were subjected to single-strand conformation polymorphism (SSCP) analysis in 40 mL of 10% non-denaturing PAGE (acrylamide and bis-acrylamide, 29:1) gel, using 1X TBE buffer in a vertical gel electrophoresis unit (Cleaver, UK). The gels were silver stained, as described by SAMBROOK and RUSSELL (2001). SSCP variants were recorded manually based on the number of bands and mobility shifts. Two representative samples of each unique PCR-SSCP band pattern were custom sequenced using an automated ABI DNA Sequencer (Amnion Biosciences Pvt. Ltd., Bengaluru, India). SNPs were detected by analysis of sequence data using BioEdit Software (Version 7.0.5) (HALL, 1999) and Clustal-W multiple sequence alignment software.

Statistical analysis. The statistical analysis was carried out using repeated measures of the GLM (General Linear Model) procedure (SAS 9.3). The data were classified

according to the season of semen collection (three groups), the age group of the bulls (three groups - up to 3 years, 3-4 years and 4-5 years), and also based on genotypes. The three groups of seasons were categorised as: winter (November to February), summer (March to June) and rainy (July to October). Moreover, the number of the bulls categorised in different age groups were 30 (up to 3 years), 29 (3-4 years) and 10 (4-5 years). Two different fixed models were used for association studies. The first model was used for association of the SSCP pattern of different polymorphic exons with semen parameters, i.e. volume, concentration, motility and post thaw motility, and the other model was used for association of the SSCP pattern with sperm viability and HOS reactivity.

Model 1: $Y_{ijkl} = \mu + Gi + Aj + Sk + e_{ijkl}$

Model 2: $Y_{ijk} = \mu + Gi + Aj + e_{ijk}$

where,

Y_{ijkl} is observation of the l^{th} bull with i^{th} genotype and j^{th} age in k^{th} season
Y_{ijk} is observation of the k^{th} bull with i^{th} genotype and j^{th} age
μ is the overall mean
G_i is the fixed effect of i^{th} genotype
A_j is the fixed effect of j^{th} age (j = 3, 4 and 5)
S_k is the fixed effect of k^{th} season of collection (1-winter, 2- summer and 3-rainy)
e_{ijkl} is the random error, which is NID (0, σ_e^2)

Results

PCR-SSCP variants. The PCR-SSCP analysis of the AQP7 gene revealed varying degrees of genetic polymorphisms in exons 1, 4, 5, 7 and 8 in Murrah buffaloes. Exons 2, 3 and 6 were found to be monomorphic. The SSCP analysis showed 17 SSCP band patterns in Murrah bulls. Exons 1 and 4 revealed 3 band patterns while 5, 4 and 2 band patterns were observed for exons 5 (Fig. 1), 7 and 8, respectively. The frequencies of SSCP variants for polymorphic exons in 69 Murrah bulls genotyped in the present study are summarised in Table 2.

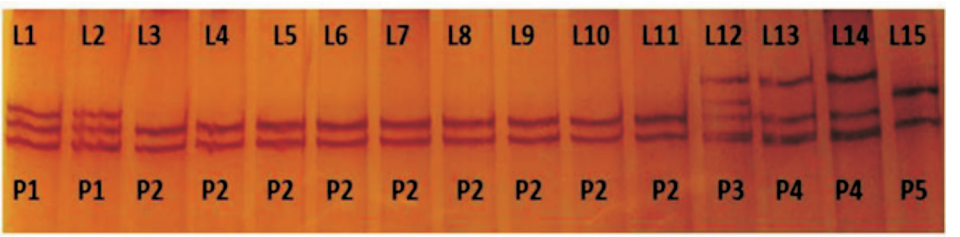

Fig. 1. Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) patterns observed in exon 5 of aquaporin 7 gene in Murrah bulls. (L- lane; P - pattern of SSCP band).

Table 2. Frequencies of Single Strand Conformation Polymorphism (SSCP) variants in exons 1, 4, 5, 7 and 8 of the aquaporin 7 gene in Murrah bulls

Exon	Pattern	No of observations	Frequency of SSCP variants
Exon 1	Pattern 1 (E1P1)	38	0.5507
	Pattern 2 (E1P2)	15	0.2174
	Pattern 3 (E1P3)	16	0.2319
Exon 4	Pattern 1 (E4P1)	21	0.3034
	Pattern 2 (E4P2)	15	0.2174
	Pattern 3 (E4P3)	33	0.4783
Exon 5	Pattern 1 (E5P1)	7	0.1015
	Pattern 2 (E5P2)	25	0.3623
	Pattern 3 (E5P3)	14	0.2029
	Pattern 4 (E5P4)	14	0.2029
	Pattern 5 (E5P5)	9	0.1304
Exon 7	Pattern 1 (E7P1)	20	0.2899
	Pattern 2 (E7P2)	16	0.2319
	Pattern 3 (E7P3)	21	0.3043
	Pattern 4 (E7P4)	12	0.1739
Exon 8	Pattern 1 (E8P1)	38	0.5507
	Pattern 2 (E8P2)	31	0.4493

SNP identification. Representative samples were custom sequenced to confirm the mobility shift in each pattern. The retrieved sequences representing each of the unique PCR-SSCP patterns were analyzed by comparing these sequences with the bovine AQP7 gene reference sequence (Ensemble Ref Seq: ENSBTAG00000020105), using Bio Edit

software and Clustal-W multiple sequence alignment (Figs. 2, 3) for detecting single nucleotide polymorphism (SNPs) and their respective deduced amino acid variations. Eleven nucleotide sequences (partial) were deposited with the NCBI gene bank. A total of 20 SNPs were observed in Murrah bulls. Out of 20 SNPs, 8 were in the exonic region (Table 3), of which 7 SNPs brought about change in the amino acid in the transformed products, and one was present in non-coding exon 8. In the intronic region 12 SNPs were detected (C88OA, A14714T, G14730A, G14909A, C14917T, C15424A, G15428A, A14736G, C15866T, G15883A, G15922A and insertion of G at 14901bp). Nucleotide change T15259C, G15292A and C15328T in exon 5 and C15427G in partial intron 5 were present in all the Murrah bulls, as compared to cattle (COWS??).

Table 3. Summary of single nucleotide polymorphisms observed in the aquaporin 7 gene in Murrah bulls

Region	Transversion	Transition	Loci (SNPS)	Amino acid change
Exon-1	T/G		T677G	Leu→Arg
		T/C	T753C	Pro→Thr
Exon -4	A/C		A14808C	Met→Leu
	T/A		T14832A	Leu→Met
Exon-5	T/A		T15265A	Cys→Ser
		G/A	G15316A	Val→Ile
Exon -7	G/T		G15959T	Ala→Ser
Exon -8	T/A		T16788A	-
Total	6	2	8	7

Table 4. Estimates of different semen parameters in Murrah bulls (Mean ± SE)

Traits		Mean ± SE	Range
Sperm concentration (millions of cells /mL)		904.73 ± 15.81	150-1920
Motility (%)		56.01 ± 0.66	25-73
Semen volume per ejaculate (mL)		2.61 ± 0.04	1.5-7.45
Sperm viability	Fresh semen (%)	87.89 ± 1.75	52-98
	Frozen semen (%)	57.73 ± 2.53	31-81
Hypo osmotic swelling reactivity	Fresh semen (%)	64.86 ± 2.6	38-85
	Frozen semen (%)	45.77 ± 1.93	30-65
Post thaw motility (%)		46.29 ± 0.38	25-55

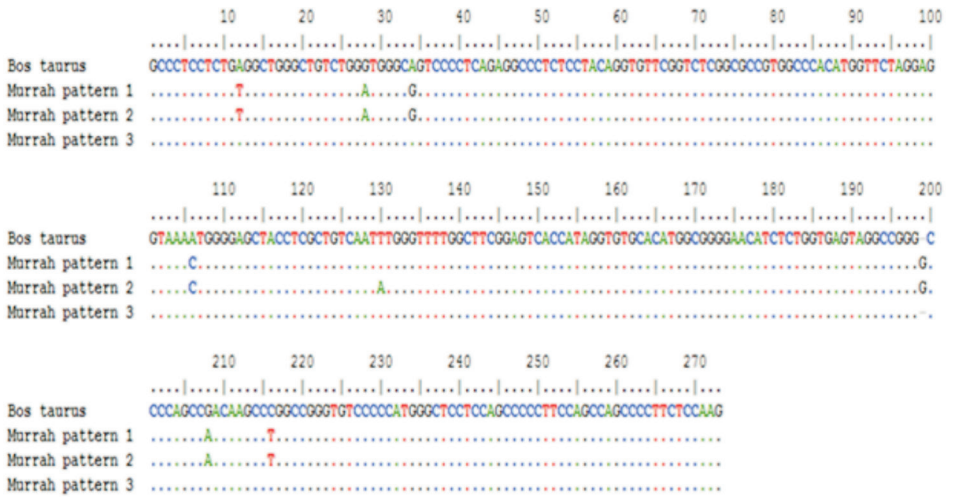

Fig. 2. Clustal-W multiple alignment of exon 4 (AQP7 gene) showing eight single nucleotide polymorphisms (SNPs) in three samples in Murrah Bulls

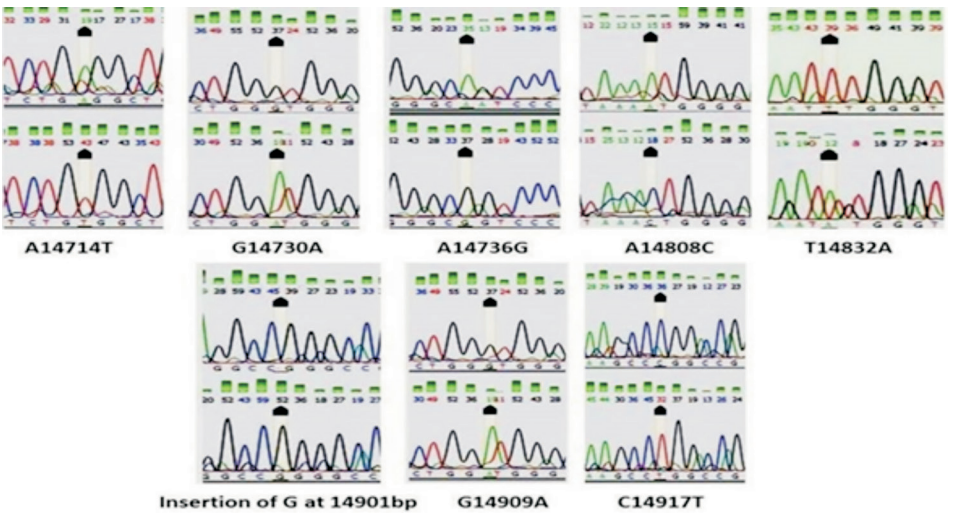

Fig. 3. Sanger Trace figure of Single Strand Conformation Polymorphism (SSCP) variant sites of exon 4 in Murrah bulls

Table 5. Least squares means for sperm concentration and sperm viability for different patterns of exon 5 in Murrah bulls

Pattern	Sperm concentration (millions of cells/mL)	Sperm viability (%)	
		Fresh	Frozen
Pattern E5P1	$777.27^{b} \pm 98.64$	$82.00^{b} \pm 3.99$	$48.67^{b} \pm 6.44$
Pattern E5P	$1158.77^{a} \pm 77.11$	$90.87^{a} \pm 1.59$	$57.32^{ab} \pm 2.56$
Pattern E5P3	$841.77^{b} \pm 73.52$	$83.50^{b} \pm 3.10$	$54.00^{b} \pm 4.99$
Pattern E5P4	$918.99^{ab} \pm 68.58$	$88.08^{a} \pm 2.62$	$70.29^{a} \pm 4.22$
Pattern E5P5	$751.62^{b} \pm 76.41$	$81.88^{b} \pm 3.46$	$57.25^{ab} \pm 5.58$

Means with different superscripts were significantly different from each other at ($P<0.05$).

Effect of SSCP variants on semen quality. The mean SCON (millions of cells /mL), MOT (%), VOL (mL), PTM (%) and sperm viability (%) and HOS reaction (%) in both fresh and frozen samples from Murrah bulls are summarised in Table 4. SSCP variants of exon 5 had a significant ($P<0.01$) effect on SCON. Exon 5 SSCP variants had a significant ($P<0.05$) effect on sperm viability in both fresh and frozen semen, and SSCP variants of exon 8 had a significant ($P<0.01$) effect on PTM in Murrah bulls.

The least square means of SCON (millions of cells /mL) and sperm viability (in percentage) for exon 5 in Murrah bulls are summarized in Table 5. Bulls with Pattern E5P2 of exon 5 were found to produce semen with the highest SCON (1158.77 ± 77.11 millions of cells/mL) and sperm viability (90.87 ± 1.59%). The least squares means of PTM (in percentage) for exon 8 in patterns E8P1 and E8P2 are 44.76 ± 0.98 and 47.61 ± 0.72, respectively. The pattern E8P2 was significantly different from the pattern E8P1.

Discussion

DQPs are known to be involved in the early stage of spermatogenesis, in the secretion of tubule liquid, and in the concentration and storage of spermatozoa (HUANG et al., 2006). AQP7 relates to the cell volume reduction of spermatids by mediating the efflux of water from spermatids during spermiogenesis (CALAMITA et al., 2001). A role for AQP7 in the morphological change of secondary spermatocytes to spermatids has been suggested by KAGEYAMA et al. (2001). The cDNA of AQP7 have been reported, in the male reproductive and gastrointestinal tracts (KOYAMA et al., 1997; MA et al., 1997). The ontogeny and distribution of AQP7 in rat testes suggest its involvement in major physiological changes in testes development and spermatogenesis (CALAMITA et al., 2001). The present study was designed to identify genotype patterns in Murrah bulls. In this study, 20 SNPs in 17 different SSCP band patterns were detected in the AQP7 gene of Murrah bulls. PCR-SSCP variants of the AQP7 gene were found to be associated with semen parameters in Murrah bulls. Earlier reports indicated the association of the AQP7

gene with semen quality in cattle (ZHAO et al., 2009; MA et al., 2011; KUMAR et al., 2014).

Exon 2 and 3 were found to be monomorphic in the Murrah bulls. MA et al. (2011), reported two SNPs on exon 2 (A264G) and exon 3 (G371C) of the AQP7 gene in Simmental and Charolaise bulls, using the PCR-SSCP technique. In the present study, exon 5 was associated with SCON and sperm viability. Bulls with pattern 2 of exon 5 were found to produce more viable and concentrated semen. Bulls with pattern 2 of exon 8 were found to produce semen with high PTM. Associations of different SNPs in the AQP7 gene were reported earlier. MA et al. (2011) reported an association of the observed SNPs with 10 different semen quality parameters, a significant association of A264G transition with acrosome integrity ($P<0.01$) and motility ($P<0.05$) in frozen semen, and G371C transversion with acrosome integrity ($P<0.05$), percentages of viable sperm ($P<0.05$) and motility ($P<0.01$) in frozen semen were also found. In a similar study, KUMAR et al. (2014), reported the same SNPs (A264G, G371C) in Frieswal cattle and observed a significant effect of A264G (in exon 2) on sperm motility, as well as post-thaw motility and G371C (in exon 3) on semen volume, sperm motility, and post-thaw motility. Both transition and transversion were found to influence the quality parameters positively. The present results were reported for the first time in buffaloes, so no earlier reports are available to compare these findings with those in buffalo bulls.

It is known that the genotype of an individual SNP may affect other SNPs and thus the genotype combination effect is an indication of interaction between multiple SNPs (ZHENG et al., 2011). The present results indicate that there is a high variability in the AQP7 gene and genetic variants are associated with semen parameters in Murrah buffaloes. These SSCP variants with an association with semen parameters offer an opportunity for identification of markers for semen quality in buffaloes. However, further studies using a larger number of bulls have to be carried out before using these SSCP variants in the Marker Assisted Selection (MAS). The present study could be a step towards identification of genetic markers for selecting buffalo bulls with high fertility.

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SAŽETAK

Gen akvaporin 7 (AQP7), koji pripada akvagliceroporinima, analiziran je kombinacijom lančane reakcije polimerazom i utvrđivanjem polimorfizma jednolančane konformacije (PCR-SSCP) s ciljem da se istraži povezanost genskih varijanti s kvalitetom sjemena u Murrah bivola. Genomska DNA izdvojena je iz krvi bivola (n = 69) high salt metodom. Za otkrivanje polimorfizma pojedinačnih nukleotida (SNP) sekvencije su analizirane programom Bio Edit (verzija 7.0.5). Statistička analiza provedena je GLM procedurom ponovljenih mjerenja (SAS 9.3). Analiza je pokazala 17 različitih SSCP varijanti AQP7gena u Murrah bivola. Tri jedinstvena SSCP rasporeda linija utvrđena su u egzonima 1 i 4. Egzoni 5, 7 i 8 pokazali su pet, četiri i dva jedinstvena SSCP rasporeda linija. Sekvenciranje je pokazalo ukupno 20 SNP-a (8 u egzonima i 12 u intronima). PCR-SSCP varijante egzona 5 statistički su značajno povezane ($P<0,01$) s koncentracijom sjemena. SSCP varijante egzona 5 i 8 značajno su povezane ($P<0,05$) s vijabilnošću spermija i njihovom pokretljivošću nakon odmrzavanja. Istraživanje je pokazalo visok stupanj genetičke varijabilnosti akvaporina 7 u Murrah bivola. Utvrđene SSCP varijante koje su povezane s pokazateljima kakvoće sjemena upućuju na mogućnost upotrebe akvaporina 7 gena kao kandidatnog gena za identifikaciju biljega kvalitete sjemena bivola.

Ključne riječi: gen AQP7; Murrah bivol; PCR-SSCP; SNP