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RESEARCH ARTICLE

IDENTIFICATION OF MOLECULAR MARKERS IN BMP15 GENE OF PAKISTANI GOAT BREEDS

Ahmad Nawaz[1], Masroor Ellahi Babar*[2], Tanveer Hussain[3], Asif Nadeem[3], Farah Bilal[3], Khalid Javed[2], Khushi Muhammad[4]

1. Lasbela University of Agriculture, Water and Marine Sciences, Utthal, Balochistan, Pakistan.
2. Department of Livestock Production, University of Veterinary and Animal Sciences Lahore, 54000, Pakistan.
3. Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences Lahore, 54000, Pakistan.
4. Department of Microbiology, University of Veterinary and Animal Sciences Lahore, 54000, Pakistan.

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Abstract

This research was performed for screening polymorphism of Bone Morphogenic Protein 15 (BMP15) in two goat breeds Teddy and Beetal of Pakistan. Teddy is the more prolific than other breeds of goat found in the country. To find out molecular markers associate with fertility, the selection of animals based on single birth (Beetal) and multiple births (Teddy) history were collected. Forty five samples were collected from each breed, direct sequencing was done identify the genetic variation. While the mutations in the candidate gene associate with fecundity in sheep were not identified in investigated Pakistani goat breeds. Although the sequencing data showed six novel polymorphic sites in Teddy breed. Two intronic mutations in base No. 982 with T>C and 5572 with A>G and four exonic mutations at nucleotide position 6280 T>G, 6353 G>A, 6443 T>C and 6492 A>G were identified. All these mutations were reported and registered in NCBI with accession no. of JN655669 - JN655670. These finding furnished significant explanations for the conclusion of BMP15 gene may be a major gene which affects the prolificacy in Teddy goat. This study could provide basic molecular data on the reproductive characteristics of local breeds of Pakistan and a scientific basis for the conservation and utilization of goat breeds.

Introduction

Three significant genes (*GDF9, BMPR1B and BMP15*) have been studied collectively called fecundity genes (Fec) that genetically regulated the ovulation rate (OR) and litter size (LZ) of the domesticated goat (*Capra hircus*). Bone Morphogenic Protein 15 (*BMP15*) is also known as GDF9B/FecX is a member of transforming growth factor β (TGFβ) superfamily, contains more than 35 proteins have been found have a crucial task in both growth as well as differentiation (Juengel *et al.,* 2004). A number of mutations in fecundity genes have been found increased fecundity in different breeds of sheep, namely, Belclare ($FecX^B$) Hanrahan *et al.,* 2004; Inverdale ($FecX^I$) Galloway *et al.,* 2000; Galway ($FecX^G$) Hanrahan *et al.,* 2004; Hanna ($FecX^H$) Galloway *et al.,* 2000; Lacaune ($FecX^L$) Bodin *et al.,* 2003 and in Rasa Aragonesa sheep ($FecX^R$) there is a 17 bp mutation in the functional gene (Monteagudo *et al.,* 2009). It was accomplished that all these mutations were significantly associated with ovulation rate in different sheep breeds. It has been illustrated that heterozygosity in *BMP15* gene increase ovulation rate (OR) and litter size while the animals homozygous for this gene are infertile (Hanrahan *et al.,* 2004). *BMP15* gene is X-linked expresses in oocytes involved in regulation of granulose cell proliferation and differentiation by promoting granulose cell mitosis, suppressing follicle stimulating hormone receptor expression and engaged in the stimulation of kit ligand expression, All of which contributing a significant role in female fertility in mammals (Juengel *et al.,* 2002; Moore and Shimasaki, 2005). Currently the hypothesis based on the non-covalently bond heterodimers and homodimers of the *BMP15* proteins regulated the fertility in small ruminants (Hanrahan *et al.,* 2004).

Pakistan is the third largest goat producing country in the world after China and India. At present, there are 61.5 million goats in Pakistan and their population is increasing at the rate of more than 3% per annum. The importance of small ruminants in general and high prolific animals in particular, is greatly increased in Pakistan due to ever increase in the population growth rate during the last decade. Low production and high demand have double the price of mutton in the last 7 years. Due to bird flu, there is severe crisis in poultry industry and the entire load is shifted to red meat. As a result the price of mutton went out of reach of poor or middle class community. In order to decrease the gap of demand and supply, there is dire need to work on small ruminants particularly in identifying genes responsible for more birth per conception and also in life time of the animal. Such kinds of studies have produced good results through better production of prolific breeds. It is an established fact that an animal producing twins or triplet contributes more than 1.5 times toward meat than the animals producing single offspring per lambing. Among the indigenous goat breeds, Teddy goats are more prolific than other breeds of goats found in Pakistan. According to (Anonymous, 1996) twining percentage in goat breeds is 56.4, 26.5, 27.5 and 22.5 in Taddy, Beetal, Nachi and Dera Din Pannah respectively. Due to low heritability of litter size attempts to increase litter size by selection within a breed results in slow progress (Morris, 1990). Therefore, the detection of major genes which have great effects on ovulation rate and litter size has generated substantial interest among small ruminants producing breeders and scientists.

Markers that appreciably contribute to the variance of trait expression in livestock have been increasingly a focus in the field of livestock genetics. If such markers can be identified in Pakistani goat breeds, identification and planned breeding of high prolific animals will result in fast vertical expansion of small ruminants. There is comparatively less scientific knowledge available on small ruminants in this regard, and not a single study is done in Pakistan. Therefore the present research work is proposed to study the fecundity gene Bone Morphogenetic Protein 15 (*BMP15*). Results of this will help to increase the mutton production and uplift the socio economic condition of small ruminant's farmers in the country.

Material and Methods

Experimental animals

The present study was conducted on two goat breeds Teddy and Beetal of Pakistan. A total of 90 ewes comprised of 45 ewes of Teddy with history of twinning and 45 ewes of Beetal with history of single birth from different Government Livestock farms i.e. Small Ruminants Research Institute Patoki, Punjab and Livestock Experimental Station Chak Katora Hasil pur, Punjab and their respective home tracts.

Blood collection and DNA isolation

Ten ml blood sample was collected aseptically from jugular vein of selected goat into 50 ml falcon tubes containing 200 ul anticoagulants i.e. Ethylene diamine tetra-acetic acid (0.5 M EDTA). DNA was extracted by using the standard protocol of Sambrook and Russel, 2001 by phenol-chloroform extraction procedure.

Primer designing

Twenty nine specific primer for the amplification of BMP 15 gene primers were designed using software Primer3 (Steve and Skaletsky, 2000) (Table 1) and Insilico PCR of UCSC Genome browser web facility using the already reported sequence of BMP 15 gene of Capra hericus (GeneBank Accession# EU743938.1) available at NCBI.

PCR amplification

Polymerase chain reactions were carried out in a 25 uL reaction mixture containing approximately 2.5 uL of 10× PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100], 2.5 mM of MgCl2, 200 uM of each dNTP, 2 uM of each primer, 50 ng of ovine genomic DNA, and 1Uof Taq DNA polymerase (Farmantos). The amplification conditions for primers of the BMP15 gene were as follows: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s; with a final extension at 72°C for 10 min, on a BioRad Thermocycler.

Statistical analysis

The amplified regions were sequenced using an ABI 3130 genetic analyzer (Applied Biosystem, USA). The sequences were blast against reference sequence by using BioEdit 7.0.9.0 (Hall, 1999). DnaSP 5.1 software was used for sequence variation sites (Librado and Rozas, 2009) and MEGA 5 program for phylogenetic analysis using Neighbor-joining method (Kumar *et al.*, 2011). SNPs associations with fecundity were performed using the SNPator package (http://www. snpator.org). Nucleotide sequences of BMP15 gene from Teddy and Beetal have been submitted to the GenBank database libraries and given the accession number of JN655669 - JN655670 respectively.

Result and Discussion

The Teddy and Beetal females have multiple and single birth respectively were screened for Bone Morphogenic Protein 15 (BMP15) mutations in the present study. The whole gene was sequenced containing complete exon 1 (328bp) and exon 2 (857bp). Out of six mutations a total of four mutations in Teddy were discovered in exon 2 at nucleotide No. 6280 with T>G, 6353 with G>A, 6443 with T>C and 6492 with A>G (Figure 2). When BMP15 gene sequences of Teddy goat are compared with Jining Grey goat (EU743938) one transversion and five transition mutations were identified. Two transition mutations observed in intron at position T982C and A5572G. One synonymous (T6280G) and three non-synonymous (G6353A, T6443C and A6492G) were identified which corresponded the amino acid changes Glycine301Serine, Tyrosine331Histidine and Aspragine347Serine respectively. All the nucleotide changes were seen novel in exon 2 of BMP15 gene in Teddy goat in the current study, which were different from reported in previous studies. BLAST analysis comparison of coding sequences of Teddy with Beetal and Jining Grey goat showed 99% homology.

There are five point mutations have been identified in sheep e.g. $FecX^H$ in Hanna (Galloway *et al.,* 2000), $FecX^I$ in Inverdale (Galloway *et al.,* 2000), $FecX^G$ in Galway (Hanrahan et al. 2004), $FecX^L$ in Lacaune (Bodin *et al.,* 2003) and $FecX^B$ in Belclare (Hanrahan *et al.,* 2004), all corresponding a foremost effect on ovulation rate. The ovulation rate would be boost up among heterozygous ewes for any of these mutations while the homozygous ewes are infertile due to malfunction of normal ovarian follicular development (Bodin *et al.,* 2003 and Hanrahan *et al.,* 2004). In the recent few years, an incredibly immense concentration has been given to studying the candidate genes of fecundity in goat. Exon two of BMP15 gene in goats showed several polymorphic sites other than those reported in sheep have been published in numerous studies. Markhoz goat showed three polymorphisms at exon 1, C200T and two at exon 2, G573A and T755G (Ghoreishi *et al.,* 2011). Further A963G mutation was found in exon 2 of BMP15 gene in Jining Grey goats has three genotypes (AA, AG and GG), Mongolia Cashmere, Angora and Liaoning Cashmere were observed to have only AA genotype while two genotypes AG and GG found in Boar goats (Feng *et al.,* 2009). Jiao *et al.,* 2007) further reported two polymorphic sites (A963G and C1050G) in exon 2 of BMP15 gene. Wang *et al.,* (2011) implemented PCR-SSCP and DNA sequencing in two local Chinese goats Funiu white goat and Taihang black goat and found three genotypes (AA, BB and AB) and two genotypes (AB and BB) were detected respectively. The result showed four mutations (T456G, C466G, C510T and T511C) in genotypes BB of Funiu white goats and no similar mutation was observed in Taihang black goat. Seven substitutions were identified in mature peptide sequence of BMP15 gene in white goat population of China (Ran *et al.,* 2009).

All these studies showed that the BMP15 gene has been found a major gene that influences the fecundity in goats. To determine the molecular marker concern with fecundity the samples selection was based on the single birth (Beetal) and multiple births (Teddy). This type of selection of animals provides a unique research material to identify molecular markers associated with fertility. The mean litter size was observed 2.87 and 1.13 in Teddy and Beetal respectively. In the present study high fecundity breed, Teddy was not associated with any point mutations in BMP15 gene with low fecundity breed Beetal. This materialize to harmonize with the previous views concerning with goats that low fecundity exhibited no correlation with point mutations in this gene, whilst high fecundity goats may be linked the mutations.

Teddy and Beetal also compared with each other and with other animals through deduced amino acids sequences of BMP15 gene (Figure 1). It is interesting that the amino acids substitutions occurs in Teddy are corresponding with sheep (AAF81688). Differentiation was detected between Teddy and Beetal at all positions. After comparison high frequency of deletion mutations was observed in BMP15 gene in Chicken. One to fifteen amino acids deletion mutations were found in chicken of BMP15 mature peptide. These finding revealed the ovulation in BMP15 gene existed in all observed mammals and demonstrated the divergence in the regulation of fecundity in vertebrates.

The complete coding sequence of BMP15 gene of Teddy (AEX09409) and Beetal (AEX09410) was compared with other published sequences of BMP15 gene of Sheep (AAF81688), Cattle (AAS99651), Buffalo (ABN05299), Yak (ELR54422), Human (EAW89914), Mice (NP033887) and Chicken (AAU94351) (Figure 3). Both goat breeds (Teddy and Beetal) constituted a branch in the phylogenetic tree. As expected corresponding to evolutionary point of view, in the phylogenetic tree goats are closely related to sheep to form a common cluster. The chicken is far most from all other published sequences due to high frequency of deletion mutations in the polypeptide chain. The sequences of same region was compared, it was observed that the BMP15 gene sequence similarity between both goat breeds and with sheep was more than 99.9%, where it showed approximately 97% and 93% of similarities with sequence of Human/Mice and Chicken respectively (Table 2).

Table 1: Oligonucleotide primers used for the amplification of specific loci of BMP15 gene

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Teddy    MVLLSILRILLLWGLVLFMEHRVQMTQVGQPSIAHLPEAPTLPLIQELLEEAPGKQQRK-PRVLGHPSRYMLELYQRSAD  79
Beetal   MVLLSILRILLLWGLVLFMEHRVQMTQVGQPSIAHLPEAPTLPLIQELLEEAPGKQQRK-PRVLGHPSRYMLELYQRSAD  79
Sheep    MVLLSILRILL-WGLVLFMEHRVQMTQVGQPSIAHLPEAPTLPLIQELLEEAPGKQQRK-PRVLGHPLRYMLELYQRSAD  78
Cattle   MVLLSILRILLLWGLVLFMEHRVQMTQVGQPSIAHLPEAPTLPLIQELLEEAPGKQQRK-PRILGHPLRYMLELYQRSAD  79
Buffalo  MVLLSILRILLLWGLVLFMEHRVQMTPVGQPSIAHLPEAPTLPLIQELLEEAPGKLQRK-PRVLGHPLRYMLELYHRSAD  79
Yak      MVLLSILRILLLWGLVLFMEHRVQMTQVGQPSIAHLPEAPTLPLIQELLEEAPGKQQRK-PRILGHPLRYMLELYQRSAD  79
Mice     MALLTILRILL-WGVVLFMEQRVQMAKPGWPSTALLADDPTLPSILDLAKEAPGKEMKQwPQ--GYPLRYMLKLYHRSAD  77
Human    MVLLSILRILFLCELVLFMEHRAQMAEGGQSSIALLAEAPTLPLIEELLEESPGEQPRK-PRLLGHSLRYMLELYRRSAD  79
Chicken  MALLRPFTALLLLTVLLSWA---------------ASQTPPLPLLQALRAQAPGSQGWRgGAASGQPLRYMLELYQRAAD  65

Teddy    ASGHPRENRTIGATMVRLVRPLASVARPLRGSWHIQTLDFPLRPNRVAYQLVRATVVYRHQLHLTHSHLSCHVEPWGQKS  159
Beetal   ASGHPRENRTIGATMVRLVRPLASVARPLRGSWHIQTLDFPLRPNRVAYQLVRATVVYRHQLHLTHSHLSCHVEPWGQKS  159
Sheep    ASGHPRENRTIGATMVRLVRPLASVARPLRGSWHIQTLDFPLRPNRVAYQLVRATVVYRHQLHLTHSHLSCHVEPWVQKS  158
Cattle   ASGHPRENRTIGATMVRLVRPLASVARPLRGSWHIQTLDFPLRPNRVAYQLVRATVVYRHQLHLTHSHLSCHVEPWVQKS  159
Buffalo  ASGHPRENRTIGATMVRLVRPLASVARPLRGSWHIQTLDFPLRPNRVAYQLVRATVVYRHQLHLTHSHLSCHVEPWVQKS  159
Yak      ASGHPRENRTVGATMVRLVRPLASVARPLRGSWHIQTLDFPLRPNRVAYQLVRATVVYRHQLHLTHSHLSCHVEPWVQKS  159
Mice     PHGHPRENRTIGAKMVRLVKPSANTVRPPRGSWHVQTLDFPLASNQVAYELIRATVVYRHQLHLVNYHLSCHVETWVPKC  157
Human    SHGHPRENRTIGATMVRLVKPLTNVARPHRGTWHIQILGFPLRPNRGLYQLVRATVVYRHHLQLTRFNLSCHVEPWVQKN  159
Chicken  HEGRPRRGRSLSTNTVRLVQAASHGGQPWAGRWYVQPLTYRLDAQSEAEHLLRVTVAYPQSLPLPRGRLLCAVE----LP  141

Teddy    PTNHFPSSGRGSPKPSLLPKTWTEMDIMEHVGQKLWNHKGRRVLRLRFVCQQPRGSEVLEFWWHGTSSLDTVFLLLYFND  239
Beetal   PTNHFPSSGRGSPKPSLLPKTWTEMDIMEHVGQKLWNHKGRRVLRLRFVCQQPRGSEVLEFWWHGTSSLDTVFLLLYFND  239
Sheep    PTNHFPSSGRGSSKPSLLPKTWTEMDIMEHVGQKLWNHKGRRVLRLRFVCQQPRGSEVLEFWWHGTSSLDTVFLLLYFND  238
Cattle   PTNHFPSSGRGSSKPSLLPKAWTEMDIMEHVGQKLWNHKGRRVLRLRFVCQQPRGSEVREFWWHGTSSLDTVFLLLYFND  239
Buffalo  PTNHFPSSGRGSTKPSLSPKAWTEMDIMEHVGRKLWNHKGRRVLRLRFVCQQPTGSEVREFWWHGTSSLDTGFLLLYFND  239
Yak      PTNHFPSSGRGSSKPSLLPKAWTEMDIMEHVGQKLWNHKGRRVLRLRFVCQQPRGSEVREFWWHGTSSLDTVFLLLYFND  239
Mice     RTKHLPSSKSGSSKPSPMSKAWTEIDITHCIQQKLWNRKGRSVLRLRFMCQQQKGNETREFRWHGMTSLDVAFLLLYFND  237
Human    PTNHFPSSEGDSSKPSLMSNAWKEMDITQLVQQRFWNNKGHRILRLRFMCQQQKDSGGLEL-WHGTSSLDIAFLLLYFND  238
Chicken  PAAKAPAVLLSPTAPS--RHGWAEADITPYLSPA--NSSSGGTLTLRHICVRSGRAATA-----APPSPADPFLLLFLND  212

Teddy    T-QSVQKTKPLPKGLKEFTEKDPSLLLRRARQAGSIASEVPGPSREHDGPESNQCSLHPFQVSFQQLGWDHWIIAPHLYT  318
Beetal   T-QSVQKTKPLPKGLKEFTEKDPSLLLRRARQAGSIASEVPGPSREHDGPESNQCSLHPFQVGFQQLGWDHWIIAPHLYT  318
Sheep    T-QSVQKTKPLPKGLKEFTEKDPSLLLRRARQAGSIASEVPGPSREHDGPESNQCSLHPFQVSFQQLGWDHWIIAPHLYT  317
Cattle   T-QSVQKTKPLPKGLKEFTEKDPSLLLRRARQAGSIASEVPGPSREHDGPESNLCSLHPFQVSFQQLGWDHWIIAPHLYT  318
Buffalo  T-QSVQKTKPLPRGLKEFTEKDPSLLLRRARQAGSIASEVPGPSREHDGPESNQCSLHPFQVSFQQLGWDHWIIAPHLYT  318
Yak      T-QSVQKTKPLPKGLKEFTEKDPSLLLRRARQAGSIASEVPGPSREHDGPESNLCSLHPFQVSFQQLGWDHWIIAPHLYT  318
Mice     T-DDRVQGKLLARGQEELTDRESSFLMRSVRQACSIESDASCPSQEHDGSVNNQCSLHPYKVSFHQLGWDHWIIAPRLYT  316
Human    ThKSIRKAKFLPRGMEEFMERES--LLRRTRQADGISAEVTASSSKHSGPENNQCSLHPFQISFRQLGWDHWIIAPPFYT  316
Chicken  T-----RSGTLPEPR-----------RSRREAGTLLHDLPGYLRDAGGDKS-DCSLRSFPVSFAQLGWDHWIIAPHRYN  274

Teddy    PNYCKGVCPRVLHYGLNSPNHAIIQNLVSELVDQNVPQPSCVPYKYVPISILLIEANGSILYKEYEGMIAQSCTCR  394
Beetal   PNYCKGVCPRVLYYGLNSPNHAIIQNLVNELVDQNVPQPSCVPYKYVPISILLIEANGSILYKEYEGMIAQSCTCR  394
Sheep    PNYCKGVCPRVLHYGLNSPNHAIIQNLVSELVDQNVPQPSCVPYKYVPISILLIEANGSILYKEYEGMIAQSCTCR  393
Cattle   PNYCKGVCPRVLHYGLNSPNHAIIQNLVNELVDQSVPQPSCVPYKYVPISILLIEANGSILYKEYEGMIAQSCTCR  394
Buffalo  PNYCKGVCPRVLHYGLNSPNHAIIQNLVNELVDQSVPQPSCVPYKYVPISILLIEANGSILYKEYEGMIAQSCTCR  394
Yak      PNYCKGVCPRVLHYGLNSPNHAIIQNLVNELVDQSVPQPSCVPYKYVPISILLIEANGSILYKEYEGMIAQSCTCR  394
Mice     PNYCKGICTRVLFYGLNSPNHAIIQSLVNELVNHSVPQPSCVPYNFLPMSILLIETNGSILYKEYEGMIAQSCTCR  392
Human    PNYCKGTCLRVLFDGLNSPNHAIIQNLINQLVDQSVPRPSCVPYKYVPISVLMIEANGSILYKEYEGMIAESCTCR  392
Chicken  PRYCKGVCPRLLFDGYHAPNHAVVQNLVHQLVDANVPRPSCVPYRYSPISVLMIQHDGSILYKEYENMIAESCTCR  350
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Figure 3: Phylogenetic analysis on the basis of complete coding sequence of BMP15 gene using Neighbor-joining method, bootstrap consensus inferred from 1000 replicates.

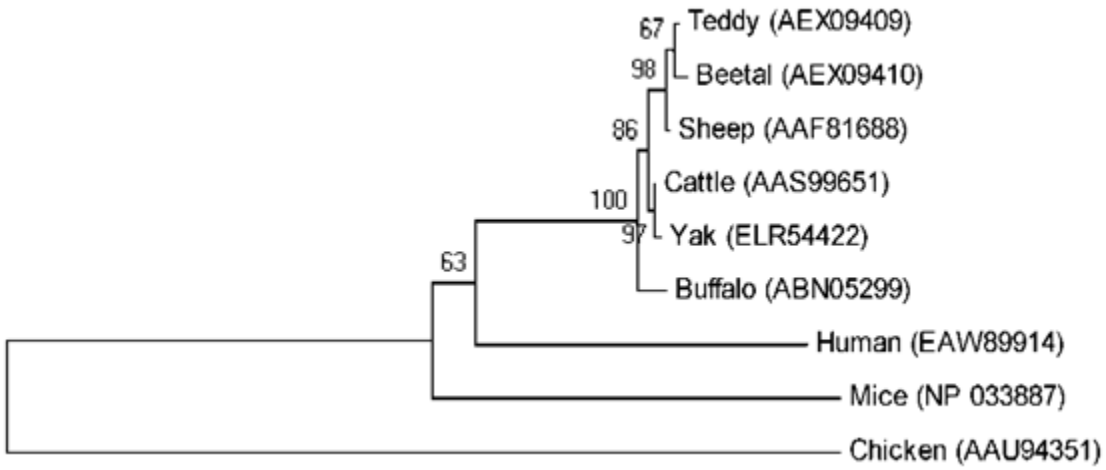
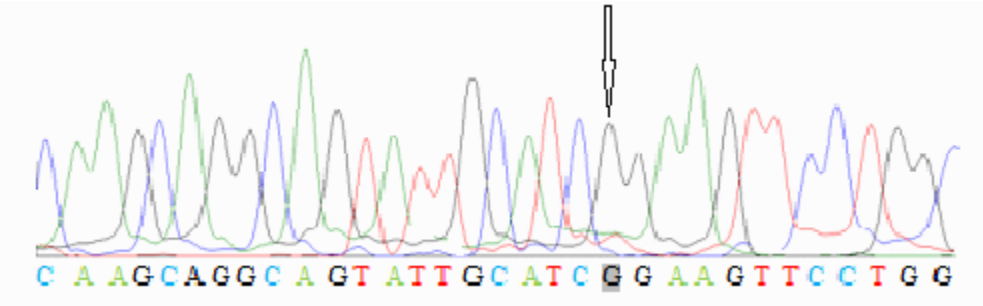
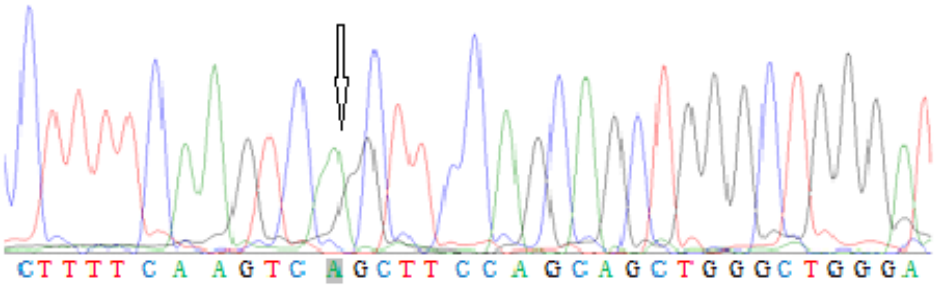


Table 2: Percentage of divergence of BMP15 gene of Teddy (AEX09409) and Beetal (AEX09410) was compared with other published sequences of BMP15 gene of Sheep (AAF81688), Cattle (AAS99651), Buffalo (ABN05299), Yak (ELR54422), Human (EAW89914), Mice (NP033887) and Chicken (AAU94351)

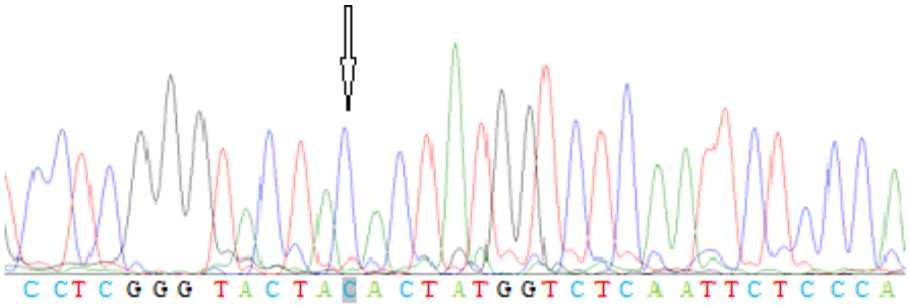
	Teddy	Beetal	Sheep	Cattle	Buffalo	Yak	Human	Mice	Chicken
Teddy									
Beetal	0.009								
Sheep	0.006	0.015							
Cattle	0.020	0.023	0.015						
Buffalo	0.035	0.038	0.032	0.023					
Yak	0.023	0.026	0.017	0.003	0.026				
Human	0.337	0.337	0.329	0.313	0.313	0.317			
Mice	0.274	0.274	0.267	0.259	0.267	0.263	0.421		
Chicken	0.765	0.771	0.759	0.765	0.771	0.765	0.849	0.809	



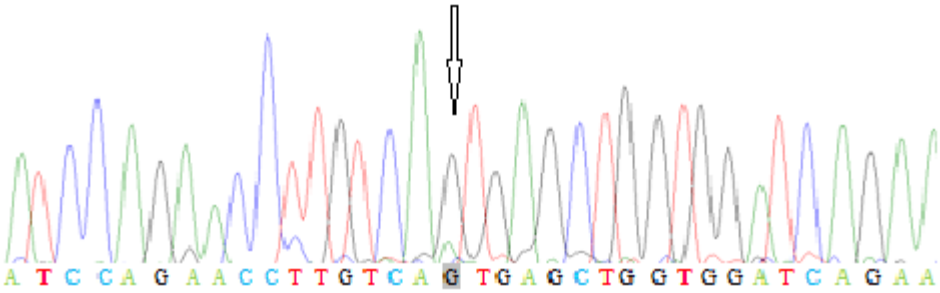
A: 6280(T>G)



B: 6353(G>A)



C: 6443(T>C)



D: 6492(A>G)

Figure 2: Mutations in exon 2 of BMP15 gene A: 6280(T>G); B: 6353(G>A); C: 6443(T>C) and D: 6492(A>G)

Conclusion

Two indigenous goat breeds (Teddy and Beetal) differing in prolificacy were used for direct DNA sequence analysis to find genetic variations in BMP15 gene. Although the sequence alignment showed that the mutations in the candidate gene associate with fecundity in sheep were not identified in Pakistani Teddy and Beetal goat breeds, however polymorphisms that may be potentially meaningful were found in exon 2 of BMP15 gene in Teddy goat breed. These SNPs may be further evaluated and selected as markers of fecundity in goat breeds in future.

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