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Research Article

Study of *FecX*^{G} Polymorphism in Beetal Goat and Its Phylogenetic Relationship

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Abstract

The objective of the current study was to detect the incidence of *FecX*^{G} mutation in 141bp fragment of exon 2 of BMP-15 gene in Beetal goat and to determine its phylogenetic relationship with other goat breeds. In this study 60 Beetal goats were randomly selected and divided equally into two groups on the basis of their body weights. The PCR-RFLP and DNA sequencing techniques were employed to explore polymorphism in exon 2 of BMP-15 gene. Upon digestion with Hinf1, the purified product showed heterozygous carriers with two bands (111bp, and 54bp) in all animals. The analysis of polymorphism for *FecX*^{G} in Beetal goat indicates that the genetic factor responsible for difference in growth rates is not related to the reported mutated alleles of BMP-15 gene. Therefore, it is suggested that polymorphisms in the other regions of BMP-15 gene should be explored which might be responsible for the difference of growth rates in Beetal goat. Phylogenetic tree of mRNA of BMP-15 gene was constructed and the results revealed Teddy as outgroup. Moreover, among all the breeds used in this analysis Teddy is also phenotypically different from other due to its smaller size and greater prolificacy. However, all the remaining breeds were present in a single large clade, with Black Bengal and Markhoz goat in a further single sub-clade and showing maximum divergence from the common ancestor. The Beetal was next to Black Bengal and Markhoz and along with their common ancestor they were present in a single clade.

Keywords: *FecX*^{G} *polymorphism, BMP-15 gene, Beetal goat, PCR-RFLP, Phylogenetic relationship*

Beetal Keçisinde *FecX*^{G} Polimorfizmi ve Filogenetik İlişkisinin İncelenmesi

Öz

Bu çalışmanın amacı Beetal keçisinde BMP-15 geninin ekzon 2'sinde 141 bp fragmanında *FecX*^{G} mutasyonunun insidansını ve diğer keçilerle olan filogenetik ilişkisini belirlemektir. Çalışmada 60 Beetal keçisi rastgele seçildi ve vücut ağırlıklarına göre eşit iki gruba ayrıldı. BMP-15 geninin ekzon 2'sindeki polimorfizmi araştırmak amacıyla PCR-RFLP ve DNA sekanslama teknikleri kullanıldı. Tüm hayvanlarda, Hinf1 ile kesilme sonrasında elde edilen saf ürün iki bantlı (111bp ve 54bp) heterozigot taşıyıcılar olarak gözlemlendi. Beetal keçilerinde *FecX*^{G} polimorfizm analizi, büyüme oranlarındaki farkın oluşmasından sorumlu olan genetik faktörlerin BMP-15 geninin mutasyonlu alleli ile ilgili olmadığını gösterdi. Bu nedenle, BMP-15 geninin diğer bölgelerindeki polimorfizmin, Beetal keçilerinde büyüme oranlarındaki farklılığının oluşmasından sorumlu olabileceği düşüncesi ile araştırılması önerilmiştir. BMP-15 geninin mRNA'sının filogenetik ağacı oluşturuldu ve elde edilen sonuçlar Teddy'nin dışgrup olduğunu ortaya koymuştur. Ayrıca, bu çalışmada kullanılan tüm ırklar arasında, Teddy daha küçük boyutu ve daha fazla üretkenliği nedeniyle diğer ırklardan fenotipik olarak farklıydı. Ancak, geri kalan tüm ırklar tek bir büyük kuşakta bulunurken Siyah Bengal ve Markhoz keçisi başka bir alt kuşakta yer aldı ve ortak atadan azami sapma gösterdiler. Beetal, Siyah Bengal ve Markhozdan sonra ortak ataları ile birlikte tek kuşakta yer almaktadır.

Anahtar sözcükler: *FecX*^{G} *poliformizm, BMP-15 geni, Beetal keçisi, PCR-RFLP, Filogenetik akrabalık*

INTRODUCTION

Goats are vital indigenous assets of Pakistan and have an ample share in the annual production of milk and meat besides providing enough income to rural families [1]. Beetal is one of the most famous goat breed of Punjab province of Pakistan; moreover, it is also present in some parts of Indian (East) Punjab. It is very popular due to its greater body size and higher potential for meat and milk yield especially in rural areas of Punjab province [2]. However, information regarding the genetic potential of Beetal for meat production is scarce which merit some well-

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planned genetic and genomic studies. There is substantial need to explore the candidate genes for growth traits in this goat breed. Bone morphogenetic protein-15 (BMP-15), Growth hormone (GH), and Insulin like growth factor-1 (IGF-1) genes are reported as candidate genes for growth and skeletal development in goat [3-5].

The BMP family of proteins is the largest subgroup of the transforming growth factor-ß (TGF-ß) superfamily [3] and act as a major molecule in tissue and organ development [5,6]. It plays a vital role in the functioning of connective tissue, brain, kidney, and muscle [7]. The BMP-15 is the one of the crucial members of BMP family which is famous for affecting reproductive [4,8,9], and growth traits [10,11] in small ruminants. It is reported to enhance proliferation and differentiation of cells by promoting mitosis and controlling gene expression [12,13]. Some mutations in the BMP-15 gene have been reported to be associated with the ovulation rate in different sheep breeds, especially the *FecX^B* mutation in Booroola breed and *FecX^G* in Inverdale breed [7,14-16]. The present study is based on the hypothesis that *FecX^G* polymorphism in BMP-15 gene might be associated with difference in growth rate viz body weight and body measurements in Beetal goat. Therefore, primarily the study was aimed to identify polymorphism at the *FecX^G* loci in BMP-15 gene in the Beetal goat using PCR-Restriction Fragment Length Polymorphism (RFLP) method. Moreover, the secondary objective was the determination of relationship of this precious genetic resource of Pakistan with other goat breeds of the region (South Asia) through phylogenetic analysis.

MATERIAL and METHODS

Ethics Statement

Ethical permission (number DR/151) was obtained from the Ethical review committee for animal research of University of Veterinary and Animal Sciences, Lahore, Pakistan, before start of the study.

Experimental Population

In total, 60 healthy unrelated female Beetal goats of about one year of age were randomly selected from Small Ruminant Training and Research Centre, University of Veterinary and Animal Sciences, Lahore, Pakistan. Animals were equally divided into two groups on the basis of difference in their body weight and body measurements i.e. 30 in higher body weight group and 30 in lower weight group. The body weight of higher weight category animal ranged from 55-60 kg while low weight category animals were having weight of 40-45 kg. All animal were stall fed and additionally one kg concentrate was also offered to each animal on daily basis. The animals were reared in the sub-tropical climatic condition of central Punjab of Pakistan. Phenotypic data for different morphometric traits including heart girth, body height, body length, and body weight were recorded.

DNA Extraction

Blood samples with a volume of 5 mL were taken from jugular vein of each goat by using sterile 5ml syringe having a 22 guage needle fixed to it. The blood sample was immediately poured, after removing the needle, into 50 mL falcon tubes containing 160 µL of 0.5 M EDTA. 100 µL of each blood sample were transferred into Eppendorf tubes. 1000 µL TEB buffer (Tris HCL 10 mM, EDTA 2 mM) was added and samples were vortexed for 5 min and then centrifuged at 1000 rpm for 5 min. A pellet was made at the bottom of eppendorf. After discarding the remaining solution, 20 µL proteinase K enzyme was added along with 60 µL SDS (10%) solution and 100 µL of TNE buffer (Tris HCL 10 mM, NaCL 400 mM, EDTA 2 mM). Samples were vortexed for 5 min and placed in water bath at 58°C for overnight digestion. In fully digested samples, PCL (Phenol 25: Choloroform 24: Iso-amyle 1) was added. Three layers were formed after centrifugation at 1000 rpm for 10 min. Samples were washed with 70% ethanol and again centrifuged at 1000 rpm for 10 min. A solid pellet of DNA was formed and dissolved in 100 µL double distil water. DNA was stored at 4°C or -20°C. The concentration of DNA was measure in Nanodrop spectrophotometer (ThermoFisher) instrument.

PCR Amplification

Polymerase chain reaction (PCR) was carried out in a final reaction volume of 20 µL in C1000 Touch™ Thermal Cycler (BIO-RAD, USA). The primers for the amplification of exon 2 of BMP-15 are given in *Table 1*. The reaction mixture consisted of 150 µM dNTPs, 1.2 mM $MgCl_2$, 2.0 mM 10 × buffer, 30 ng each forward and reverse primer and 1 Unit of Taq DNA polymerase. The PCR reaction cycle protocol was 5 min at 95°C; 30 cycles at 94°C for 45 s, annealing at 60°C for 30 s, extension at 72°C for 45 s. The PCR products were visualized in Gel Doc™ EZ imager (BIO-RAD, USA) following electrophoresis through 2% agarose gel.

Table 1. *Primers and PCR amplification parameters used to amplify BMP-15 gene*

Gene	Sequence	[1]Tm (°C)	Size of Amplicon (bp)	Reference
BMP-15E2F	CACTGTCTTCTTGTTACTGTATTTCAATGAGAC	68.17	141	[17]
BMP-15E2R	GATGCAATACTGCCTGCTTG	62.41		

E2: Exon 2; F: Forward Primer; R: Reverse Primer; [1]Tm Melting temperature (°C)

PCR-RFLP Analysis

The digestion of PCR products was done with restriction enzyme HinfI at 37°C for 12 h. After digestion, the samples were stored at 4°C. The digested product was visualized in Gel Doc™ EZ imager (BIO-RAD, USA) following electrophoresis through 2% agarose gel.

Sequencing

The amplified region was sequenced using an ABI 3130 Automated DNA Sequencer (Applied Biosystems, USA). Sequence data were aligned using CLUSTALW algorithm in MEGA 7 software [18].

Phylogenetic Analysis

The mRNA (1184bp) sequences of BMP15 gene of seven different goat breeds including Beetal goat, Black Bengal goat, Markhoz goat, Tibetan goat, Lezhi black goat, Ganjam goat, and Teddy goat were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/) and used to construct phylogenic tree in Mega7 software [18]. The accession numbers of these goat breeds are given in *Fig. 5*. The phylogenetic tree was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [19]. The tree was made by using Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 7 nucleotide sequences.

RESULTS

A 141bp amplified DNA fragment was subjected to 2% agarose gel electrophoresis. The results showed that amplified fragment size was consistent with the expected size as determined from the gene sequence information *(Fig. 1)*.

The amplicons showed a 141bp product in the present study *(Fig. 1)*. The RFLP analysis revealed two bands. One band was appeared at 111bp and other band was at 54bp. All the tested Beetal goats were found carriers of *FecX*^{G} mutation as the restriction site for Hinf1 was observed in all the samples *(Fig. 2)*. The restriction site of Hinf1 enzyme is showed in *Fig. 3* from the chromatogram produced for Beetal goat in the current study and same mutation is shown in the sequences of Beetal goat in *Fig. 4*.

The 1184bp long mRNA of BMP15 gene of some Chinese, Indian, Iranian, and Pakistani goat breeds including Beetal, Black Bengal, Markhoz, Tibetan, Lezhi black, Ganjam, and Teddy goat were retrieved from NCBI to construct phylogenic tree. The phylogenetic tree showed Teddy goat as outgroup and its sequence was different from other goat breeds when clustered at 1*100 nucleotide substitution level *(Fig. 5)*. However, all the remaining breeds were present in a single large clade, with Black Bengal and Markhoz goat in a further single sub-clade. The Beetal was next to Black Bengal and Markhoz and along with these two breeds and the common ancestor they were present in a single clade.

DISCUSSION

The present study was carried out to find the polymorphism of *FecX*^{G} of BMP-15 gene in Beetal goat by PCR-RFLP method and also by sequencing. From the study, it was found that all the genotype of Beetal goat showed two bands of 111bp and 54bp after digestion with Hinf1 enzyme. It's suggested that although digestion was done at the said locus indicating the presence of mutation but this mutation was observed in the all the tested animals. Out of the total 60 animals, tested in this study, half of them

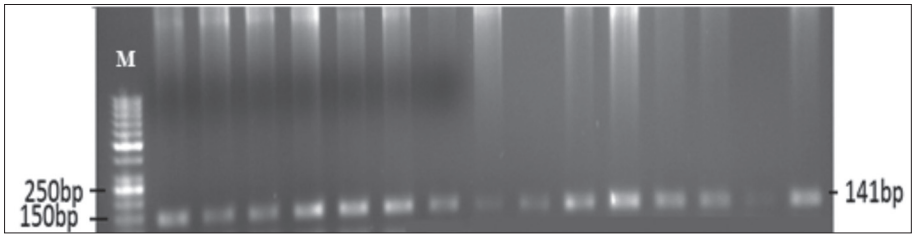
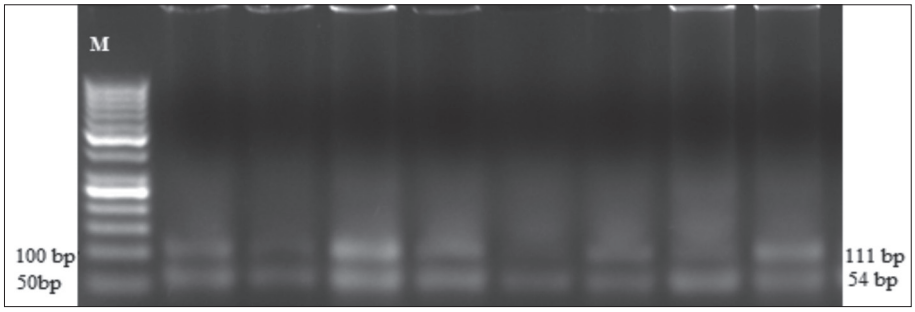


Fig 1. Amplified products of exon2 of BMP-15 gene by PCR. In Lane1 the letter 'M' represent the Ladder

Fig 2. Hinf1-RFLP patterns of BMP-15 gene. This pattern was observed in all tested animal. In Lane1 the letter 'M' represent the Ladder (50bp)



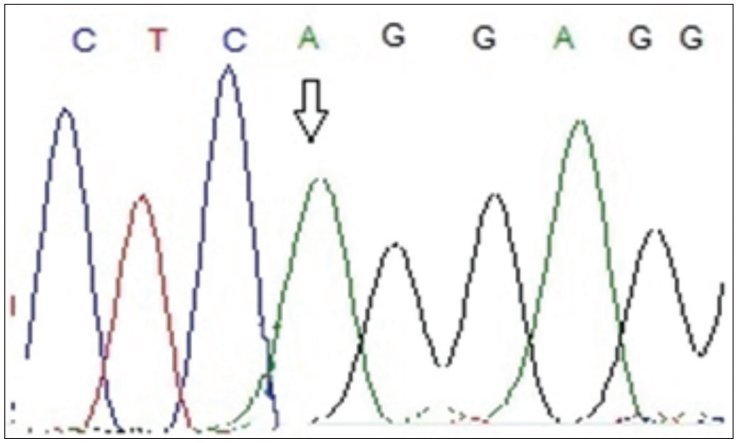


Fig 3. Showing the *FecX*^{G} mutation in the Beetal goat

no evidence of mutation in *FecX*^{G} in Iranian goats by using PCR-RFLP technique and all animals were monomorphic. Similarly, He, Chu [22] also observed absence of mutation in BMP-15 gene in six breeds of Chinese goats. However, contrarily mutations at five different points in exon 2 of BMP-15 gene were found associated with prolificacy and growth in some breeds of sheep [23]. This might be due to difference in the biological effect of the mutations among different species and it had also been suggested recently that difference in BMP-15 mutation may be associated with the differences in ovulation rate and growth rate among various species [24]. In current study, monomorphic nature of *FecX*^{G} suggests that current mutation in BMP-15 gene cannot be regarded as the major polymorphism

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                ....|....| ....|....| ....|....| ....|....| ....|....|
....|....|
B_H             TTTGTCTCGT CGAATGCAGT GAGGGTCTCA GGAGGGTAAC TCTTTCAGGC
CTTTAGGGAG
B_H              TTTGTCTCGT CGG-TGCAGT GAGGGTCTCA GGAGGGTAAC TCTTTCAGGC
CTTTAGGGAG
B_H              CTGGTATCAT CG-GTGCAGT GAGGGTCTCA GGAGGGTAAC TCTTTCAGGC
CTTTAGGGAG
B_L              CTGCAATC-T CG-GTGCAGT GAGGGTCTCA GGAGGGTAAC TCTTTCAGGC
CTTTAGGGAG
B_L              GTTCAGTACG CGTGCGAACT ACACTACTCA GGAGGGTGCC TCTTTCAGGG
CTTTAGGGAG
B-L              GTTCAGTACG CGTGCGAACT ACACTACTCA GGAGGGTGCC TCTTTCAGGG
CTTTAGGGAG

               ....|....| ....|....| ....|....| ....|....| ....|....|
....|....|
B_H              AGGTTTGGTT TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
B_H              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
B_H              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
B_L              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
B_L              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTACCTCG
AAGACAGTGA
B_L              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
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Fig 4. Sequence of Beetal goat showing the mutation of *FecX*^{G} in all the tested Beetal goat (B). B_H are the high weight Beetal goat and B_L are the low weight Beetal goat

were of low body weight and other half was having higher body weight. The results showed that the variation in body weight of animals was not due to *FecX*^{G} mutation because *FecX*^{G} was observed in both heavy and low body weight animals. It is very likely that some other variants in BMP-15 or in some other candidate genes might be involved in affecting/controlling body weight in Beetal goat. In agreement with the findings of present study the Polley [20] found that all known point mutations in BMP-15 and GDF9 genes were monomorphic in the Black Bengal goat. Likewise, Deldar-Tajangookeh, Shahneh [21] observed associated with growth traits of goat. Further investigation should be directed at other loci of BMP-15 gene and also in some other genes by using larger sample size.

Phylogenetic tree of mRNA (1184bp long) of BMP-15 gene indicated the Teddy goat as outgroup from the other breeds *(Fig. 5)* of China, India, Iran, and Pakistan. The difference of the Teddy with other breeds was based on the seven nucleotides sequences, out of total 1184bp used to construct the phylogenetic tree. However, all the remaining six breeds including Black Bengal, Markhoz,

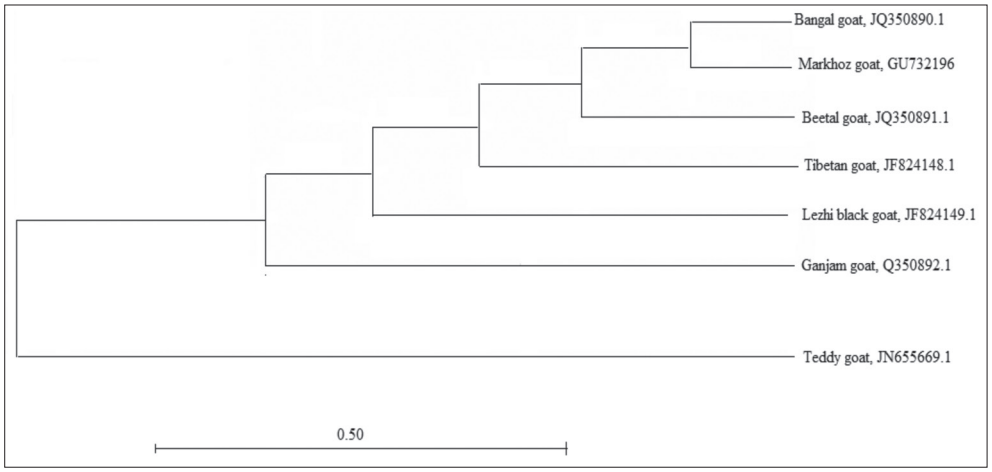

Fig 5. Neighbour-joining phylogenetic tree of mRNA of BMP-15 gene of different goat breeds

Beetal, Lezhi Black, and Ganjam goat breeds were grouped into a single mega clade. However, Black Bengal and Markhoz goat were clustered together in a single sub-clade and both of them showed the maximum divergence from the common ancestor followed by Beetal goat. The phylogenetic tree in this study was made on the basis of mRNA sequence of exonic regions of BMP15 gene which is a famous candidate gene for fecundity in small ruminants; therefore, it is likely that the difference shown among the breeds by the phylogenetic tree is the difference in the sequence of exons of BMP15 genes, in narrow sense, and in their prolificacy in broader sense. In agreement with the litter size in the six breeds present in a single larger clade is about 1.3 to 1.8 [25-27] whereas Teddy is the most prolific breed of Indian sub-continent and famous for producing triplet and quadruplet in addition to twins [28].

However, the Ganjam goat was closer to Teddy goat in the tree compared with other goat breeds. The reason for this might be that both share some similarities in their phenotypic appearance like coat color, medium size body height, shape of head and face, and bear etc. Moreover, in addition to these two breeds the other breeds in this study are mainly black coat color. Additionally both of them are meat-type breeds only whereas the remaining breeds are mainly used for dairy and hair/fiber (mohair) production. It is highly recommended that phylogenetic trees of these goat breeds should be developed in future studies by using larger genetic data sets (whole genome sequence) will better explain their relatedness and relationship with each other.

Based upon present finding, it may be concluded that polymorphism in *FecX*^{G} i.e genetic factor responsible for difference in growth rates is not related to the reported mutated alleles of BMP-15 gene in Beetal goat. Therefore, polymorphisms in the other regions of BMP-15 gene should be explored which might be responsible for the difference of growth rates in Beetal goat. Phylogenetic tree of mRNA of BMP-15 gene of different goat breeds revealed that all breeds goat were present in a single large clade, with Black Bengal and Markhoz goat in a further single sub-clade and showing maximum divergence from the common ancestor. The Beetal was next to Black Bengal and Markhoz and along with their common ancestor they were present in a single clade. Teddy goat act as outgroup and phenotypically different from other due to its smaller size and greater prolificacy.

Author Contribution

AB and IZ designed the study. MI and MS did do the Lab work, whereas AB and IZ did run all the analyses. Finally AB, and IZ drafted the manuscript and all authors read and approved the final draft.

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