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Detection of 19-bp indel of *PLAG1* gene and its effects on morphometric traits in indigenous and crossbred cattle of Bangladesh

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ABSTRACT

Stature and live weight are economically important traits in livestock species and polygenic in nature. Pleomorphic adenoma gene 1 (*PLAG1*) is a member of the *PLAG* family of zinc finger transcription factors which has a specific association with growth related traits in human and various livestock species including cattle. This study investigated the 19-bp indel (insertion/deletion) polymorphism of the *PLAG1* gene and its possible association with morphometric traits in indigenous and Holstein-Local (H×L) crossbred cattle of Bangladesh. A total of 160 blood samples were collected from the aforementioned cattle populations whereas data on morphometric traits were recorded only from H×L crossbreds. PCR amplification fragment harboring 19-bp indel detected three genotypes II (366 bp), ID (366/347 bp) and DD (347 bp). The II, ID and DD genotype frequencies were 0.00, 0.09 and 0.91 respectively in indigenous cattle populations and the corresponding frequencies were 0.14, 0.43 and 0.43, respectively in H×L crossbred populations. None of the investigated morphometric traits had a significant association with 19-bp indel genotypes. However, non-genetic factors such as herd, year and parity had significant effects only on hip height trait in crossbred cattle (P<0.001). In conclusion, the identified 19-bp indel polymorphism was temperate breed Holstein specific and was absent in indicine populations that could be utilized only for crossbred cattle of Bangladesh upon validation with large a number of samples.

Introduction

Cattle are the most trustworthy components of the crop-livestock mixed farming system of Bangladesh that governs a significant role in our economy. Nearly 24.50 million heads of cattle are distributed throughout the country (DLS, 2019), with an average growth rate of 0.37% over the last one decade (DLS, 2016). They have multipurpose use for providing milk, meat, organic fertilizer and fuel, and are strongly linked with the rural livelihoods (Hamid *et al.,* 2017). The cattle population is comprised of mainly various indigenous varieties along with the resultant crossbreds derived from crossing indigenous with various exotic breeds such as Holstein-Friesian (H×L), Sahiwal (SL) and recently introduced Jersey at a different level of genetic combinations. Among the indigenous cattle varieties Red Chittagong (RCC), Pabna (PC), North Bengal Grey (NBG) and Munshiganj (MC) perform

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better compared to the existing Non-descript Deshi (DES) under a low input production system (Bhuiyan *et al.,* 2021). The indigenous cattle have large phenotypic variation from individual to individual both for qualitative and quantitative traits. There are significant differences observed in the body height traits of the cattle population in our country (Hadiuzzaman *et al.,* 2010). Morphometric traits like body length, shoulder height, heart girth and hip height etc. are good indicators for growth performance in any livestock population including cattle. Genetic polymorphism has been reported for great influences on the improvement of cattle morphometric traits. Stature and body weight are both highly heritable, and large-scale genetic studies have identified chromosomal regions impacting these traits in cattle (Vargas *et al.,* 2000). Therefore, genetic selection would create an immediate opportunity to improve morphometric traits of economic importance. Polymorphisms of some gene families greatly enhance the growth traits of cattle where the pleomorphic adenoma gene (*PLAG*) family is noteworthy. *PLAG1* and *PLAGL2* belong to the *PLAG* gene family (Smith *et al.,* 2002). Earlier studies have manifested that the *PLAG* family proteins regulate the expression of many important genes in the body. *PLAG1* is consisted of four exons and the total length is 8534 bp which is located in chromosome 14 (GenBank Accession: NC_037341.1).

Single nucleotide polymorphisms (SNPs) located within or nearby the *PLAG1* gene were associated with economically important traits in cattle (Lee *et al.,* 2013; Zhong *et al.,* 2018). Moreover, studies showed that the *PLAG1* gene plays a regulatory role in adult human height traits (Gudbjartsson *et al.,* 2008) as well as potential roles in milk production, reproductive performance, muscle formation and body height of livestock species (Fink *et al.,* 2017). A 19-bp insertion/deletion (indel) polymorphism (rs523753416) was detected in the intron of *PLAG1* gene that had a significant association with several morphometric traits in Qinchuan cattle (Xu *et al.,* 2018). There were significant differences also observed between 15 bp indel variation and body weight, chest circumference and body height traits ($P<0.05$) in Shaanbei White Cashmere goat (Wei *et al.,* 2021). Until to date, there is no information available on *PLAG1* gene polymorphisms and their possible effects on the phenotypic performance traits (growth and morphometry) of Bangladeshi cattle populations. Considering the above stated scenario, the distribution of 19-bp indel polymorphism was investigated in indigenous and crossbred cattle of Bangladesh. Alongside, the possible association was evaluated between the identified indel and morphometric traits in H×L crossbred cattle aiming to develop an effective marker for growth traits of crossbred cattle in Bangladesh.

Materials and Methods

Blood sampling and DNA extraction

In total, 160 samples were collected from Government institutional herds, university managed herds, commercial dairy farms and from the farmers' herds of different geographic regions. In particular, sampling for indigenous cattle was performed from different areas of Mymensingh, Sirajganj, Naogaon and Rajshahi as well as from the indigenous herds that have been maintained at Bangladesh Livestock Research Institute (BLRI). Besides, the morphometric data and blood samples of crossbred cattle those having at least 1st parity records were collected from commercial dairy herds of Manikganj and Gazipur in 2020. DNA extraction was performed from the whole blood using the PrimePrep Genomic DNA Extraction kit, (Add Bio Inc, South Korea) according to the manufacturer's instructions. The amount and purity of extracted DNA were measured by a Nanodrop spectrophotometer (ND2000, Thermo Fisher Scientific) and preserved at -20°C for further use.

Primer selection and PCR amplification:

One pair of primers was selected for this study from the previous report of Zhou et al. (2019). Primer synthesis was performed by Macrogen, South Korea and is shown in Table 1. PCR amplification was carried out by Biometra T-Gradient thermocycler in a 16 µl reaction volume comprising 1x Buffer, 1.5mM dNTP, 10mM of each primer, 3 U of Tag polymerase, ~100 ng genomic DNA. The thermal cycles were as follows; 95°C for 10 min followed by 37 cycles of 95°C for 30 sec,

Table 1: Primers used in PCR amplification

Gene fragment	Primer name	Sequence of primer	Amplicon size (bp)
PLAG1	*PLAG1*-F	5'-AAAAGAGTCCGCGTTTACTGC-3'	347/366
	PLAG1-R	5'- CGATGAACTCTCCACCTGCG-3'	

Table 2: Genotypic and allelic frequencies of bovine *PLAG1* gene for 19-bp indel in indigenous and crossbred cattle of Bangladesh

Population*	N	Genotype frequency			Allelic frequency	
		II	ID	DD	I	D
RCC	22	0.00 (0)	0.05 (1)	0.95 (21)	0.02	0.98
MC	27	0.00 (0)	0.11 (3)	0.89 (24)	0.06	0.94
PC	29	0.00 (0)	0.10 (3)	0.90 (26)	0.05	0.95
SL	10	0.00 (0)	0.10 (1)	0.90 (09)	0.05	0.95
Pooled data (zebu)	88	0.00 (0)	0.09 (8)	0.91 (80)	0.05	0.95
H×L crossbred	72	0.14 (10)	0.43 (31)	0.43 (31)	0.35	0.65

**RCC, Red Chittagong cattle; MC, Munshiganj cattle; PC, Pabna cattle; SL, Sahiwal; HF, Holstein Friesian and L, Non-descript local cattle. Values of parentheses represent the number of samples belong to each genotype.*

Table 3: Association of 19-bp indel of *PLAG1* gene with morphometric traits in Holstein-Local crossbreds

Trait	Genotype*			Level of significance**
	DD (25)	ID (24)	II (8)	
Body length (cm)	147.62±1.48	145.84±2.09	145.10±1.76	NS
Heart girth (cm)	188.26±1.45	190.08±1.86	190.50±3.72	NS
Hip height (cm)	136.45±1.41	136.42±1.44	136.84±2.12	NS
Live weight (kg)	484.89±9.88	489.21±13.48	487.93±18.80	NS

**DD, Deletion type; ID, Heterozygous; II, Insertion type; NS, **Non-significant (P>0.05). Values of parentheses indicate the number of samples included in each genotype.*

57°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 10 min.

Gel documentation and genotyping

Agarose gel electrophoresis was performed using 5 µl of each PCR product for checking the successful amplification of the target sequence fragment of *PLAG1* gene. Gel images were captured by the Digital Gel Documentation system (Sunil Bio, South Korea) to know the insertion or deletion type of each individual in the target fragment of *PLAG1* gene. Three different band patterns were observed

Table 4: Test of significance of various factors and their interactions on morphometric traits of H × L crossbred cattle

Factor	Body length (cm)	Heart girth (cm)	Hip height (cm)	Live weight (kg)
Genotype (G)	NS	NS	NS	NS
Herd (H)	**	NS	***	NS
Parity (P)	NS	NS	***	NS
Year (Y)	NS	NS	***	NS
G*H	NS	NS	NS	NS
G*P	NS	NS	NS	NS
H*P	NS	NS	NS	NS

*NS: P>0.05; *: P<0.05; **: P<0.01; ***: P<.001*

Table 5: Least-squares means (LSM) with standard errors (SE) of body length (cm), heart girth (cm), hip height (cm) and live weight (kg) of H×L crossbred according to parity, year and herd

Factor	N	Body length (cm)	Heart girth (cm)	Hip height (cm)	Live weight (kg)
		LSM±SE	LSM±SE	LSM±SE	LSM±SE
Parity					
1	10	149.60±2.34	187.45±3.63	140.46^{a}±1.37	487.47±19.66
2	8	146.69±3.21	190.18±3.13	141.92^{a}±1.39	492.40±22.00
3	19	147.05±2.07	191.70±1.78	138.23^{a}±1.15	500.92±12.80
4	13	144.98±2.22	186.98±1.56	132.08^{b}±1.80	469.12±10.94
5	4	143.51±5.13	185.42±4.52	128.91^{b}±3.00	458.96±37.18
6	3	143.09±4.71	193.89±6.11	127.00^{b}±2.54	500.25±48.32
Year					
2012	11	141.09±2.28	188.19±2.79	126.77^{c}±1.26	464.13±19.15
2013	4	150.50±3.65	184.79±1.22	133.99^{bc}±1.91	475.59±17.43
2015	9	147.60±2.26	189.65±2.78	136.03^{b}±1.47	492.91±20.53
2016	10	150.62±3.83	190.50±3.05	140.72^{ab}±1.70	506.92±21.36
2017	18	146.47±1.52	190.78±2.01	138.71^{ab}±0.99	493.49±10.67
2018	5	145.29±3.63	187.45±4.13	144.27^{a}±0.95	474.10±28.77
Herd					
ADL	28	150.50^{a}±1.45	187.51±1.76	136.61^{a}±1.02	491.14±11.56
AFBL	29	142.68^{b}±1.36	191.11±1.35	134.44^{b}±1.38	483.26±9.74

N, no. of observation; the different values in the same column differ significantly (P<0.05).

as insertion (II = 366 bp), deletion (DD=347 bp) and heterozygote (ID = 366/347). According to the band patterns, the type of genotype was identified and recorded.

Morphometric data collection and Statistical analysis:

Growth and body conformation related morphometric measurements like body length, heart girth and hip height (cm) were recorded from H×L crossbreds. Live weights (kg) were estimated based on the recorded information using Shaeffer`s formula (Khan et al., 2003). Genotypic and allelic frequencies based on the resultant PCR genotypes were calculated according to Falconer and Mackay (1996). Single marker association analysis was performed in order to evaluate the relationships between the resultant genotypes of *PLAG1* gene polymorphism and morphometric traits in crossbred cattle populations using the GLM procedure of SAS for windows 9.1.3 according to the following model. Mean separation was performed using Duncan's Multiple Range Test (DMRT).

$Y_{ijk} = \mu + P_i + Y_j + F_k + e_{ijk}$

Where, Y_{ijk} = the dependent variable; μ = the overall mean; P_i = the effect of i^{th} parity; Y_j = the fixed effect of j^{th} year; F_k = the fixed effect of k^{th} farm; e_{ijk} = the random residual error of primer specific fragments (347/366 bp)

Result

PCR amplification for detection of 19-bp indel of PLAG1 gene

PCR amplification showed the size of 347/366 bp indel fragments that indicated the primer specific amplification (Figure 1).

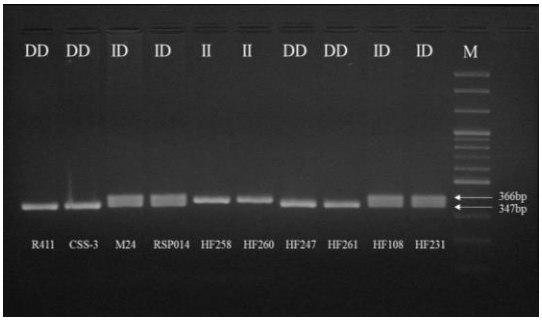

Figure 1: *Image of PCR product of the PLAG1 gene fragment. M = 100 bp DNA marker, DD = deletion type (347 bp), II = Insertion type (366 bp) and ID = heterozygous (347/366 bp). Animal identity of each individual is given beneath the banding patterns*

Three different genotypes were detected based on the amplified fragments of 19-bp indel of *PLAG1* gene. The homozygous insertion (II), homozygous deletion (DD) and heterozygous (ID) genotypes possessed 366 bp/366 bp, 347 bp/347 bp and 366 bp/347 bp, respectively (Figure 1) as revealed by the agarose gel documentation. Notably, the present results depicted that the 19-bp insertion was species specific, absent in *Bos indicus* (zebu population) but detected predominantly in the crossbred populations of Bangladesh. Therefore, trait specific association analysis was limited only for H×L crossbred cattle.

Genotypic and allelic frequency

Genotypic and allelic frequencies of the *PLAG1* gene for 19-bp indel are shown in the Table 2. The homozygous II genotype ranged between 8 to 20% in the crossbred populations whereas it was absent in the investigated indigenous cattle samples of Bangladesh. The indigenous cattle possessed predominantly the DD genotype that varied between 89 to 95% whereas in the H×L crossbreds it was only 43%. However, only 9% of the indigenous cattle were heterozygote (ID) in nature whereas 43% of crossbred cattle samples had ID genotype. Moreover, the I and D alleles frequencies varied from 0.02 to 0.06 and 0.94 to 0.98 among the four different cattle populations of indigenous origin and were largely differed in H×L crossbreds ranging from 0.31 to 0.40 and 0.60 to 0.69, respectively.

Trait specific association analysis

Table 3 represents the association between 19-bp indel derived *PLAG1* genotypes and three different morphometric traits and live weight. Association analysis revealed none of the traits differed significantly among the genotypes of the studied crossbred population. However, the dominant DD genotype had a higher body length (147.62±1.48 cm) compared to ID (145.84±2.09 cm) and II (145.10±1.76 cm) genotypes. In contrast, larger heart girth was measured in the recessive II genotype (190.50±3.72 cm) than the ID (190.08±1.86 cm) and DD (188.26±1.45 cm) genotypes. There were no discernable differences

for hip height among the three genetic groups of *PLAG1* polymorphism.

The effects of non-genetic factors (parity, year and herd) and their interactions on studied morphometric traits and live weight are presented in Table 4. Body length had significant effects (P<0.01) only at different herds. Hip height trait differed significantly (P<0.01) for the factors of herd, parity and year. However, heart girth and live weight did not differ significantly for any of the factors and their interactions. The highest hip heights were observed in 1st (140.46±1.37 cm) and 2nd (141.92±1.39 cm) parities while the lowest heights were found in older cattle at 5th (128.91±3.00 cm) and 6th parities (127.00±2.54 cm) (Table 4). Likewise, there were increasing trends in hip heights as the progression of years. The older cows in the year 2012 had significantly lower hip height (126.77±1.26 cm) than the young cows in the year 2018 (144.27±0.95 cm). The mean body length varied from 142.68±1.36 cm to 150.50±1.45 cm among the dairy herds.

Discussion

Genotype and allele frequencies

PLAG1 is a widely investigated candidate gene that plays a pivotal role in the growth and stature of different mammals. Previous studies reported that SNPs or indel polymorphisms of *PLAG1* gene were associated with morphometric features, live weight and stature in cattle (Li *et al.,* 2013; Zhou *et al.,* 2019), in human (Gudbjartsson *et al.,* 2008), in sheep (Pan *et al.,* 2021) and in goat (Wei *et al*., 2021). The present finding is comparable with Zhou *et al.* (2019) who reported that the heterozygous ID genotype had comparatively higher frequency (0.50) than those of the homozygous DD (0.16) and II (0.34) genotypes, respectively in Yunling cattle of China. Moreover, the I and D allelic frequencies were found 0.59 and 0.41, respectively in the aforementioned cattle population. In another investigation involving 38 Chinese origin cattle breeds, Hou *et al*. (2019) found that the Q and q alleles frequencies were 0.13 and 0.87, respectively for g.25015640G>T SNP of *PLAG1* gene. Likewise, Chinese Xianan (XN) and Yunling (YL) cattle had Q allele frequencies 0.74 and 0.55 respectively (Li *et al.,* 2013). Moreover, the relatively higher frequencies of Q allele are associated with height in XN and YL cattle populations that may have introgressed paternally and these investigations support the present findings. Pan *et al.* (2021) reported the frequencies of I and D alleles were 0.56 and 0.44 respectively for 30-bp indel of *PLAG1* gene in sheep and for 45-bp indel, I and D allele frequencies were 0.85 and 0.15 respectively. There were considerable differences observed for genotypic and allelic frequencies between previously reported findings and the present study. In general, genotypic and allelic frequency is population or breed specific and largely depends on the number of investigated samples, breed purity and distribution of alleles in the respective breed or population. Until to date, there was no published report on indel polymorphisms of the *PLAG1* gene and their association in zebu cattle breeds. However, the present investigation gave some insight information at the population level for this mutation.

Effect of genotype

Several body size traits such as chest circumference, body and cross height had a significant association with the 19-bp indel of *PLAG1* gene in Yunling cattle (Zhou *et al.,* 2019) and also significantly associated with growth traits in four cattle breeds of China (Xu *et al.,* 2018). More particularly, the latter investigation reported significant association in Pinan cattle for rump length and hip width (P<0.05) whereas heart girth and cannon bone circumference differed significantly in Xianan cattle (P< 0.01) and the heart girth, hucklebone width, rump length, hip width, height at the sacrum and chest depth of the Jiaxian cattle (P< 0.05). These two findings contradict the present investigation. In addition, Zhong *et al.* (2018) reported that a novel SNP of *PLAG1* gene showed significant association for body height, chest circumference and other morphometric traits but body shape had insignificant effects. This result partially agrees with the present findings. In another investigation, Hou *et al.* (2019) reported that the g.25015640G>T

SNP had significant association with body height trait. A small sample size of this study with large deviations in morphometric traits might be one of the reasons for this inconsistency.

Effects of non-genetic factors

Significant effects of parity on growth traits of cattle were reported by Hoka *et al.* (2019) in crossbred Friesian dairy cows and Patterson *et al.* (2002) in Charolais beef cattle. They mentioned that the growth was maximum in earlier parity than the latter parity and is in agreement with our findings. Amasaib *et al.* (2011) and Meikle *et al.* (2004) found significant effects of parity on the performance, body weight and body condition scores of crossbred dairy cows and are in close relation to the present study. The hip height of older animals with multiple parities was higher than their younger counterparts. This result pinpointed towards the increment of exotic inheritance gradually in the descendants that also incurred with larger body size. The year had highly significant ($P<0.001$) effects on hip height and is supported by the previous findings of Nahar *et al.* (2016) and Hernendez *et al.* (2011). They reported that growth and body weight gain were highly influenced by the effect of year. In this study, the effects of the year on hip height having a trend of 2018>2016>2017>2015>2013>2012 which supports strongly with the findings of Curtis *et al.* (2018) and Amasaib *et al.* (2011). Most of the earlier studies reported higher growth performance in the later years than the previous one in dairy cattle populations. Herds had also significant effects on two morphometric traits body length and hip height and are consistent with the previous findings of Mezgebe *et al.* (2018) in Begait cattle of Northern Ethiopia; Zhou *et al.* (2019) in Yunling cattle of China and Morris (2012) in Beef cattle. They found a significant association between *PLAG1* gene polymorphisms and the traits of hip height, growth performance and average daily gain in different cattle breeds. The herd differences for those traits might be due to the genetic composition of the crossbred animals and selection direction towards large versus medium sized cows.

Conclusions

None of the investigated morphometric traits had a significant association with 19-bp indel genotypes in H×L crossbred cattle of Bangladesh despite some non-genetic factors herd, parity and year had significant association with body length and hip height. Here we first report the distribution of indel polymorphisms in available cattle genetic resources of Bangladesh and found temperate breed Holstein oriented polymorphism. In conclusion, our results suggest that, the identified 19-bp indel polymorphism in the *PLAG1* gene could be used as a marker for growth and morphometric traits of H×L crossbred cattle of Bangladesh upon validation with a large number of samples.

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