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Identification and analysis of a 5-bp indel of a porcine BMP7 gene promoter

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ABSTRACT. Bone morphological protein7 (BMP7), a member of the transforming growth factor-β (TGF-β) family, was first identified because of its ability to induce ectopic chondro-osteogenesis *in vivo*. It also plays a crucial role in the growth, development, and physiological functioning of the reproductive system. Among intra-ovarian growth factors, many studies have shown that BMP7 plays a pivotal role in regulating the early phases of follicular growth. We detected a 5-bp insertion-deletion at 602 bp upstream from the transcription start site of the BMP7 gene promoter among 258 pigs of 3 breeds. Along with 2 homoduplex DNAs, another 4 previously unknown bands (named A, B, C, and D) were detected by non-denaturing polyacrylamide gel electrophoresis. By DNA sequencing, we found that PCR products from heterozygotes contained 2 homoduplexes and 4 heteroduplexes. Genetic polymorphism analysis revealed 3 genotypes (AA, AB, and BB) at this site; the distribution of these genotypes followed Hardy-Weinberg equilibrium. A was the dominant allele (0.715), and AA was the dominant genotype (0.500). The polymorphism information

content value was calculated to be 0.325, the expected heterozygosity was 0.407, and effective number of alleles was 1.688, indicating an intermediate degree of polymorphism and good potential for selection and breeding. Highly significant differences were found between different breeds and distributions of genotypes. Based on correlation analysis, the 5-bp indel site does not significantly affect porcine reproductive traits (total number of births, number of piglets born alive, litter birth weight, and litter weight at 21 days; $P > 0.05$), which was consistent with the results of genetic variation analysis.

Key words: BMP7; Promoter; 5-bp indel (insertion and deletion); Heteroduplex DNA

INTRODUCTION

Bone morphogenetic protein7 (BMP7) is a member of the transforming growth factor super-family, which stimulates the differentiation of osteoblasts and induces ectopic bone formation. Recent studies have found that BMP7 also induces the differentiation of granulose cells, stimulates synthesis of follicle-stimulating hormone, and inhibits the production and secretion of progesterone. Additionally, BMP7 is involved in the formation and development of numerous organs.

In 1989, Nagamine et al. discovered 2 non-target heteroduplexes while studying murine testis-determining genes (Zfy-1 and Zfy-2), which derived from an 18-bp deletion of Zfy-2 (Ozkaynak et al., 1990). Meanwhile, when amplifying DNA sequences from double heterozygotes of human sickle hemoglobin and hemoglobin C, Sheffield et al. (1989) identified 2 heteroduplex bands consisting of sickle hemoglobin and hemoglobin C strands by denaturing gradient gel electrophoresis.

Currently, it is difficult to differentiate genotypes with insertion-deletion (indel) polymorphisms of only a few nucleotides by regular denaturing or non-denaturing polyacrylamide gel electrophoresis (PAGE). However, heteroduplexes may serve as a reference for genotyping and also facilitate the identification of potential indel polymorphisms in unknown gene sequences (Liu et al., 1991). In this study, we investigated a 5-bp indel polymorphism in the BMP7 gene at 602 bp upstream from the transcription start site (TSS) in farmed pigs by non-denaturing PAGE. We found that in addition to 2 target bands, all heterozygous individuals produced 4 non-target electrophoretic bands.

MATERIAL AND METHODS

Methods

Genomic DNA samples were obtained from 258 pigs belonging to 3 breeds (Landrace, N = 25; Duroc, N = 85; Large White, N = 148). They were reared in the Zhengyang Pig Breeding Center, Henan Province of China. About 20-30 hairs with follicles were collected from the back of each pig and stored in 2-mL centrifuge tubes containing 75% (v/v) ethanol and stored at -20°C for subsequent analysis.

Primer design and PCR amplification

The porcine BMP7 promoter sequence was obtained from the GenBank sequence (CR956362). Forward and reverse primers (5'-CTGTGCAGCCGCAGTGCCTACC-3' and 5'-ATCCCTCCCCACCCCACCATCT-3', respectively) were designed with the Oligo Primer Analysis Software (Molecular Biology Insights, Cascade, CO, USA). The target PCR product was expected to be 277 bp. DNA was extracted from hair follicles by a phenol-chloroform extraction method (Zhou et al., 2011). The extracted DNA was amplified by PCR in a 10-μL reaction consisting of 3.5 μL double-distilled water, 0.5 μL primers, 0.5 μL (approximately 50 ng) DNA template, and 5 μL TaqMix. The cycling protocol was 5 min at 94°C, 30 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. PCR products were confirmed by 1% agarose gel electrophoresis.

PAGE and cloning sequencing

Polyacrylamide gels (12%) were prepared by mixing 9.0 mL 30% polyacrylamide solution (29:1), 3 mL 10X TBE buffer, 0.21 mL 10% ammonium persulfate, 10.5 μL TEMED, and 17.79 mL water. PCR products were separated by PAGE on these gels. Bands were stained with silver nitrate and photographed for further analysis. The above PAGE results were preliminarily analyzed, and PCR products from individuals with differing genotypes were extracted and purified using an agarose gel extraction kit (Tiangen Biotech, Beijing, China). Extracted PCR products were ligated into a pUC19 vector and sequenced by Taihe Biotechnology (Beijing, China).

Analysis of non-target bands

Homozygotes of differing genotypes were identified based on PAGE and sequence analysis, and their DNA extracts were then combined. Non-target bands from heterozygotes were excised with a sterile scalpel and recovered following a published method (Castillo et al., 2006). Briefly, the band was mixed with 50-100 μL TE buffer, incubated at 37°C for 12 h, and then centrifuged at 7378 *g*. The supernatant was collected and 2 μL was used as a template for PCR amplification with the primers described above. PCR products were confirmed by 1% agarose gel electrophoresis and then separated by PAGE on 12% gels.

Data analysis

Data were analyzed for the 5-bp indel polymorphism in the porcine BMP7 promoter. Different allele and genotype frequencies were calculated. The distribution of genotypes among the porcine breeds was analyzed by the Fisher exact test. If a global difference was detected, genotypes were further analyzed by partition of the chi-squared test (χ^2). The effects of genotype on reproductive traits were analyzed (overall significance and multiple comparisons) by a general linear model procedure (SPSS 16.0; SPSS, Chicago, IL, USA). The following model was constructed considering influential factors of reproductive traits and also the conditions of the present study:

$$Y_i = \mu+R+A+S+B+P+G_i+E_i$$

where Y_i is the reproduction record of an individual, μ is the mean value of the population, R

represents the effects of porcine breed, *A* represents the effects of years, *S* represents the effects of seasons, *B* represents the effects of birth order, *P* represents the effect of various gestational periods, G_i represents the fixed effects of the 5-bp indel site, and E_i represents random errors.

RESULTS

PCR product identification and PAGE separation of the BMP7 gene promoter

DNA samples from porcine hair follicles were amplified by PCR and confirmed by 1% agarose gel electrophoresis. Electrophoretograms (Figure 1) showed bands at 277 bp as expected. PCR products were further analyzed by PAGE. PAGE electrophoretograms (Figure 2) showed 3 genotypes (named AA, AB, and BB) associated with the 5-bp indel. As shown in Figure 2, lane 2 was identified as AA, lanes 3 and 4 were BB, and lanes 1 and 5 were AB. Heterozygotes (lanes 1 and 5) produced 2 additional non-target bands. Furthermore, non-target bands from differing heterozygotes showed 2 combinations, designated A, B, C, and D.

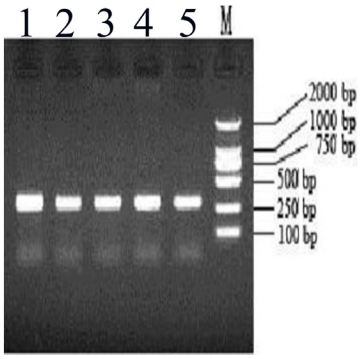

Figure 1. Agarose gel electrophoretograms of PCR products amplified from DNA samples collected from porcine hair follicles. *Lane M* = molecular weight marker; *lane 1-5* = positive products.

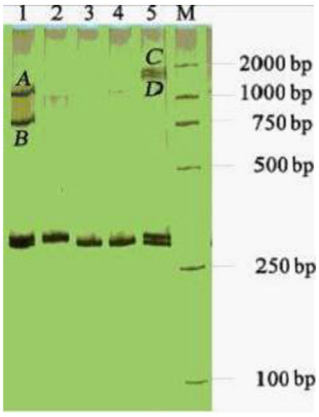

Figure 2. PAGE electrophoretograms of PCR products amplified from DNA samples collected from porcine hair follicles. *Lanes 1* = AB heterozygotes and two non-target bands; *lane 2* = AA homozygotes; *lanes 3* and *4* = BB homozygotes; *lane 5* = AB heterozygotes and two non-target bands; *lane M* = molecular weight marker. A, B, C, and D = non-target bands from differing heterozygotes showing 2 combinations.

Analysis of non-target bands

To identify the origins of non-target bands, bands from AA- and BB-type homozygotes were cloned and sequenced. Sequence analysis (Figure 3) revealed that AA-type porcine was insertion homozygote (Figure 3, top panel), BB-type porcine was deletion homozygote (Figure 3, middle panel) and AB-type porcine was heterozygote (Figure 3, botton panel). Moreover, another 5-bp region (GAAAA, dashed lines in Figure 3) was found immediately before the 5-bp indel site (GAAAA, solid line in Figure 3).

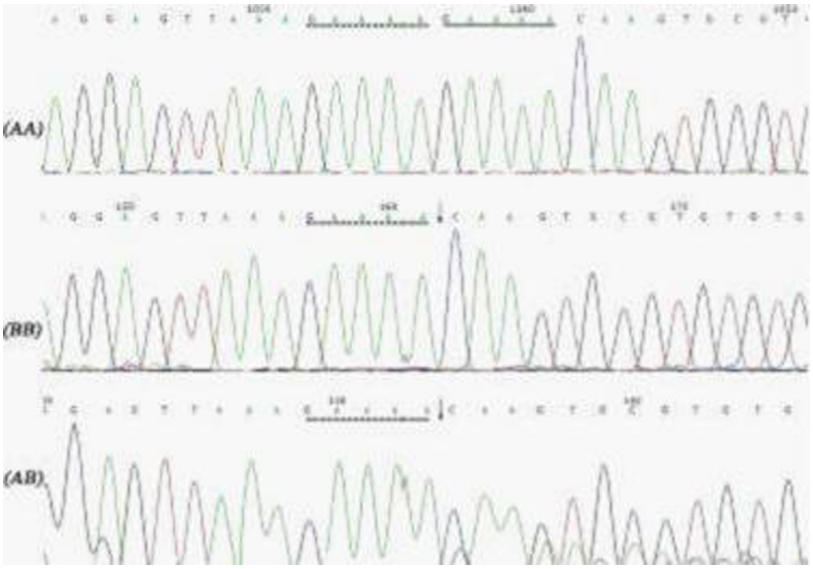

Figure 3. Sequencing analysis of the 5-bp indel site in porcine with various genotypes.

DNA samples from various homozygotes were combined and re-amplified by PCR and agarose gel electrophoretograms (data not shown) of PCR products showed single target bands similar to those in Figure 1. PCR products were also separated by PAGE on 12% gels. As shown in Figure 4, lanes 13, 14, and 19 were the PCR products of mixed DNA samples from homozygotes. Lanes 1, 5, 7, 8, and 10 were AB-type individuals. Lanes 4 and 11 were AA-type individuals. Lanes 2, 3, 6, 9, 12, 15, 16, 17, 18, and 20 were BB-type individuals. All lanes related to heterozygotes (regardless of being individual DNA samples or mixed samples) showed 2 non-target bands of 2 combinations. Size estimation based on the DNA marker confirmed that the 4 non-target bands were the previously identified bands A, B, C, and D (Figure 2). A, B, C, and D bands in Figure 5 were recovered, re-amplified by PCR and separated by denaturing PAGE (Figure 5). Lanes 1-4 in Figure 5 were PAGE bands of recovered re-amplified C and D bands, which contained only 2 target bands and the non-target bands C and D. Lanes 5-8 were PAGE bands of recovered re-amplified A and B bands, and similarly contained only 2 target bands and the non-target bands A and B. These results confirmed that the non-target A, B, C, and D bands were not produced by non-specific PCR amplification.

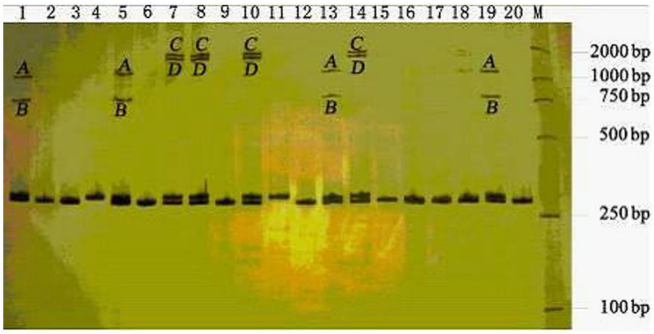

Figure 4. PAGE electrophoretograms of PCR products amplified from mixed DNA samples from homozygote. *Lanes 1*, *5*, *7*, *8*, and *10* = AB-type individuals; *lanes 2*, *3*, *6*, *9*, *12*, *15*, *16*, *17*, *18*, and *20* = BB-type individuals; *lanes 4* and *11* = AA-type individuals; *lanes 13*, *14*, and *19* = PCR products of mixed DNA samples from homozygotes; *lane M* = molecular weight marker.

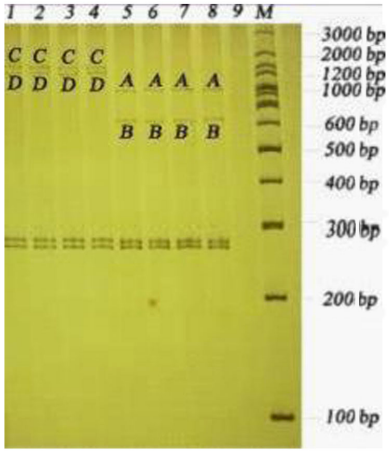

Figure 5. Denaturing PAGE electrophoretograms of re-amplified PCR products from recovered A, B, C, and D bands. *Lanes 1-4* = PAGE bands of recovered re-amplified C and D bands; *lanes 5-8* = PAGE bands of recovered re-amplified A and B bands; *lane 9* = blank control; *lane M* = molecular weight marker.

Population genetic characteristics of the 5-bp indel polymorphism in the BMP7 gene promoter

Genotype and allele frequencies, as well as polymorphic indicators associated with the BMP7 gene promoter, were calculated based on PAGE results and analyzed by the Hardy-Weinberg equilibrium test to determine the distribution of genotypes among porcine breeds (Tables 1 and 2).

The population (Table 1) was characterized by 2 alleles (A and B) and 3 genotypes (AA, AB, and BB) in terms of the 5-bp indel polymorphism. A was found to be the dominant allele and AA was the dominant genotype. χ^2 was less than $\chi^2_{0.05(1)}$ (3.84), indicating the absence of significant differences ($P > 0.05$) in the population.

Table 1. Genotype frequency, allele frequency, and polymorphic indicator of the BMP7 promoter.

	Genotype frequency			Allele frequency		χ^2	PIC	Nei's H_E	N_E
	AA	AB	BB	A	B				
5-bp indel	0.50 (129)	0.43 (111)	0.07 (18)	0.715	0.285	0.753^{ns}	0.325	0.407	1.688

PIC = polymorphism information content; Nei's H_E = Nei's expected heterozygosity; N_E = effective number of alleles; ns = nonsignificant. Values of χ^2 are from tests of the distribution of various genotypes for Hardy-Weinberg equilibrium in different microsatellites.

Table 2. Distribution of genotype frequencies in terms of the 5 bp indel site among porcine breeds.

Breed	Genotype frequency			Gene frequency		χ^2	PIC	Nei's H_E	N_E
	AA	AB	BB	A	B				
Landrace	0.480 (12)	0.520 (13)	$0.000\ (0)^{AC}$	0.740	0.260	2.811^{ns}	0.311	0.443	1.795
Large White	0.420 (62)	0.500 (74)	$0.080\ (12)^{C}$	0.669	0.331	2.333^{ns}	0.345	0.385	1.626
Duroc	0.647 (55)	0.282 (24)	$0.071\ (6)^{AB}$	0.788	0.212	2.206^{ns}	0.278	0.334	1.501

For abbreviations, see legend to Table 1.

Therefore, the population was in Hardy-Weinberg equilibrium. Sequence analysis showed that allele B was derived from allele A by insertion of a 5-bp sequence (GAAAA) at 602 bp upstream from the TSS.

Frequencies of the various genotypes related to the 5-bp indel polymorphism (Table 2) varied significantly between the porcine breeds ($P = 0.007$). Partition of chi-squared showed that Landrace and Large White pigs had similar genotype distributions ($P > 0.05$). The differences between Landrace and Duroc pigs were almost statistically significant ($P = 0.053$). Notably, the differences between Duroc and Large White pigs were highly significant ($P = 0.003$).

Association between the 5-bp indel polymorphism and reproductive traits in pigs

As it shown in table 3, dominant effects existed between the A and B alleles, and the dominant effects on the total number of birth (TNB), number of piglet born alive (NBA), litter birth weight (LBW) and litter weight at 21 days were 0.174 head, 0.200 head, 0.703 kg and 0.203 kg, respectively. The number of pigs with the BB genotype was the highest for the TNB, NBA, LBW and litter weight at 21 days compared with those with the AA and AB genotypes. However, there were no significant differences among the three genotypes.

Table 3. Effects of the 5-bp indel polymorphism on the reproductive traits of porcine.

Traits	5-bp indel			P	Additive	Dominant
	AA	AB	BB		effect	effect
Total number of births	8.481 ± 0.603	8.411 ± 0.610	8.689 ± 0.694	0.732	0.104	-0.174
Number of live births	8.063 ± 0.610	7.979 ± 0.616	8.295 ± 0.701	0.670	0.116	-0.200
Litter birth weight (kg)	11.516 ± 0.892	11.143 ± 0.902	12.176 ± 1.026	0.103	0.330	-0.703
Litter weight at 21 days (kg)	46.189 ± 3.184	47.691 ± 3.219	49.598 ± 3.663	0.104	1.705	-0.203

Data are reported as means ± standard error. Values in each line with different lower case superscripts are at $P < 0.05$; and with capital superscripts different at $P < 0.01$. Non marked values mean no difference.

DISCUSSION

Indel polymorphism is a common gene polymorphism in population variation. Indel sequences vary in size and can be as small as a single base. Indel polymorphism is often responsible for trait differences between individuals. Alberts et al. (1997) studied the relationship between a 6-bp (GGGGA) indel polymorphism in exon 7 of the endoglin gene and intracerebral hemorrhage (ICH) in Western individuals and found that significantly more ICH patients showed the homozygous form of the indel and significantly less patients showed the heterozygous form, compared with control subjects. Thus, it was concluded that this gene polymorphism was a genetic susceptibility factor for ICH.

A heteroduplex is a double-stranded DNA molecule formed by recombination of single, not completely complementary strand from differing sources. In the present study, heterozygous pigs with regard to the 5-bp indel in the porcine BMP7 promoter showed 4 non-target bands in 2 combinations (AB and CD). The 4 bands were identified as heteroduplexes that originated from deletion of a 5-bp sequence (GAAAA) in the BMP7 gene of BB homozygotes at 602 bp upstream from the TSS.

In contrast to the results of Nagamine et al. (1989), heterozygotes in the present study showed heteroduplexes in 2 combinations, which are probably related to the structure of the BMP7 gene. Sequence analysis of the BMP7 gene promoter revealed that varying copies of the GAAAA sequence at this site created the indel polymorphism. The BMP7 gene in heterozygotes carried two copies of the GAAAA indel polymorphism, AA-type pigs carried 1 copy of the GAAAA indel polymorphism, and BB-type pigs carried 2 copies. Therefore, heterozygotes formed 2 heteroduplex combinations during PCR, as shown in Figure 6.

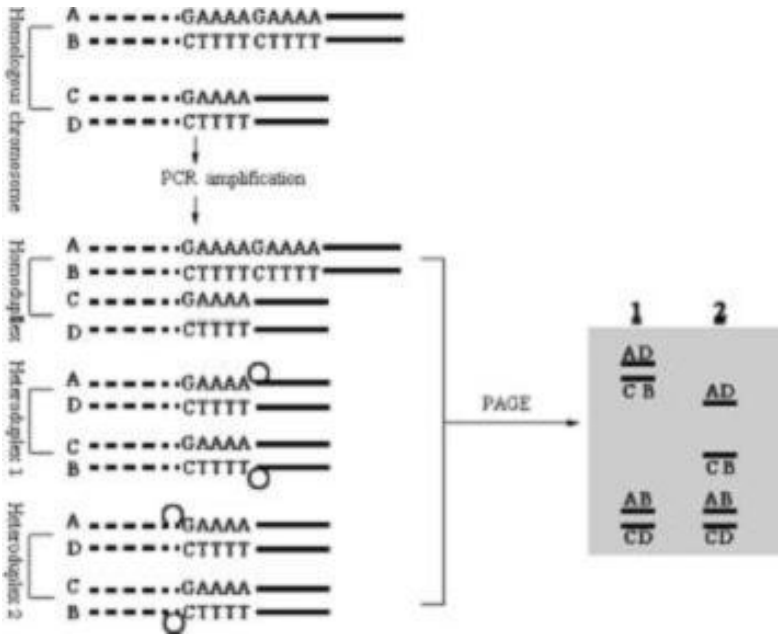

Figure 6. Schematic of the process of heteroduplex formation.

Amplification of the DNA sequence containing the indel site created positive A strands carrying 2 copies of GAAAA, negative B strands carrying 2 copies of CTTTT, positive C strands carrying 1 copy of GAAAA, and positive D strands carrying 1 copy of CTTTT. After renaturation, these strands hybridized in four combinations. A-D and C-B combinations produced 2 heteroduplexes (AD and CB), while A-B and C-D combinations produced 2 homoduplexes (AB and CD). Additionally, if a strand carrying 2 copies of GAAAA (or CTTTT) hybridized with a strand carrying 1 copy of CTTTT (or GAAAA), a local loop or bubble was formed, because of their sequence mismatch. The loop-bubble structure reduced the migration speed of the double strand during electrophoresis and therefore produced 4 bands on PAGE electrophoretograms.

Additionally, after a strand carrying 2 copies of GAAAA (or CTTTT) combined with a strand carrying 1 copy of GAAAA (or CTTTT), the unpaired GAAAA (or CTTTT) sequence appeared either in the 5'-terminus (broken line in Figure 6) or the 3'-terminus (solid line in Figure 6). Because these sequences at both sides of the indel site are conservative, the appearance of unpaired GAAAA at the 3'- or 5'-terminus led to differing spatial configurations. Different spatial configurations resulted in differing migration speeds during electrophoresis, thus showing 2 heteroduplex combinations on PAGE electrophoretograms.

The 5-bp indel site in the porcine BMP7 promoter was found to be polymorphic. A polymorphism information content (PIC) > 0.5 indicates high polymorphism, 0.25 < PIC < 0.5 indicates intermediate polymorphism, and PIC < 0.25 indicates low polymorphism (Kijas et al., 1995). For a given population, a high PIC relates to a large number of alleles and high heterozygosity, indicating a high degree of genetic variation and excellent potential for breeding and selection. In this study, the 5-bp indel site showed a PIC value of 0.325, which indicated intermediate polymorphism. Nei's expected heterozygosity value was 0.407, indicating considerable heterozygosity and reasonable opportunities for selective breeding.

Genetic polymorphism at a locus reflects the distribution of a mutation in a population. The distribution is affected by both natural and artificial selection. Theoretically, a locus under no selection complies with the Hardy-Weinberg law (Ouyang et al., 2007). In this study, the 5-bp indel site was in Hardy-Weinberg equilibrium, and correlation analysis suggested that the indel site was not significantly associated with reproductive traits (P > 0.05). These findings excluded the effects of natural and artificial selection on the determination of this 5-bp indel site. The alleles of this indel site affected reproductive traits (TNB, NBA, LBW, and litter weight at 21 days) primarily via dominant effects. Therefore, the genetic and correlation analysis produced consistent results.

We also analyzed the porcine BMP7 gene promoter sequence containing the indel site with the Promoter 2.0 Prediction Server. The results showed that in pigs with the BMP7 gene carrying 2 copies of GAAAA, the BMP7 gene promoter contained 2 copies of sex-determining region Y. In pigs with the BMP7 gene carrying 1 copy of GAAAA, the BMP7 gene promoter contained 1 sex-determining region y copy. These results suggested that this indel site may be implicated in sexual differentiation during embryonic development. However, this inference needs to be verified by further studies.

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