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The Effect of PIS Region on Horned or Polled Phenotype Traits in China Guanzhong Dairy Goats

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Key words
Guanzhong dairy goat, Horned phenotype trait, Polled phenotype trait, Polymorphism sites, Polled intersex synderome, Micro RNAs, CpG

ABSTRACT

Studies have reported goat Polled Intersex Syndrome(PIS) a 11.7-kb deletion triggers intersexuality and polledness in goats. To determine the effect of PIS region on horned phenotype of Guanzhong dairy goats, four specific primers (PIS1-1, PIS1-2, PIS2 and PIS3) covering PIS fragment were designed, and four DNA fragment were amplifed and sequenced. A 12.814kb sequence was assembled containing the whole goat PIS region both in horned goats and in polled goats, which mean there were no 11.7 kb deletion in the Guanzhong dairy goats.The polymorphisms sites in PIS region of horned and polled Guanzhong dairy goats were identified, and a total of 30 polymorphisms sites were confirmed (A648T, T652C, G1107C, T2594C, G2820A, G3556A, A3573G, T3621C, A5329T, T5392G, G5878A, C5996T, A6187G, C6228A, G6969T, G7045T, T7057C, C7266G, C7417T, T8327G, T9029G, C10034A, A10846G, del 10861-10862TT, G11842A, G12157A, G12104A, del 12431-12432AT, G12665A and del 12771-12772TT) in the 12.814 kb sequence. The further annotation results showed that a tRNA was located at 4218-4290bp, STR1 (TGTGTGTGTGTG) and STR2 (ATATATATATAT) were located at 9473-9484bp and 12451-12462bp, respectively. Three micro RNAs, dme-mir-1, dme-mir-263a and cbr-mir-354 were confermed to locate at 3002-3023bp, 9078-9094bp and 10866-10882bp, respectively. There is a CpG island at 4390-4540 bp. Our results showed that the PIS regions have no effect on polled phenotype of Guanzhong dairy goat, genetic diversity in PIS region of the goat were identified and the key gene polymorphism sites still waiting to be determined.

INTRODUCTION

The Guanzhong dairy goat, an important milk and meat livestock breeding mostly in west of China, has characteristics like strong fertility, good adaptability, high physical strength, strong disease resistance, easy to manage and high milk yield. It is a crossbreed generated in 1937 by local goats cross-fertilizated with Saanen dairy goats in Shanxi Province of China. In 1990 this variety was officially selected for breeding purpose by the state and was produced in the Guanzhong area of Shanxi Province, and hence was named as Guanzhong dairy goat (Chen, 2010). It has now been introduced all over the country.

Guanzhong dairy goats are both polled and horned goats but polled goats are better to manage than the horned goats in feeding management. They do not hurt the keepers and also do not cause any serious damage to the guardrails. Polled goats are docile and do not fight with the other members within the same group, which is conducive to the output of production performance. The horn growth in goats consumes a lot of nutrients, and hence considering feed conversation rats the cultivation of polled goats is favorite in and has important production value.

At present, a few reports have shown that the polled and intersex traits of goats are closely linked. Intersex goats are usually polled, a trait which follows autosomal dominant inheritance, and has recessive masculinity effect

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(Pailhoux *et al*., 2001; Pannetier *et al*., 2012). Pailhoux *et al*. (2001) found in positional cloning in 2001 that the intervertebral syndrome of alpine and Saanen goats was caused by a deletion of sequence 1 to 11.7 Kb.

Zhang (2010) and Pailhoux *et al*. (2001) found that there was no complete deletion of ~11.7Kb in the clone of the inter-sex Tangshan dairy goats in 2010, and found partial deletion and a large number of single nucleotide polymorphisms (SNPs) mutation. Kijas *et al*. (2013) through a genome wide association analysis of Boer goats, Australian grassland sheep, and Kashmir sheep using a gene chip found that a 769 kb region is associated with a polled trait, which contains PIS fragments. The 11.7 kb fragment therefore seems to be closely related to polled traits of goats.

In the present study, the PIS fragment sequence was amplified and sequenced in polled/horned Guanzhong dairy goats to determine SNPs associated with the polled traits and the sequence annotation. This study provides key to the production of polled Guanzhong dairy goats, and it will also be helpful in understanding the molecular mechanism of intersexual goats.

MATERIALS AND METHODS

Guanzhong dairy goats were collected from the Sheep Farm of Guangxi Animal Husbandry Institute (horned goats: n=3 and polled goats: n=3). From jugular vein 5 mL blood sample was take in anticoagulation tube and stored at -20 ºC for DNA extraction and PCR amplification.

The genomic DNA was extracted by using phenol-chloroform extraction method, and the extraction quality was detected by 1% agarose gel electrophoresis. The purity (OD260/2801.7~1.9) and concentration were detected by UV spectrophotometer.

Primer design and synthesis

According to goat PIS sequence (GenBank accession number: AF404302), four sepecific pairs of primers (PIS1-1, PIS1-2, PIS2, PIS3) were designed by using oligo6.0 software and primer5.0 software to amplify the full-length PIS sequence. A pair of primers (PIS whole) was also designed to detect a complete deletion of 11.7 kb. If 11.7 kb was completely deleted, a 1387 bp product was amplified, and if there was no complete deletion of 11.7 kb, there was no amplification product. The primer sequences are shown in Table I, and the primers were synthesized by Beijing Aoke Dingsheng Biotechnology Co., Ltd.

PCR amplification and sequencing

PCR reaction system 25μL:2×LA Taq Mix 12.5μL, 1μL of each of the upstream and downstream primers at 10 μmol/L, template DNA 100 ng, and 25μL ultrapure water was added. PCR amplification procedure comprised pre-denaturation at 95°C for 3 min; denaturation at 95°C for 30s, annealing (annealing temperature is shown in Table I) 30s, extension at 72°C for 5 min, 35 cycles; extension at 72°C for 5 min; store at 4°C. The amplification results were detected by 1.0% agarose gel electrophoresis, and the UV detector was observed and analyzed. The PCR amplification product was sent to the Biotech Engineering (Shanghai) Co., Ltd. Guangzhou Branch for sequencing.

Sequencing results were spliced, blasted and analyzed for polymorphism by using seqman software.

The tRNAscan-SE was used to predic tRNA sequence online site (http://lowelab.ucsc.edu/tRNAscan-SE/). SSRHunter1.3 software was used to predict microsatellite sites. The MIRAlign website (http://bioinfo.au.tsinghua.edu.cn/miralign/) was used to predict microRNA. And the microRNA structure was predicted by using the RNAstructure online website (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html). The conservative domain analysis was performed by using the ncbi website (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The MethPrimer was used to predicts CpG

Table I. Primer sequences of PIS.

Fragments	Primer sequences (5`-3`)	Length (bp)	Temperatures (°C)
PIS whole	F:ACTGTGACTTATCGCCTCC R:GCAGAAATTCGACCTATCCAA	1387	54
PIS1-1	F:TCTTAGGGCTTTGCATGTGGTA R:GGCCTTGAATGTGGAATGTAG	2865	59
PIS1-2	F:ATTATCTGCGTCGTGAA R:TTTGAGTCGCTATCCTG	3398	50
PIS2	F:ACTGGCATACATTCCACTGCT R:TGCGCAGAGGAAGAAACTCG	5071	55
PIS3	F:TTTGCCAGAGAAGTATGAGG R.TGGATTCAGGAAAGGAGA	2892	50.5

island (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Geng *et al*., 2015). The inverted repeat sequence was predicted by using the EMBOSS online website (http://emboss.bioinformatics.nl/cgi-bin/emboss/einverted). And the tandem repeat sequence was predicted on the EMBOSS online website (http://emboss.bioinformatics.nl/emboss-explorer/output/389561/).

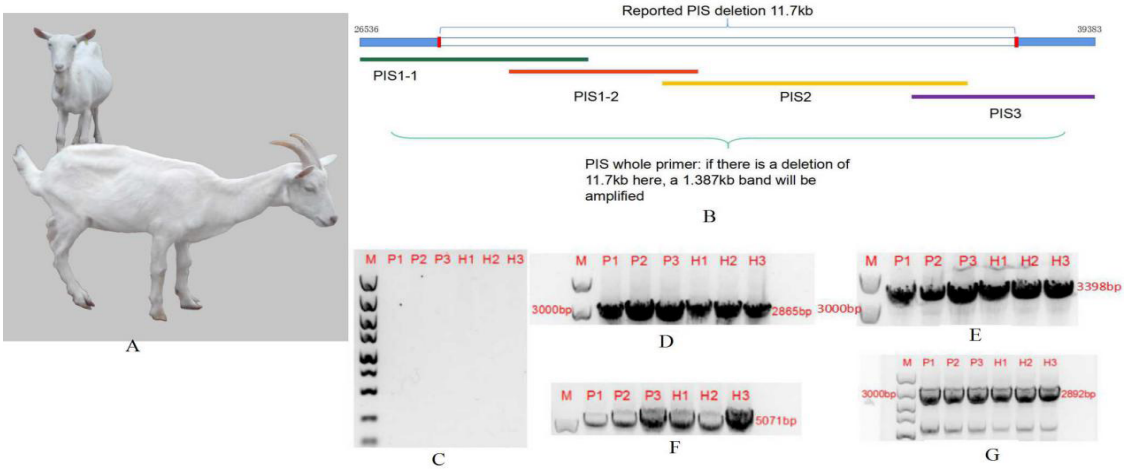

Fig. 1. PCR amplified fragments of PIS in Guanzhong dairy goats.
A, Horned and polled goats; B, The distribution of the primers in goat PIS region; C~G, PCR products of PIS whole; PIS1-1, PIS1-2, PIS2, PIS3. M, DL5000 Marker; P1-P3, PCR products of polled goats; H1-H3, PCR products of hornedgoats.

Capra hircus sex-specific gonadal PISRT1 mRNA, complete sequence

Sequence ID: AF404302.1 Length: 48420 Number of Matches: 22

Range 1: 26587 to 39371 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
22652 bits(12266)	0.0	12640/12839(98%)	79/12839(0%)	Plus/Plus

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Query  1      AGTTAGTTCACTCAGATTGCCATCGGGGCATTCAGGCCAGATCCTTACTCTAAGCAATGC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  26587  AGTTAGTTCACTCAGATTGCCATCGGGGCATTCAGGCCAGATCCTTACTCTAAGCAATGC  26646

Query  61     AGCCCATGCCTCCCTGCCCAGCCCCCACTTGCTACTGGCAGGTGCAGGCGTCTGTGCTGC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  26647  AGCCCATGCCTCCCTGCCCAGCCCCCACTTGCTACTGGCAGGTGCAGGCGTCTGTGCTGC  26706

Query  121    TTCTCCACTGGCAGAGTACTGTTGGGCTCGTAATCGGTGGGTTTTGATTATTTATTTATT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  26707  TTCTCCACTGGCAGAGTACTGTTGGGCTCGTAATCGGTGGGTTTTGATTATTTATTTATT  26766

Query  181    TTTCCTCCCTGTTATGTTGCTCTCTGTGCTTCCAAGGCTTGCCACAGACTCAGCAGTGAG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  26767  TTTCCTCCCTGTTATGTTGCTCTCTGTGCTTCCAAGGCTTGCCACAGACTCAGCAGTGAG  26826

Query  241    AGTGTTTCCCGGTGTTTGGAAACTTCTCTCTTTTTAAGACTGCCTTCCTGGGACGGAGCT  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  26827  AGTGTTTCCCGGTGTTTGGAAACTTCTCTCTTTTTAAGACTGCCTTCCTGGGACGGAGCT  26886

Query  301    CCGTCCCTACCTCttttgcctctttttttgtctttatatttttttCCTGCCTCCTTTCAA  360
              ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  26887  CCGTCCCTACCTCTTTTGTCTCTTTTTTTGTCTTTTATATTTTTTCCTGCCTCCTTTCAA  26946

Query  361    AGACAATGGGCTGCTTTTCTGTGTGCCTGATGTTCTTTGCCGGCATTCAGAAGTTGTTTT  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  26947  AGACAATGGGCTGCTTTTCTGTGTGCCTGATGTTCTTTGCCGGCATTCAGAAGTTGTTTT  27006

Query  421    GTGGAATTTCAGTTCAGTTCAGTTCAGTCACTCAGTCATGTCCGACTCTTTGCGACCCCA  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27007  GTGGAATTTCAGTTCAGTTCAGTTCAGTCACTCAGTCATGTCCGACTCTTTGCGACCCCA  27066

Query  481    TGAATTGCAGCACGCCAGGCCTCCCTGTCCATCACCAACTCCTGGAGTTCACTCAGATTC  540
              ||||||||||||||||||||||| ||||||||| ||||||| ||||||||||||||||||||
Sbjct  27067  TGAATTGCAGCACGCCAGGCCTTCCTGTCCAT-ACCAACTTCTGGAGTTCACTCAGATTC  27125

Query  541    ATGTCCATTGAGTCCGTGATGCCATCCAGCCATCTCATCCTCTGTCGTCCC--CT--TCT  596
              |||| |||||||||||||||||||||||||||||||||||| ||||||||  ||  |||
Sbjct  27126  ATGT-CATTGAGTCCGTGATGCCATCCAGCCATCTNATCCTCTGNCGTCCCTTCTCCTCT  27184
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Fig. 2. Blast results of the 12.814kb sequence (screenshot of partial results).

RESULTS

PIS fragment in Guanzhong dairy goats

Sepecific DNA fragment were successfully amplified and sequenced with PIS1-1, PIS1-2, PIS2, and PIS3 primers both in horned and polled Guanzhong dairy goats. But no sepecific DNA fragment was amplified with PIS whole primers both in horned and polled Guanzhong dairy goats, which mean there were no 11.7 kb deletion in the Guanzhong dairy goats (Fig. 1). The further assemble of the four amplified sequences, a 12.814 kb sequence was done by Seqman software, and which was 98% similar with the reported goat PIS sequences using NCBI for blast alignment (Fig. 2). Our results showed that there were no 11.7 kb deletions both in horn and polled Guanzhong dairy goats.

Genetic polymorphism analysis

The polymorphic sites of the 12.814 kb sequence were analyzed by seqman software from the sequences of 3 horn and 3 polled Guanzhong dairy goats. A total of 30 polymorphic sites were found, namely A648T, T652C, G1107C, T2594C, G2820A, G3556A, A3573G, T3621C, A5329T, T5392G, G5878A, C5996T, A6187G, C6228A, G6969T, G7045T, T7057C, C7266G, C7417T, T8327G,

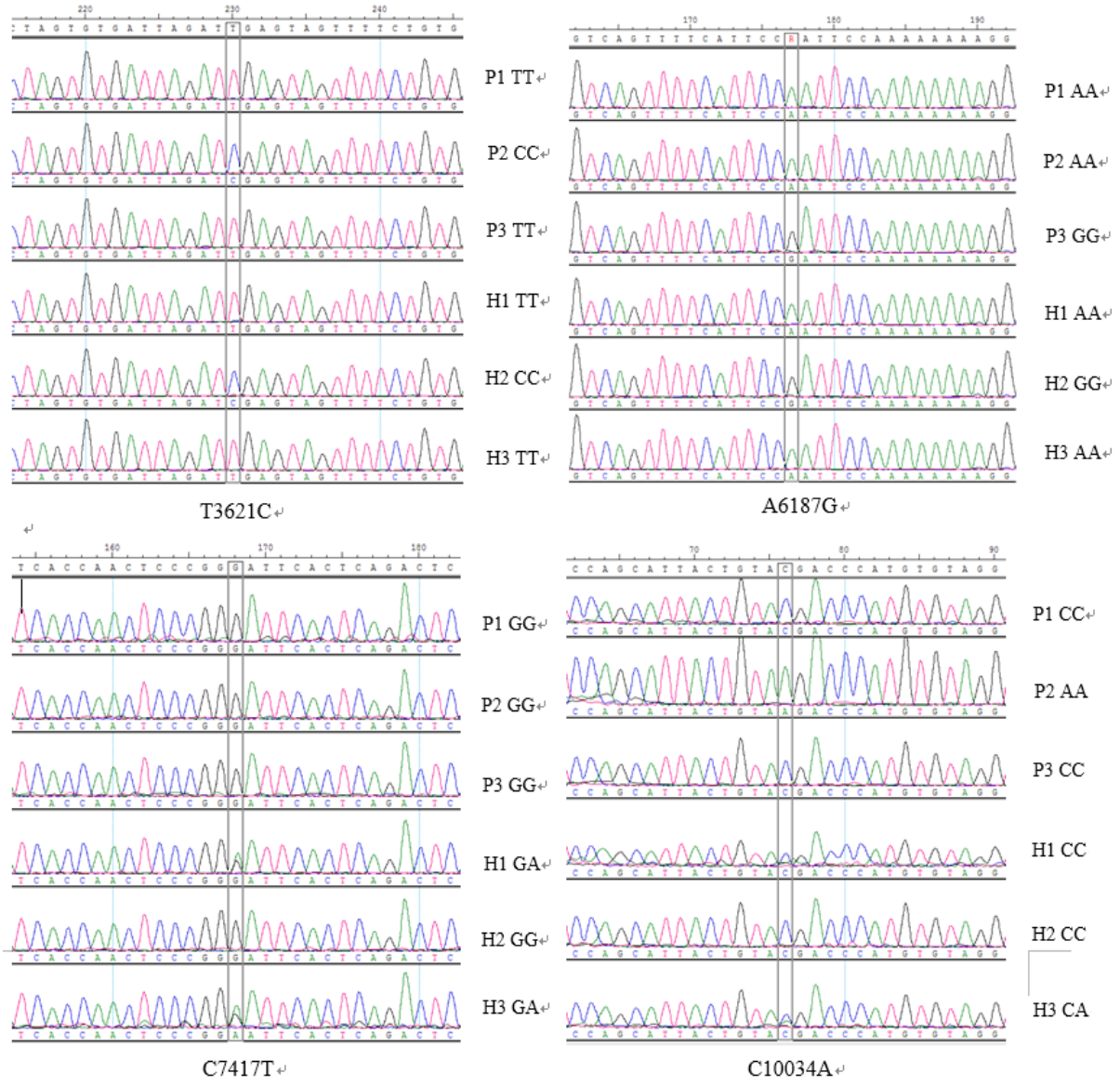

Fig. 3. Some polymorphic sites of 12.814kb. P1-P3, Polled goats; H1-H3, horned goats.

T9029G, C10034A, A10846G, del 10861-10862TT, G11842A, G12157A, G12104A, del 12431-12432AT, G12665A and del 12771-12772TT. The first 26 polymorphic loci sites were located in the PIS region, but no significant differences in polymorphic loci or large DNA fragment deletions were found in both polled and horned goats (Fig. 3). Key polymorphic gene sites affecting the horn phenotype of Guanzhong dairy goats still need to be determined.

PIS region annotation of Guanzhong dairy goats

The whole 12.814kb DNA fragment containing PIS region of goat was systematically annotated. A tRNA was predicted to exist in 4218-4290bp by tRNAscan-se online website, and its structure was also constructed. Two STRs were found in the region, STR1 (TGTGTGTGTG) and STR2 (ATATATATATAT) at 9473-9784bp, 12451-12462bp respectively by SSRHunter1. Three microRNAs, dme-mir-1, dme-mir-263a, and cbr-mir-354 were found to be located at 3002-3023 bp, 9078-9094 bp, and 10866-10882 bp, respectively using MIRAlign, and their structures were also constructed. There was a CpG island at 4390-4540 bp. The invert1 (429-2540 bp) and invert2 (5409-7514 bp) were predicted to belong to reverse repeat sequences by using EMBOSS online website, and tandem 1 (3932-5071bp) and tandem 2 (7699-10682 bp) were predicted to be two tandem repeat sequences (Fig. 4). All annotated results are shown in Figure 5.

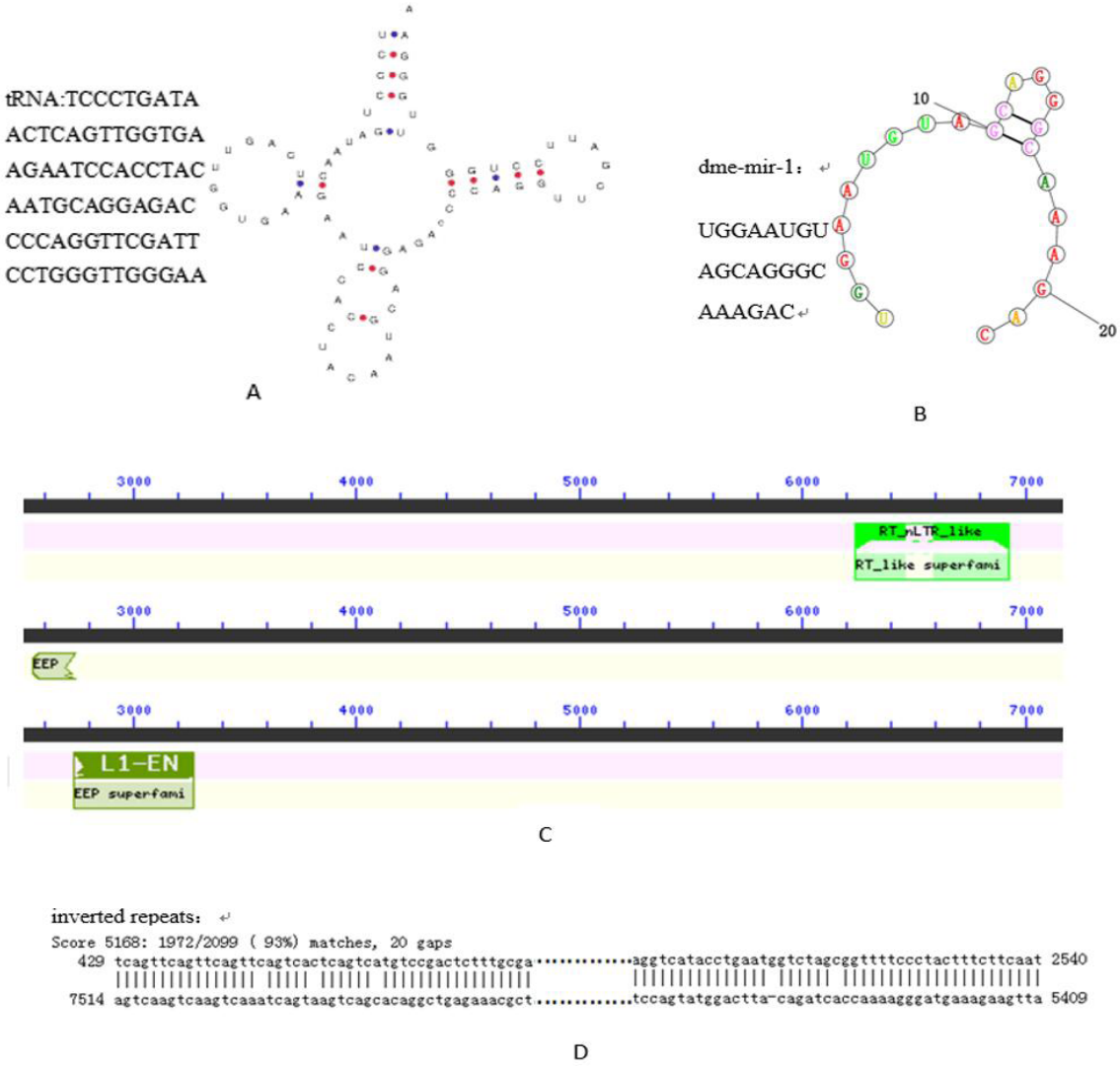

Fig. 4. The prediction result of 12.814kb sequence. A, prediction result of tRNA; B, Prediction result of microRNA; C, Prediction result of conserved domains; D, Prediction result of inverted repeats.

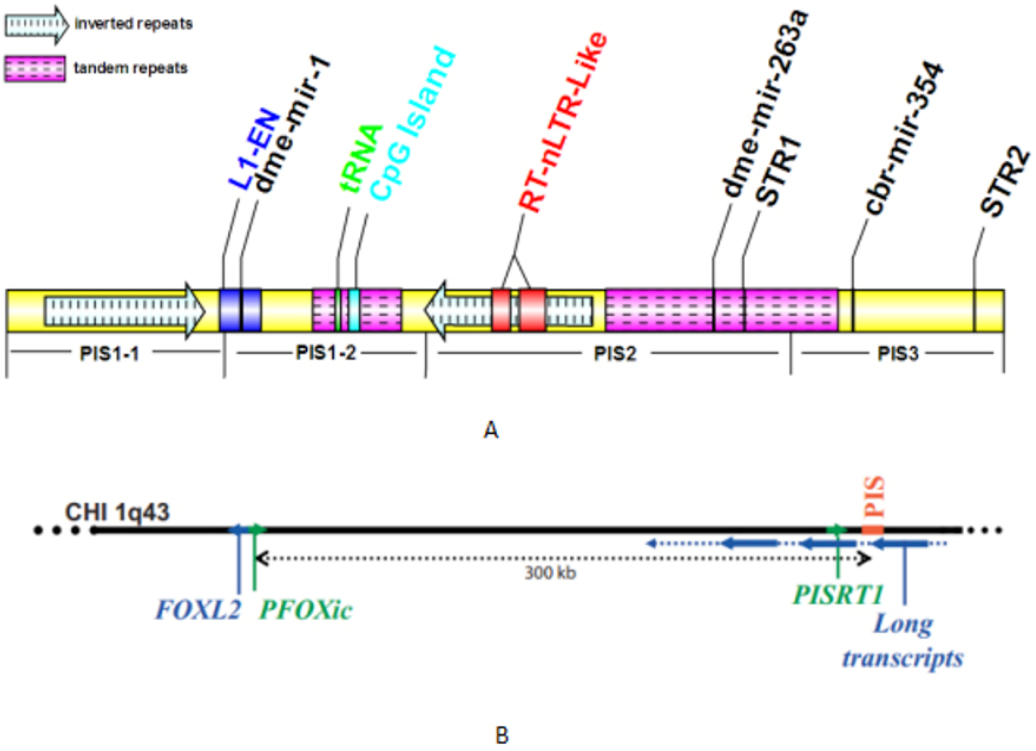

Fig. 5. Annotation result of 12.814kb sequence. A, Annotation result of 12.814kb sequence; B, the location information of PIS in chromosome.

DISCUSSION

The PIS is associated with a ~11.7 kb deletion that affects at least the expression of three nearby genes (FOXL2, PISRT1, PFOXIC). PIS are about 30 kb PISRT1 and about 300 kb in FOXL2. PISRT1 and PFOXic are long non-coding RNAs, and only FOXL2 has the function of encoding proteins (Pailhoux *et al*., 2005).

Boulanger *et al*. (2008) used nuclear transfer technology to produce intersex goats transgenic with PISRT1. Immunofluorescence analysis of 41dpc and 46dpc of intersexual goat fetuses revealed that the intersex goat fetal phenotype transferred PISRT1 gene was consistent with the intersex fetal phenotype, PISRT1 gene did not reverse the intersex traits of goats, and it was speculated that PISRT1 gene had nothing to do with the intersex traits of goats. FOXL2 encodes a fork head transcription factor that is expressed primarily in the ovary, eyelids, and pituitary. The FOXL2 gene is considered a female sex determining gene in goats.

Boulanger *et al*. (2014) used zinc finger nuclease technology to mutate the goat's fertilized ovum FOXL2 gene and then performed embryo transfer. Observation of the fetus on the 40th to 50th day of pregnancy revealed that #921 female goat have a male testicular-like organ and the eyelid was missing. It was hypothesized that the FOXL2 gene is intersexually related to the goat. In this article, quantitative analysis showed that PFOXic has a linear relationship with FOXL2 expression, while PFOXic gene shares a bidirectional promoter with FOXL2 gene. PFOXic may be only a transcriptional byproduct of FOXL2 gene.

According to the above literature reports, it was considered that the deletion of ~11.7 KB affects the expression of FOXL2 gene 300kb away from it, affecting the intersexual traits of goats. Whether 11.7 kb regulates the horned/polled traits of goats, this experiment aims to explore the molecular mechanism of the polled traits of Guanzhong dairy goats. According to the experimental results, this study did not find any complete or large fragment deletion of 11.7 KB in Guanzhong horned or polled dairy goats. A total of 30 polymorphic sites were detected, and 26 polymorphic sites were found in the PIS region, and no significant differences in polymorphic sites were found, which may be due to the small number of samples.

Sequencing results of 12.814 kb gene annotation found that there were large reverse repeat sequences (429-2540 bp, 5409-7514 bp), tandem repeat sequences (3932-

5071 bp, 7699-10682 bp) and L1-EN domain (2734-3264 bp), RT-nLTR-like domain (6235-6469 bp, 6585-6924 bp) in Guanzhong dairy goats, but the results were slightly different from those reported by Dongfeng (2009) and Dongfeng *et al*. (2008).

This experiment predicted the inclusion of STR, microRNA, CpG Island and other components, enriching people's understanding with this sequence. Where in L1-EN is the endonuclease domain of the retrotransposon long-spreading element LINE-1. RT-nLTR-like is the domain of non-long terminal repetitive sequence of retrotransposon reverse transcriptase, which indicates that this sequence contains many retrotransposon elements.

In addition to the effect of transposition on adjacent genes, transposition also triggers gene duplication or deletion of large fragments, which affects the stability of the genome and ultimately triggers various diseases (Liu, 2016). It has been reported that a large number of evidence of line-1 transposings have been found in cancer tissues such as colon cancer (Lee *et al*., 2012; Solyom *et al*., 2012) lung cancer (Iskow, 2010), prostate cancer, ovarian cancer (Solyom *et al*., 2012) and liver cancer (Shukla *et al*., 2013).

The LINE-1 methylation status is also associated with a variety of diseases. Irahara *et al*., 2010 reported that hypomethylation of LINE-1 is closely related to colorectal adenocarcinoma and has important implications for the prognosis and survival time of colorectal adenocarcinoma. Wang *et al*. (2011) reported that decreased methylation of LINE-1 can increase the risk of fetal neural tube defects. Methylation is thought to fight against transposable elements in the genome. Earlier, Burden *et al*. (2005) reported the treatment of 3T3 cells with 5-azacytidine (a pyrimidine analog) reduced DNA methylation and increased the number of LINE-1 transcripts. LINE1 promoter methylation inhibits reflex activation and transcription, so the degree of methylation of LINE1 can be used as a marker for measuring genomic stability and oncogene activity (Asada *et al*., 2006).

CONCLUSION

The above results showed the PIS region have no effect on polled phenotype of Guanzhong dairy goat, a few ofpolymorphic sites were identified in PIS region of the goat, but the key gene polymorphism sites affect polled phenotype of the goat still waiting to be determined.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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