INTRODUCTION

The mammary gland is comprised of several ductal systems and acini and is an important structure that distinguishes mammals from other animals. Mammary gland development and function are tightly orchestrated, and its main function is to produce and deliver milk to offspring (Plante et al., 2011). Teats (or nipples), epidermal appendages on the udders or breasts of mammals (Pumfrey et al., 1980), play an important role in mammalian reproduction and offspring growth.

In addition to normal teats, some mammals, such as swine, cows, and sheep, also have supernumerary teats. In pigs, teat number is a reproductive trait that directly affects the lactation rate of sows and the survival rate of piglets. Previous studies have reported that teat number is increased due to the strong effect of an insertion mutation in the Vertebrae Development Associated (VRTN) gene. Some studies have also shown that teat number is increased in other species such as swine and sheep. However, research on the genetics of teat number in shepherds and other livestock is still relatively limited. In this study, we used a genome-wide association study to identify genetic markers and genes related to the number of teats in the Hu sheep population, a native Chinese sheep breed. A single marker method and several multi-locus methods were utilized. A total of 61 SNPs were found to be related to the number of teats. Among these, 11 SNPs and one SNP were consistently detected by two and three multi-locus models respectively. Four SNPs were concordantly identified between the single marker and multi-locus methods. We also performed quantitative real-time PCR testing of these identified candidate genes, identifying three genes with significantly different expression. Our study suggested that the LHFP, DPYSL2, and TDP-43 genes may be related to the number of teats in sheep. The combination of single and multi-locus GWAS detected additional SNPs not found with only one model. Our results provide new and important insights into the genetic mechanisms of the mammalian multiparous teat phenotype. These findings may be useful for future breeding and understanding the genetics of sheep and other livestock.
gene on Sus scrofa chromosome 7 (SSC7) in Landrace and Korean pigs (Lee et al., 2014), Duroc pigs (Arakawa et al., 2015), Erhualian pigs (Wang et al., 2017a), and Large White pigs (Duijvesteijn et al., 2014). Different genes, SPRED2, MKX, TMSB4X, and ESR1, are involved in this trait in Chinese Sushan pigs (Zhou et al., 2019). In cattle, some interesting genes related to teat number have been identified. For instance, the inheritance of supernumerary teats in Holstein cattle depends on a QTL on chromosome 20 and a polygenic part (Joerg et al., 2014). Another study found that the gene LG5R on chromosome 5 was a candidate for the presence of supernumerary teats (Butty et al., 2017). In contrast to numerous studies in pigs and cattle, a study showed that BBX and CD47 on chromosome 1 were commonly identified as significant by genome-wide association study (GWAS) in Wadi sheep (Peng et al., 2017). However, genetic variants associated with teat number in Hu sheep are not known.

Teat number is a typical polygenic quantitative trait. Currently, use of the Bonferroni correction not only controls the false-positive rate for single-locus GWAS but also excludes some important loci with small effects (Zhang et al., 2019). Population structure and genetic correlations have been widely analysed by mixed linear models (Sul et al., 2018). Several recently developed multi-locus models, including the fast multi-locus random-SNP-effect EMMA (FASTmrMLM) (Tamba & Zhang, 2018), polygenic-background-control-based least angle regression plus empirical Bayes (pLARmEB) (Zhang et al., 2017), polygenic-background-control-based Kruskal–Wallis test plus empirical Bayes (pKWmEB) (Ren et al., 2018) and Iterative Sure Independence Screening (ISIS) EM–Bayesian LASSO (Tamba et al., 2017) have been shown to effectively resolve this issue.

Hu sheep, a descendant of Mongolian sheep, is a famous lambing breed in China. In ewes of the Hu sheep, TT (individuals with two normal teats) and MT (individuals with two normal teats and one or two supernumerary teats) account for 76–62% and 38–24% respectively. For MT ewes, the supernumerary teats are smaller than the normal teats, but some can produce milk. In the current study, experiment animals were raised in Inner Mongolia Golden Grassland Ecological Technology Group Co. Ltd (Bayannaoer, China). In brief, a total of 160 Hu sheep ewes (77 TT and 83 MT sheep) born within a month were analyzed. All sheep were subjected to the same growth and feeding condition. Ear marginal tissues were collected and stored in 1.5-ml microcentrifuge tubes containing 75% ethanol. In all the cases, particular efforts were made, based on both pedigree information and the knowledge of local herdsman, to ensure that the animals were as distantly related as possible.

**Sample collection and phenotyping**

In the current study, experiment animals were raised in Inner Mongolia Golden Grassland Ecological Technology Group Co. Ltd (Bayannaoer, China). Principal component analysis (PCA) and linkage disequilibrium (LD) analysis were performed using the quantified SNPs to investigate the population structure.
among the TT and MT sheep. PCA was performed with GCTA (Yang et al., 2011). LD among SNPs were estimated as the squared correlation ($r^2$) of alleles with a window size of 5000. The average LD decay distance ($r^2=0.05$) was calculated by PLINK v1.90 (Purcell et al., 2007) for the whole genome of the Hu sheep. Moreover, Weir & Cockerham Fst analysis was performed with the filtered SNPs to estimate Within–breed genetic differentiation using VCFtools (Danecek et al., 2011).

**Single–locus GWAS**

In the present study, GEMMA software (Zhou & Stephens, 2012) was used to implement LMM for single–locus GWAS of teat number. GEMMA calculated the genomic relatedness matrix between TT and MT individuals within each population to account for population structure. The first two principal components calculated using the top two eigenvectors of PCA; (Fixed effects) including the intercept; $X$ is the vector of all marker genotypes; $\beta$ is the effect size of the marker and is an estimate of the marker/SNP additive effect; $u$ is the vector of random effects; $\epsilon$ is the vector of errors; $\tau^{-1}$ is the variance of the residual errors; $\lambda$ is the ratio between the two variance components; $K$ is a known relatedness matrix; $I_n$ is the identity matrix; $\text{MVN}_n$ denotes the $n$–dimensional multivariate normal distribution; and $n$ refers to the number of sheep.

Given that Bonferroni correction is a stringent criterion, false discovery rate (FDR) was used to determine the threshold $p$ values of single–locus GWAS (Wang et al., 2017b). In the present study, FDR was set as 0.005, and the threshold $p$ value was defined as $p = \text{FDR} \times N/M$, where $N$ represents the number of SNPs with $p$ value $<0.005$ in the results of GWAS and $M$ refers to the total number of qualified SNPs for teat number in Hu sheep. The adjusted $p$ value that limited the FDR was calculated using the Benjamini–Hochberg method (Benjamini & Hochberg, 1995).

**Multi–locus GWAS**

Multi–locus GWAS was performed with four models, including FASTmrMLM (Tamba & Zhang, 2018), pLARmEB (Zhang et al., 2017), pKWmEB (Ren et al., 2018), and ISIS EM–BLASSO (Tamba et al., 2017). All four multi–locus models were implemented in the R package mrMLM (Wang et al., 2016) to detect SNPs associated with teat number in Hu sheep. Q (population genetic structure) matrix was the same as that used in single–locus GWAS and K (genomic relatedness) matrix was calculated using R package mrMLM. All SNPs were treated as random effects in the first stage of these five methods, in which the main purpose is to select all potentially relevant SNPs (Pan et al., 2018). In the second stage, the selected SNPs were fitted into the multi–locus models and the markers with largest effects that surpassed the threshold of LOD values were regarded as promising trait associated SNPs (Wang et al., 2016). The critical $p$ value parameters were set at default values in the first step. The critical threshold of LOD score was set to 3 for SNPs at final stage.

**Annotation of candidate genes**

Based on the LD decay distances of the Hu sheep, The sheep genome assembly 4.0 named Oar_v4.0 (https://www.ncbi.nlm.nih.gov/assembly/GCA_000298735.2/) were retrieved to characterize candidate genes in targeted regions. The search to for positional candidate genes was extended 200 kb up- and downstream from the significant SNPs.

**Quantitative real-time PCR**

Six Hu sheep from the Inner Mongolia Golden Grassland Ecological Technology Group Co. Ltd were randomly selected, including three TT sheep and three MT sheep. The teats of the TT sheep and the MT sheep were collected and immediately stored in RNAlater for RNA extraction. Total RNA of each sample was isolated using RNeasy pure Tissue Kit (TIANGEN, China) according to the supplied manufacturer’s instructions. For each sample, 1 μg of RNA was used for cDNA synthesis using PrimeScript™ RT reagent Kit (cat. # RR047A; Takara) according to the supplied protocol. The primer sequences were designed using Primer Premier 5.0 (Premier Biosoft International, San Francisco, CA, USA) and listed in Table S1. Quantitative real-time PCR (qRT-PCR) was performed on a 384 Real-Time System (ABI Q7 Flex; Singapore) with a 10 μl reaction system using TB Green® Premix Ex Taq™ II (cat. # RR820A; Takara) according to the supplied protocol to examine the mRNA expression levels of LHFP, TDP-43, DPYS1L2, and TWIST1. Each sample had three technical duplicates, and GAPDH and ACTB were used as reference genes to normalize target gene expression. The thermal cycling process was as follows: 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 20 s. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Arocho et al., 2006).
RESULTS

SNP genotyping

The extracted DNA from each collected sample was genotyped using the Thermo Fisher/Affymetrix Genomic Geneseeek Profilor Ovine HD 630K at Niuqin Company (Beijing, China). The quality of genotyping of the 157 Hu sheep was examined using PLINK v1.90 (Purcell et al., 2007). The characteristics of the SNPs in Hu sheep are summarized in Table S2, and Figure S1. These SNPs were roughly proportionally distributed on all 26 chromosomes of sheep, with the longest chromosome having the largest number of SNPs. The average marker density was approximately 202.71 SNPs per Mb in Hu sheep.

Population structure and LD decay

PCA was used to assess the population structure among individuals with different teat numbers, as is shown in Figure S2. To correct the population stratification, the first two principal components were embedded as covariates in the association analysis model. In addition, Q-Q plots with genomic inflation factors ($\lambda_{GC}$) were generated to assess the influence of the population structure on the single-locus GWAS (Figure 2b). Systematic inflation of the test statistics was not observed for the GWAS of either the TT or the MT sheep. The average LD decay distance of the Hu sheep was approximately 200 kb, where $r^2$ dropped to 0.05 (Figure 1). Furthermore, the pairwise Weir & Cockerham Fst value was 0.0004 between TT and MT individuals, implying little to moderate genetic differentiation (Gorssen et al., 2020).

Single-locus GWAS for teat number

Significant SNPs detected by single-locus GWAS (LMM) for the teat number of Hu sheep are shown in Table 1 and Figure 2a. Four SNPs in Chr10, two SNPs in Chr2, two SNPs in Chr5, one SNP in Chr23, and one SNP in Chr4 surpassed the threshold ($-\log_{10}(p) = 4.69$) with the FDR controlled at 0.005.

Multi-locus GWAS for teat number

Next, we performed multi-locus GWAS using several methods including FASTmrMLM, pLARmEB, pKWmEB, and ISIS EM-BLASSO. The four multi-locus GWAS identified 55 teat number-associated SNPs with LOD scores $>3$ (Table S3; Figure 3). Among these SNPs, FASTmrMLM and pLARmEB detected the highest number of SNPs (19), followed by pKWmEB (18) and ISIS EM-BLASSO (18). Eleven and one were detected by two and three multi-locus models respectively. Moreover, one SNP on Chr2 and one SNP on Chr4 detected by single-locus LMM were also identified by multi-locus models. Venn diagrams show the distribution of the SNPs from the four multi-locus methods and highlight the concordance between the single-locus method and the different multi-locus methods (Figure 4). Two SNPs were concordantly identified between the single marker and the multi-locus methods.

Candidate genes search and functional annotation

Considering the genome-wide LD decay distance of the Hu sheep used in the present study, genomic regions within 200 kb on either side of the 61 SNPs were used to mine candidate genes for teat number. To further understand the functions of the genes implicated by the GWAS, a final set of 208 genes within the LD regions of these SNPs were functionally annotated (Table 1; Table S3). Among them, TAR DNA-binding protein 43-like (TDP-43) and twist family bHLH transcription Factor 1 (TWIST1) genes were identified.
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simultaneously by the single-locus LMM model and by all four multi-locus models. LHFPL tetraspan subfamily member 6 (LHFP) was recognized by single-locus LMM model and FASTmrMLM model. Meanwhile, dihydropyrimidinase like 2 (DPYSL2) was recognized by the single-locus LMM model and the pKWmEB model. Therefore, we preliminarily identified these four genes as candidate genes and verified them by qRT-PCR. The expression divergences of these four genes between TT and MT sheep were validated by qRT-PCR as shown in Figure 5. From Figure 5, we can see that the expression levels of the LHFP, DPYSL2, and TDP-43 genes in MT individuals were significantly higher than those in TT individuals, and the expression levels in MT individuals were more than twice as high as those in TT individuals. However, we can also see that the expression of the TWIST1 gene in individuals with MT is higher than that in individuals with TT, but it does not reach a significant level. Therefore, three genes including LHFP, DPYSL2, and TDP-43 were further highlighted as promising candidates for teat number in Hu sheep.

DISCUSSION

In this study, we carried out a GWAS of teat number with a panel of 160 Hu sheep using one single-locus model (LMM) and four multi-locus models (FASTmrMLM, pLARmEB, pKWmEB, and ISIS EM-BLASSO). The combination of the single-locus and multi-locus models significantly increased the power of GWAS and detected 61 significant SNPs. According to the results of the GWAS, four candidate genes were annotated using a series of functional annotations and qRT-PCR.
Figure 3: Manhattan plots of the four multi-locus GWAS for teat number in Hu sheep. For (a–d), the Manhattan plots indicate LOD scores for genome-wide SNPs (y-axis) plotted against their respective positions on each chromosome (x-axis), and the horizontal lines indicate the thresholds for significance (LOD score = 3).
findings provide new insight into further deciphering the genetic architecture of teat number in sheep.

Previous studies demonstrated that complex traits were focused only on single-locus GWAS models by using general linear models and mixed linear models (MLMs) (He et al., 2018; Price et al., 2006; Sant’Ana et al., 2018). Nevertheless, the multi-locus GWAS model has higher statistical ability and lower FPR and is better than the single-locus GWAS model (Segura et al., 2012; Wang et al., 2016). For instance, Cui et al. performed a GWAS to detect loci related to rice salt tolerance at the seed germination stage using six multi-locus GWAS methods (Cui et al., 2018). The six models detected 371 significant SNPs and 56 QTNs co-detected by at least three methods.

In the present study, the SNPs detected by the single-locus GWAS model did not exceed the threshold line significantly. To improve the efficiency of the study, we performed multi-locus GWAS methods on the teat numbers of Hu sheep. The SNPs detected by the single-locus and multi-locus GWAS models were considered to be significantly correlated with teat number. In this study, the single-locus method had less power for detecting SNPs with minor effects and it ignored the presence of additional QTL on quantitative traits. However, multi-locus models consider multiple QTLs and treat them as random effects (Bu et al., 2020), which is close to the real genetic model of animals and plants.

In this study, we regarded LHFP, DPYSL2, and TDP-43 as functional genes associated with teat number in Hu sheep by single-locus method, multi-locus models, and qRT-PCR. Notably, TDP-43, a highly conserved
RNA- and DNA-binding protein, can be cleaved by caspase3 to generate 25/35 kDa C-terminal fragments (Zhang et al., 2007) and it induces apoptosis through a toxic gain-of-function (Zhang et al., 2009). According to a previous study, the 35 kDa fragment of TDP-43 may serve as a potential therapeutic target to cure breast cancer (Nan et al., 2018). Interestingly, Zhao et al. (2020) conducted research and found that the expression levels of TDP-43 were positively correlated with higher milk output by using human milk samples from lactating women. Mechanistically, TDP-43 is involved in post-transcriptional regulation of Btn1a1 and Xdh mRNA stability, which are required for the secretion of lipid droplets from epithelial cells into the lumen (Zhao et al., 2020). All of this evidence suggests that TDP-43 plays an important role in the breast. In the present study, the expression levels of TDP-43 in MT individuals were significantly higher than those in TT individuals. Therefore, we inferred that MT sheep might have higher milk output than TT sheep.

LHFP, as a common competing endogenous RNA hub in breast cancer, is associated with mesenchymal differentiation in glioma (Nagaishi et al., 2012). Another study found that LHFP may be involved in the mechanisms that lead to the transformation or progression of the original tumor in human breast cancer (Levy, 2021), as well as lymph node metastasis. An earlier study found that DPYSLs are a family of proteins developmentally regulated during maturation of the nervous system, and DPYSL1, DPYSL2, and DPYSL3 may be prognostic markers in breast cancer (Levy, 2021; Zottel et al., 2020). The current findings, although in need of extensive validation, are expected to promote our understanding of the underlying regulatory mechanism of the teat number trait in sheep.

Our results revealed that teat number in sheep is a complex microeffect multigene trait. However, a number of the genes associated with teat number in cattle and pigs did not match our results (Arakawa et al., 2015; Butty et al., 2017; Duijvesteijn et al., 2014; Joerg et al., 2014; Lee et al., 2014; Wang et al., 2017a; Zhou et al., 2019). In our GWAS, we treated teat number as a disease status, which may differ from teat number in cattle and pigs. In addition, different sample sizes may identify many different candidate genes associated with teat number trait.

**CONCLUSIONS**

In conclusion, 10 and 55 SNPs were found to be associated with teat number by using single-locus and multi-locus methods respectively. The integration of single-locus and multi-locus GWAS detected additional SNPs in comparison with that using only one model. Based on the result of GWAS and qRT-PCR, three novel candidate genes, LHFP, DPYSL2, and TDP-43, were found. Our findings may provide the information resource to further understanding the complexity of the genetic mechanism of teat number trait, and a reference to optimize breeding program for improvement of reproductive performances in sheep.

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**CONFLICT OF INTEREST**

The authors have declared that no competing interest exists.

**DATA AVAILABILITY STATEMENT**

Phenotypes are available at: https://figshare.com/s/0b326ab8632bbd1c82d6. Genotypes are available at: https://figshare.com/s/197c20e3229490cc5d6.

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