Whole Genome Sequencing of Hulunbuir Short-Tailed Sheep for Identifying Candidate Genes Related to the Short-Tail Phenotype

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ABSTRACT The Hulunbuir short-tailed sheep (Ovis aries) is a breed native to China, in which the short-tail phenotype is the result of artificial and natural selection favoring a specific set of genetic mutations. Here, we analyzed the genetic differences between short-tail and normal-tail phenotypes at the genomic level. Selection signals were identified in genome-wide sequences. From 16 sheep, we identified 72,101,346 single nucleotide polymorphisms. Selection signals were detected based on the fixation index and heterozygosity. Seven genomic regions under putative selection were identified, and these regions contained nine genes. Among these genes, T was the strongest candidate as T is related to vertebral development. In T, a nonsynonymous mutation at c.G334T resulted in p.G112W substitution. We inferred that the c.G334T mutation in T leads to functional changes in Brachyury—encoded by this gene—resulting in the short-tail phenotype. Our findings provide a valuable insight into the development of the short-tail phenotype in sheep and other short-tailed animals.

Sheep were among the earliest domesticated herbivores. Sheep domestication dates back to the end of the Mesolithic period, ~11,000 yr ago (Chessa et al. 2009). Domestication and artificial selection have led to marked changes in sheep behavior, appearance, and other important traits (Megenls et al. 2008). Most mammal tails are used for balance, communication, and attack. However, in sheep, tails store energy. The fat-tail phenotype is a trait necessary for survival in harsh environments (Pourlis 2011). It is exhibited by Hulunbuir short-tailed sheep, which have been bred for the past century by local herdsmen of the Hulunbuir grassland—a world-renowned highland pasture in arid and semi-arid regions of North China characterized by a short frost-free period, long and cold winter, and dry season constituting a considerable part of the year (Yang et al. 2012). The tails of Hulunbuir short-tailed sheep (Figure 1A) are of variable length, and are generally classified into two categories: extremely short, a tail exposing the anus (Figure 1C); and moderately short, a tail covering the anus (Figure 1D).

Several candidate genes—including HES7 (Bessho et al. 2001), PAX1 (Wilms et al. 1998), T (Smith 1999), and WNT3A (Greco et al. 1996)—related to vertebral development in laboratory mice are reported to be related to the short-tail phenotype; however, the determinant genes related to the short-tail phenotype in sheep remain to be identified.

Recently, many genomic regions under putative selection—which may be related to domestication, adaptation, and other important traits—have been reported in chickens, cats, dogs, and pigs (Rubin et al. 2010; Axelsson et al. 2013; Moon et al. 2015; Xiao et al. 2016). However, detection of selection signals within a single species or population cannot determine whether the genomic region is under putative selection or related to genetic drift. Genomic regions under putative selection are identified using the fixation index (Fst), which is based on significant differences in allele frequencies between two populations. However, Fst does not identify the population wherein selection occurs; hence, it cannot be used to determine the direction of selection. Therefore, in the present study, we analyzed heterozygosity (H0) to identify the specific genomic regions under putative selection. Axelsson et al.

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(2013) combined Fst and H_{p} to locate the genomic regions under putative selection, and the corresponding genes during dog domestication. Here, we applied the methodology of Axelsson et al. (2013) to locate the genomic regions related to the short-tail phenotype in Hulunbuir short-tailed sheep.

To verify the role of the determinant genes and identify novel genes regulating the short-tail phenotype, we selected sheep with extremely short tails from a population of Hulunbuir short-tailed sheep. To confirm the mutation site, 120 short-tailed sheep and 110 Barag sheep were randomly selected for whole genome sequencing; 13.1 Gb of high-quality data were generated, achieving an average fivefold genome coverage for each individual. The clean data were aligned to the sheep reference genome.

MATERIALS AND METHODS

**Ethics statement**

All animal care and experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Inner Mongolia Agricultural University.

**Samples**

Hulunbuir short-tailed sheep and Barag sheep were selected from the Autonomous County of Evenki in the Inner Mongolia Autonomous Region, China. Barag sheep (Figure 1B), which resemble Hulunbuir short-tailed sheep but have a normal tail morphology (Figure 1E), served as controls. We randomly selected 100 short-tailed sheep and 100 Barag sheep for tail-length measurement. Caudal vertebrae were collected from 10 short-tailed sheep and 10 Barag sheep. Blood samples were collected from the following sheep—eight unrelated 2-yr-old short-tailed sheep (four males and four females) with extremely short tails, and eight unrelated 2-yr-old Barag sheep (four males and four females) with normal tails were selected for whole genome sequencing; 120 short-tailed sheep and 110 Barag sheep were randomly selected to confirm the mutation site; ~2 ml of blood was collected in EDTA-containing vacutainers and stored in liquid nitrogen (−196°C). Genomic DNA was extracted from the whole blood samples using the DNeasy Blood and Tissue Kit (Qiagen, Duesseldorf, Germany).

**X-ray analysis and specimen preparation**

X-ray images of the caudal vertebrae were captured from the front using the ClearVet DR16 Imaging System. Deposits and muscle were carefully peeled off from the caudal vertebrae. The caudal vertebrae were fixed in ethanol for 5 d, and cleared by immersing in 0.5% NaOH for 2 d. To remove fat, oil, and adipose tissue, the caudal vertebrae were immersed in petrol for 3 d. Data were recorded and saved according to the order of X-ray images.

**Sequencing and SNP calling**

A sequencing library with an average insert size of 350 bp was constructed for each sample. Sequencing was performed on the Illumina Hiseqation 2000 sequencer at Novogene Corporation to generate 150-bp paired-end reads; ~13.1 Gb of high-quality data were generated, achieving an average fivefold genome coverage for each individual. The clean data were aligned to the sheep reference genome.

Figure 1 Tail phenotypes of sheep. (A) Hulunbuir short-tailed sheep; (B) Barag sheep; (C) extremely short in Hulunbuir short-tailed sheep; (D) moderately short in Hulunbuir short-tailed sheep; (E) normal tail in Barag sheep.
(Oar_v3.1 http://asia.ensembl.org/Ovis_aries/Info/Index) using Burrows-Wheeler Aligner 0.6.1 with parameters set as “mem -k 32 -M” (Li and Durbin 2010); duplications were eliminated from the alignments using SAMtools with parameters set as “rmdup.” Single nucleotide polymorphism (SNPs) were detected using SAMtools with parameters set as “mpileup-m -F 0.002 -d 1000” and annotated using ANNOVAR (Li et al. 2009; Wang et al. 2010). SNPs with low-quality scores (GQ < 20) and inter-SNP distance of <5 bp were filtered.

Selective sweep analysis
A sliding-window approach (100-kb windows sliding in 10-kb steps) was employed for quantifying $H_p$ in the short-tailed sheep and $F_{st}$ between the short-tailed and Barag sheep (Li et al. 2014). $H_p$ was calculated using the formula $H_p = 2\sum n_{Maj} \sum n_{Min}/(\sum n_{Maj} + \sum n_{Min})^2$, where $\sum n_{Maj}$ and $\sum n_{Min}$ are the sum of $n_{Maj}$ and $n_{Min}$, respectively. We transformed the $H_p$ values into Z-scores using the formula $ZH_p = (H_p - \mu H_p)/\sigma H_p$, where $\mu H_p$ is the overall average heterozygosity and $\sigma H_p$ is the SD for all windows within the group. Genetic differentiation between the short-tailed and Barag sheep was measured using fixation index ($F_{st}$) with the formula $F_{st} = 1 - p_1 q_1 + p_2 q_2/2p_1 q_1$, where $p_1, p_2$ and $q_1, q_2$ represent the frequencies of alleles A and a in populations of Hulunbuir short-tailed sheep and Barag sheep, respectively, and $p_1$ and $q_1$ represent the frequencies of alleles A and a, respectively, in the whole population (Clark and Hartl 2007). $F_{st}$ values were Z-transformed using the formula for Z$H_p$. The $H_p$ and $F_{st}$ data were calculated based on the R language package (http://www.r-project.org/). The selected regions were defined as genetic regions in overlapping windows with extremely low $ZH_p$ values ($ZH_p < -5$) and extremely high $ZF_{st}$ values ($ZF_{st} > 4.5$).

T sequencing
The primer pairs for the amplification of T exons were designed based on the sheep genome assembly (Oar_v3.1). The primer sequences are provided in Supplemental Material, Table S1. Sanger sequences of PCR duplicates were detected by Invitrogen Corporation, Shanghai, China.

Data availability
The Illumina sequence reads were deposited in the NCBI Sequence Read Archive under the accession number SRP106953. The authors state that...
all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

Measurements

The average tail lengths of the Barag and Hulunbuir short-tailed sheep were 26.32 and 16.01 cm, respectively. The average lengths of the caudal vertebrae in the Barag and Hulunbuir short-tailed sheep were 21.12 and 13.86 cm, respectively. Deformed caudal vertebrae were observed in seven Hulunbuir short-tailed sheep (Figure 2).

Genome resequencing

Eight sheep with extremely short tails were selected from the short-tailed sheep, and eight Barag sheep with normal tails were selected. We performed genome sequencing for the 16 sheep and obtained 232.23 Gb of paired-end DNA reads. Of this, 231.07 Gb (99.50%) consisted of high-quality paired-end reads (Q20 $\geq 94.33\%$ and Q30 $\geq 88.47\%$) that could be aligned to the sheep reference genome (Oar_v3.1) (Table S2). For individual sheep, the alignment rate was 98.13–98.75%. For the reference genome (excluding N’s), the average coverage depth was 4.59–5.27×. The reads with 1× coverage depth (covered by at least one base) accounted for $\geq 94.19\%$ (Table S3). The read alignments were normal, and the reads were eligible for variation detection and other analyses.

SNP and selective sweep

In the 16 sheep, we identified 72,101,346 SNPs, of which 492,307 SNPs were localized to coding regions, and responsible for 205,408 nonsynonymous nucleotide substitutions (202,338 missense, 2905 stop-gains, and
165 stop-losses) (Table S4). According to the SNP densities in the genome and reference genome assembly, the number of SNPs in the sliding window was 20, beginning from 100 kb; this trend persisted. Additionally, the regions containing 20 SNPs were stable. Therefore, the width of the sliding window was selected as 100 kb. Alignments were performed between the two populations, using a 50%-overlapping interval as the step length. For each window, Fst was calculated and Z-transformed into a ZFst value that obeyed standard normal distribution (Figure 3A). The larger the ZFst value, the greater the genetic differentiation between the populations. Hp was evaluated for the short-tailed sheep population with the same sliding-window parameters, and it was Z-transformed to standard normal distribution for the population (Figure 3A). Based on the Z value, we observed considerable genetic differentiation in many genomic regions between the populations, including those unrelated to tail morphology. To determine whether certain genetically different genomic regions were related to tail morphology, we overlapped the small interval of ZHp and large interval of ZFst. We set ZFst = 4.5 as the threshold for identifying the genomic regions under putative selection and selected windows with ZFst values greater than this threshold (Figure 3B). Moreover, we set ZHp = 2.5 as the threshold and selected windows with ZHp values less than this threshold (Figure 3C). The overlapping regions were identified and combined into seven candidate genomic regions (20–160 kb).

| Chr | Interval | Location | ZFst | ZHp | Candidate Gene | Annotation | Gene Location |
|-----|----------|----------|------|-----|----------------|------------|---------------|
| 1   | 69800001–69950000 | 4.9462 | −5.4862 | FNBPL1 | Formin-binding protein 1-like | 69805549–69915406 |
| 1   | 69800001–69950000 | 4.7863 | −5.9525 | BCAR3 | Breast cancer anti-estrogen resistance 3 | 69930328–70015474 |
| 2   | 37440001–37580000 | 4.9422 | −5.0057 | LCORL | Ligand-dependent nuclear receptor corepressor-like | 37365236–37452332 |
| 2   | 37477001–37890000 | 8.4551 | −5.1642 | T | T, Brachyury homolog | 87796143–87805552 |
| 6   | 16940001–17060000 | 4.7804 | −6.5384 | SLC35F2 | Solute carrier family 35, member F2 | 16854039–16994650 |
| 15  | 16940001–17060000 | 4.6566 | −5.9519 | RAB39A | RAB39A, member RAS oncogene family | 166995339–17027728 |
| 15  | 17090001–17100000 | 4.7602 | −6.0915 | CUL5 | Cullin 5 | 17088942–17210813 |
| X   | 77440001–77470000 | 4.8223 | −5.1215 | IRAK1 | Interleukin-1 receptor-associated kinase 1 | 77447119–77450460 |
| X   | 77440001–77470000 | 4.8801 | −5.0186 | MECP2 | Methyl-CpG-binding protein 2 | 77461651–77467940 |

Table 1 Overlapping genes identified using ZHp and ZFst

Figure 4 Alignment of amino acid sequences of Brachyury among animals. Brachyury is a protein encoded by T. Amino acid residues where p.G112W and p.V419I were located are indicated in blue. Identical amino acids, conserved substitutions, and semiconserved substitutions are indicated in red, yellow, and white respectively. Dots represent gaps in the alignment.
G-to-A transition in exon nine (c.G1255A), which corresponded to glycine-to-tryptophan substitution at amino acid residue 112 (p.G112W) and valine-to-isoleucine substitution at amino acid residue 419 (p.V419I), respectively (Figure 4).

**Validation using Sanger sequencing**

The identified nonsynonymous nucleotide substitutions in T were verified using Sanger sequencing with a large sample size in 120 short-tailed and 110 Barag sheep. Variation data were deposited in GenBank under the accession number MF996360. We detected the heterozygous form of c.G334T in the short-tailed sheep, but not in the Barag sheep. However, we detected c.G1255A in both populations (Table 2). We inferred that the c.G334T mutation in T is the primary cause of the short-tail phenotype. However, this does not exclude the possibility that other genes control the short-tail phenotype.

**DISCUSSION**

Hulunbuir short-tailed sheep are fat-tailed sheep, with large quantities of adipose deposited in their tail regions. Most previous research studies on sheep tails have focused on adipose deposition (Moradi et al. 2012; Wang et al. 2014; Yuan et al. 2017). Liu et al. (2015) suggested that tail length in short-tailed sheep is related to adipose deposition. However, we believe that tail length is related to the length of the caudal vertebrae. This prediction was supported by our observation of deformed vertebrae in the tails of seven Hulunbuir short-tailed sheep. The specific molecular mechanism functioning in the short-tail phenotype remains to be elucidated; however, we believe that the mutated T gene plays an important role in regulating this phenotype. Of the nine investigated genes, BCAR3 (Agthoven et al. 1998), FNBP11 (Huett et al. 2009), IRAK1 (Li et al. 2015), CUL5 (Byrd et al. 1997), and RAB39A (Seto et al. 2013) are related to the immune system; MECPP2 (Tao et al. 2009) and SLC35F2 (Sonuga-Barke 2013) are related to neurodevelopment; and LCORL (Singer-Hasler et al. 2012) is related to skeletal frame size. Only the T gene is related to development of the spine, and this gene seems to be associated with a short-tail phenotype in mice (Bedington et al. 1992).

We identified seven candidate genomic regions that included T. This gene was further verified using Sanger sequencing. T encodes a transcription factor named Brachyury, which is the key regulator of mesoderm formation during early development. The Brachyury protein is an important functional transcription factor, in which ~180 amino acid residues located near the N-terminus display DNA-binding activity; this is the T domain. This region can specifically bind to a 5-bp functional domain in DNA (TCACA) (Kispert and Herrmann 1993; Palena et al. 2007; Fernando et al. 2010). Brachyury is specifically expressed in the notochord of the mesoderm during gastrulation, and it regulates growth and development of the embryonic notochord. However, Brachyury is not expressed during mid-to-late pregnancy (Sangol et al. 2011).

As early as 1927, Dobrovolskaïa-Zavadskaïa described the phenotype of a Brachyury mutation in mice (Kavka and Green 1997). Mice heterozygous for the Brachyury mutation had short and slightly curved tails. Homozygous or compound heterozygous mice died in utero after ~10 d of the embryonic period because of failure to form the dorsal cord and allantois (Showell et al. 2004). The short-tail phenotype in mice was first discovered in 1927, but it was not until 1990 that the T gene was cloned (Herrmann et al. 1990). Pennimpede et al. (2012) characterized an inducible miRNA-based on an in vivo knockout mouse model of T, which exhibited skeletal defects.

We identified two loci of nonsynonymous mutations in T using genome sequencing in short-tailed sheep, and we localized the c.G334T mutation in the T domain of the Brachury gene. Specific regions in the T domain are highly sensitive to structural changes caused by mutations, and these mutations may result in conformational changes in the protein. By combining X-ray diffraction and analysis of the DNA-binding domain in Xenopus, Muller observed high similarity between amino acids in this region and those in contact with DNA (Müller and Herrmann 1997). In the present study, we inferred that the p.G112W mutation affects the binding ability of the T domain to DNA.

We did not detect the c.T334T mutation in the short-tailed sheep population, consistent with the theory that homoyzogotes or compound heterozygotes result in embryonic lethality (Buckingham et al. 2013). The c.G334G mutation was detected in individuals with normal tails. Wu et al. (2010) observed that, when mice with moderately long tails were mated with short-tailed mice, the offspring showed short-tail and normal-tail phenotypes; however, the genotypes of the offspring were not analyzed. This may explain the phenomenon of the c.G334G mutation in individuals with normal tails in the short-tailed sheep population. Herdsmen continue to breed this group and eliminate individuals with nonideal tail types and other individuals exhibiting poor phenotypes; we propose that this explains the nonconformity of genotypic frequencies with genetic theory.

In conclusion, the results of our present study suggest that the c.G334T mutation in T directly results in the short-tail phenotype in sheep. The candidate genes identified in our study provide the basis for understanding the molecular mechanism of the short-tail phenotype in sheep and other short-tailed animals.

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