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# Comparative transcriptome analysis revealed lower genetic variations of genes in cattleyak testis*

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**Reproduction of cattleyak was greatly restricted due to male infertility. Genomic sequencing indicated higher similarity between cattle and yak and studies were undertaken to investigate the mechanisms of spermatogenic arrest of cattleyak on the molecular level, while there was no information available on the genetic variants of testis transcriptomes between cattleyak and yak. To gain insight into molecular genetic characteristics of spermatogenesis for cattleyak and yak, we investigated genetic alterations for their testis transcriptomes using RNA-seq. The total number of novel transcript units identified from cattleyak was much lower than for yak. The total number of alternative splicing (AS) events identified from cattleyak was also lower than it was in yak, among which intron retention was the prevalent type of AS and it occurred on average in 40% of all AS events in cattleyak and at an average 37% in yak. The cattleyak exhibited lower transitions than yak. The transversions identified in cattleyak were also lower than in yak. The lower AS events in the testis transcriptome of cattleyak resulted in the deficiency of mRNA/protein isoforms and their critical roles in spermatogenesis, which would lead to the spermatogenic arrest. The lower genetic polymorphisms in cattleyak could be attributed to the stagnant spermatogenic differentiation in cattleyak compared to vigorous spermatogenic differentiation in yak.**

**KEY WORDS: cattleyak / genetic variants / spermatogenic arrest / testis / transcriptome**

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Cattleyak are hybrids of cattle (♂) and yak (♀), which exhibit an outstanding adaptability to the harsh environment on the Qinghai-Tibetan Plateau and higher performance levels than yak in terms of performance traits. Although cattleyak have contributed much more to the social and economic development in the regions on the Qinghai-Tibetan Plateau, reproduction of cattleyak was greatly restricted due to male infertility. Therefore, in the past decades studies were undertaken to investigate the mechanisms of spermatogenic arrest in cattleyak on the basis of morphological anatomy, histological observations, cellular and molecular analyses [Yu *et al.* 2016]. Morphological anatomy of testes showed that the seminiferous tubule wall in cattleyak was much thinner than that of yak, while the numbers of spermatogonia, primary spermatocytes and secondary spermatocytes decreased sharply at each spermatogenic stage in cattleyak [Lu *et al.* 2014]. Although cattleyak have the same number of chromosomes (2n=60) as cattle and yak, yet the synaptonemal complexes of the autosome were abnormal and no XY bivalents were observed in the primary spermatocytes of cattleyak [Guo *et al.* 1983, Hu *et al.* 2000]. In recent years some genes associated with meiosis (*SYCP3*, *Bvh*, *Dmrt7*) were selected to investigate the molecular characteristics of spermatogenic arrest in cattleyak on the molecular level [Wang *et al.* 2012, Luo *et al.* 2013, Yan *et al.* 2014]. Genomic sequencing of yak indicated that cattle and yak genes were highly similar, with 45% of encoded protein identity and approximating 99.5% of mean protein similarity [Qiu *et al.* 2012]. In this sense, cattleyak as the hybrid of cattle (♂) and yaks (♀) should also share higher gene similarities with their parental species, and the investigation of the divergences between their testis gene expressions would give new insights into the mechanisms of male infertility in cattleyak.

Second-generation sequencing of the transcriptome (RNA-seq) is a sensitive and efficient method to detect gene expression levels of single cell or a population of cells in a specific developmental stage or physiological condition [Ozsolak *et al.* 2011, Ren *et al.* 2012]. Up to now no research has been conducted in regard to the genetic variants between cattleyak and yak testis transcriptomes. In this work we examined the genetic variants between cattleyak and yak testis transcriptomes with the aim to bring insights into mechanisms of male infertility in cattleyak.

## Material and methods

### Animals and testis sample collection

Individual cattleyak (Holstein×Maiwa yak) (n=3, named C1, C2 and C3) and yak (Maiwa yak) (n=3, named Y1, Y2 and Y3) were all 12 months old and were selected at random from a pasture in the Hongyuan county, the Sichuan province of China. Testis samples from all animals were collected by veterinary surgical operation. Fat and fascia tissues surrounding the testis were eliminated and the testicular tissues were snap frozen in liquid nitrogen, transported to a laboratory and stored at -80°C for RNA extraction.

**RNA isolation, cDNA library construction and sequencing**

The total RNA from each testis sample was isolated with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Beads with oligo (dT) were used to isolate poly(A) mRNA. Purified mRNA was then fragmented in the fragmentation buffer. Using these short fragments as templates, random hexamer-primers were used to synthesise the first-strand cDNA. The second-strand cDNA was synthesised using RNase H (Invitrogen) and DNA polymerase I (New England BioLabs). Then the cDNA libraries were prepared according to Illumina's protocols. Short double-stranded cDNA fragments were purified with a QIAquick PCR extraction kit (vendor) and resolved with the EB buffer for end reparation and single nucleotide A (adenine) addition. Afterwards the short fragments were ligated to Illumina sequencing adaptors. The suitable fragments were gel-purified and amplified by PCR. The Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System was used in the quantification and qualification of the sample library. The amplified library was sequenced on an Illumina HiSeq™ 2000 sequencing machine. Raw reads produced by Illumina HiSeqTM 2000 were filtered into clean reads and aligned to the *Bos_taurus*_ncbi database containing 63379 non-redundant sequences (http://www.ncbi.nlm.nih.gov/protein? term=txid9913[Organism]) with SOAPaligner/SOAP2. The alignment data was utilised to calculate the distribution of reads on reference genes and perform coverage analysis and other downstream analyses.

**Novel transcript prediction**

To discover novel transcribed regions, we compared our assembled transcripts and annotated genomic transcripts from reference sequences. To be reported as a novel transcript, an assembled transcript must meet three requirements, i.e. the transcript must be at least 200 bp away from the annotated gene, the length of the transcript is over 180 bp and the sequencing depth is no less than 2.

**Alternative splicing analysis**

Alternative splicing (AS), which is known to be universal in eukaryotes, leads to the generation of different mRNA transcripts ultimately translated into distinguishable proteins [Black *et al.* 2003, Lareau *et al.* 2004, Stamm *et al.* 2005]. There are seven main types of AS: A) Exon skipping; B) Intron retention; C) Alternative 5' splice site; D) Alternative 3' splice site; E) Alternative first exon; F) Alternative last exon; and G) Mutually exclusive exon. To select software for AS event detection, SOAPsplice [Huang *et al.* 2011], TopHat [Trapnell *et al.* 2009], SpliceMap [Au *et al.* 2010] and MapSplice [Wang *et al.* 2010] were evaluated based on 50 nt reads simulated by Maq [Li *et al.* 2008], in which TopHat was shown to be the best and was used in our pipeline to perform this analysis. In this work we only detected four types of AS events, which were exon skipping, intron retention, alternative 5' splice site and alternative 3' splice site. The other three types were not included in our report due to high false positive results provided by the present program.

#### SNP analysis

Single-nucleotide polymorphism (SNP) in our pipeline is referred to as a DNA/RNA sequence variation occurring when a single nucleotide —A, T, C or G— differs between samples/individuals. SOAPsnp [Saldanha *et al.* 2014], a member of the SOAP (Short Oligonucleotide Analysis Package), was used to detect SNP. The program calculated the likelihood of each genotype at each site based on the alignment of short reads to a reference sequence together with the corresponding sequencing quality scores. It then inferred the genotype with the highest posterior probability at each site based on Bayes' theorem (the reverse probability model). Thus, we took into account the intrinsic bias or errors that were common in Illumina GA sequencing data and recalibrated the quality values for use in inferring consensus sequence.

## Results and discussion

#### Testis transcriptome of cattleyak and yak

To gain insight into molecular genetic characteristics of spermatogenesis for cattleyak and yak, we investigated genetic alterations for their testis transcriptomes using RNA-seq. An average of 48368556 clean reads (range: 46683344 - 48900602) per sample were generated in this study (Tab. 1). An average of 75.82% (range: 72.28-87.92%) reads per sample was mapped to the *Bos_taurus*_ncbi database containing 63379 non-redundant sequences (http://www.ncbi.nlm.nih.gov/protein?term=txid9913[Organism]). The complete clean reads for these libraries have been uploaded to the National Center for Biotechnology Information Sequence Read Archive Web site (http://www.ncbi.nlm.nih.gov/ sra/) under accession numbers SRP074227, SRP074266, SRP074815, SRP074883, SRP074994 and SRP075157. By comparing the testis transcriptome sequences of cattleyak and yak with the *Bos_taurus*_ncbi database, we identified a range of expressed genes (from 15236 to 16020), novel transcripts (from 751 to 2585), AS events (from 41052 to 63220) and SNPs (from 157519 to 232790) among the cattleyak and yak samples (Tab. 1).

**Table 1.** Summary of data from RNA-seq for testicular samples of cattleyak (C) and yak (Y)

| Sample ID | Number of clean reads | Genome map rate | Expressed gene | Novel transcripts | Alternative splicing | SNPs |
|---|---|---|---|---|---|---|
| Y1 | 48900602 | 74.50% | 15344 | 838 | 46663 | 218829 |
| Y2 | 48553728 | 73.04% | 16020 | 1885 | 55253 | 232790 |
| Y3 | 48588332 | 73.85% | 15980 | 2585 | 63220 | 226123 |
| C1 | 46683344 | 87.92% | 15236 | 862 | 41052 | 157519 |
| C2 | 48704078 | 72.28% | 15329 | 946 | 46755 | 176964 |
| C3 | 48781254 | 73.33% | 15460 | 751 | 54285 | 182674 |

#### Novel transcripts identified from testis transcriptomes of cattleyak and yak

By comparing our assembled transcripts and annotated genomic transcripts from reference sequences, we identified 7867 novel transcript units (TUs) from testis

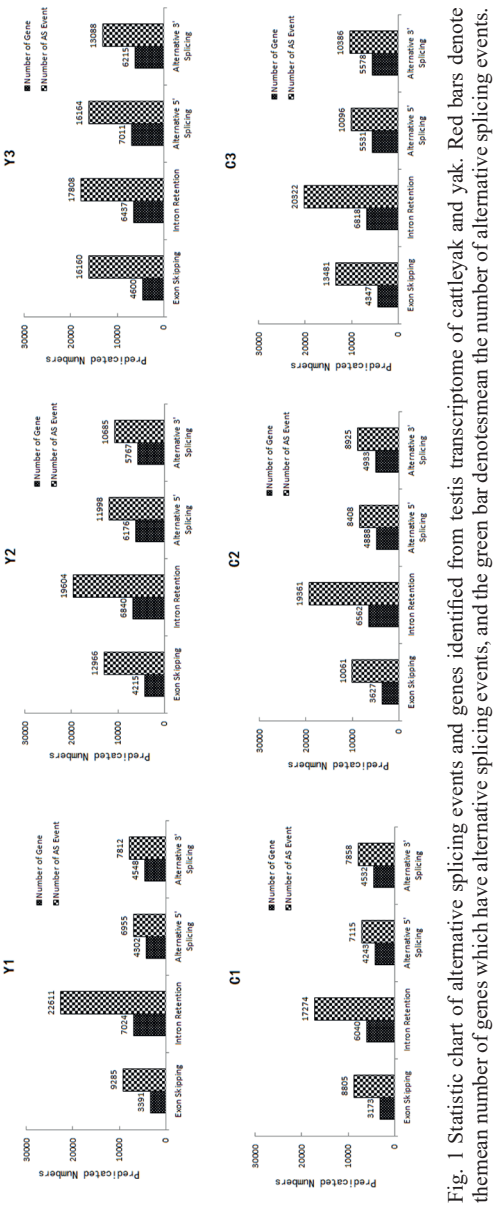


Fig. 1 Statistic chart of alternative splicing events and genes identified from testis transcriptome of cattleyak and yak. Red bars denote themean number of genes which have alternative splicing events, and the green bar denotesmean the number of alternative splicing events.

**Table 2** Number and frequency of different AS events in testis transcriptome of cattleyak (C) and yak (Y)

| Sample ID | Exon skipping | | Intron retention | | Alternative 5' splice site | | Alternative 3' splice site | |
|---|---|---|---|---|---|---|---|---|
| | number | frequency | number | frequency | number | frequency | number | frequency |
| Y1 | 9285 | 20% | 22611 | 48% | 6955 | 15% | 7812 | 17% |
| Y2 | 12966 | 23% | 19604 | 35% | 11998 | 22% | 10685 | 19% |
| Y3 | 16160 | 26% | 17808 | 28% | 16164 | 26% | 13088 | 21% |
| C1 | 8805 | 21% | 17274 | 42% | 7115 | 17% | 7858 | 19% |
| C2 | 10061 | 22% | 19361 | 41% | 8408 | 18% | 8925 | 19% |
| C3 | 13481 | 25% | 20322 | 37% | 10096 | 19% | 10386 | 19% |

transcriptome sequences of cattleyak and yak. The numbers of exons comprised in novel TUs ranged from 1 to 49 (Table S2-Table S7). The total number of novel TUs identified from cattleyak (2559) was much lower than that identified from yak (5308) (Tab. 1). This result was confirmed by our previous work, in which 120 novel miRNAs were identified from yak and 88 from cattleyak [Xu *et al.* 2018].

**Alternative splicing identified from testis transcriptomes of cattleyak and yak**

Alternative splicing (AS) plays a major role in the generation of proteomic and functional complexity in higher organisms [Blencowe *et al.* 2006]. We used TopHat in our analyses and detected four types of AS events, including exon skipping, intron retention, alternative 5' splice site and alternative 3' splice site, which were summarized in Figure 1. Among the four AS events, intron retention, in which a single intron is alternatively included or spliced, is the prevalent type of AS. Intron retention occurred averagely in 40% (range: 37-42%) of all AS events in cattleyak and 37% (range: 28-48%) in yak (Tab. 2). Exon skipping ranked second, constituting on average 23% (range: 21-25%) all AS events in cattleyak and 23% (range: 20-26%) in yak.

In our data, we found that on average 3716 (range: 3173-4347) genes per sample had undergone exon skipping in cattleyak compared to 4069 (range: 3391-4600) genes in yak. An average of 6473 (range: 6040-6816) genes per sample had undergone intron retention in cattleyak compared to 6767 (range: 6437-7024) genes in yak. On average 4887 (range: 4243-5531) genes per sample had undergone an alternative 5' splice site in cattleyak compared to 5830 (range: 4302-7011) genes in yak. In turn, an average of 5014 (range: 4532-5578) genes per sample had undergone an alternative 3' splice site in cattleyak compared to 5510 (range: 4548-6215) genes in yak. The number of genes undergoing each AS event in cattleyak was lower than that in yak.

An average of 10782 (range: 8805-13481) exon skipping cases per sample were identified in cattleyak versus 12804 (range: 1285-16160) in yak (Fig. 1). An average of 18985 (range: 17274-20322) intron retentions per sample were identified in cattleyak versus 20008 (range: 17808-22611) in yak. An average of 8540 (range: 7115-10096) alternative 5' splice sites were identified in cattleyak versus 11706 (range: 6955-6164) in yak. An average of 9056 (range: 7858-10386) alternative 5' splice sites were identified in cattleyak versus 10528 (range: 7812-13088) in yak. The total number of AS events identified from the testis transcriptome of cattleyak (142092) was lower than those from yak (165136) (Tab. 1).

**Mutations identified from testis transcriptomes of cattleyak and yak**

An average of 199150 (range: from 157519 to 232790) point mutations per sample were identified from the testicular transcriptomes of cattleyak and yak. The average occurrence of transition (A/G, C/T) per sample was 72%, which was markedly higher than that (28%) of transversion (A/C, A/T, C/G, G/T) in the testis transcriptomes of cattleyak and yak (Fig. 2, Tab. 3). The testis transcriptomes of cattleyak exhibited lower transitions (average: 124943; range: 113861-133035) than those (average: 160978; range: 156991-165412) of yak. The number of transversions identified in the cattleyak testis transcriptome (average: 47443; range: 43658-49639) was also lower than in cattleyak (average: 64936; range: 61838-67378) (Table S1).

Here, we present the genetic variants (novel TUs, AS and SNPs) between cattleyak and yak, which to date has not been documented in other studies. The most intriguing finding of this work was the lower number of genetic variants in the testis transcriptome

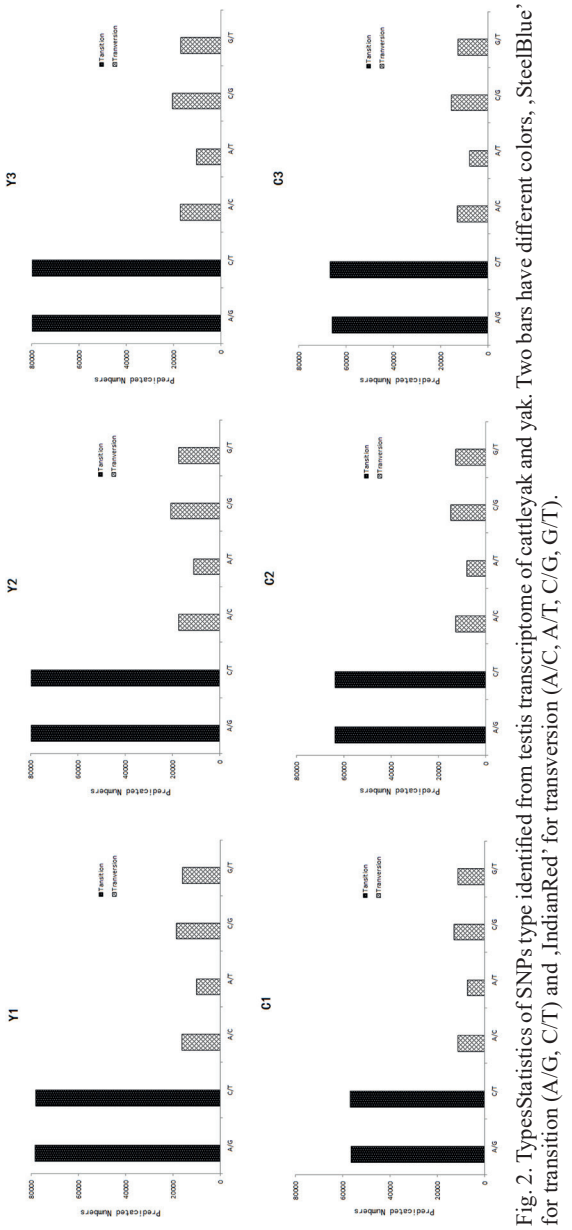


Fig. 2. TypesStatistics of SNPs type identified from testis transcriptome of cattleyak and yak. Two bars have different colors, ‚SteelBlue' for transition (A/G, C/T) and ‚IndianRed' for transversion (A/C, A/T, C/G, G/T).

**Table 3.** Number and percentage of SNP types identified from testis transcriptome of cattleyak (C) and yak (Y)

| SNP type | Y1 | | Y2 | | Y3 | | C1 | | C2 | | C3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | number | % | number | % | number | % | number | % | number | % | number | % |
| Transition | 156991 | 72 | 165412 | 71 | 160530 | 71 | 113861 | 72 | 127933 | 72 | 133035 | 73 |
| Transversion | 61838 | 28 | 67378 | 29 | 65593 | 29 | 43658 | 28 | 49031 | 28 | 49639 | 27 |
| Total | 218829 | 100 | 232790 | 100 | 226123 | 100 | 157519 | 100 | 176964 | 100 | 182674 | 100 |

of cattleyak compared to yak. Because genomic sequencing of yak indicated that cattle and yak genes were highly similar, with 45% of encoded proteins identical and mean protein similarity approximating 99.5% [Qiu *et al.* 2012], the lower number of genetic variants in the testis transcriptome of cattleyak could not be attributed to

the reference genes sequences from the *Bos_taurus*database (http://www.ncbi.nlm.nih.gov/protein?term=txid9913[Organism]) in this study. Furthermore, the similarity of genomes between yak and cattle also contributed to the similar genome map rates between cattleyak and yak. Unfortunately, the *Bos taurus* genome is not completely annotated [Zimin *et al.* 2009], which may be one of the reasons why approximately 26% of the clean reads could not be mapped.

Spermatogenesis is a complex multistage process involving many cell types，through which diploid germ cells give rise to haploid gametes. Briefly, some primitive spermatogonia A (pSGA) derived from primordial germ cells (PGC) differentiate sequentially into type A, Intermediate and type B spermatogonia through mitotic proliferation. Then, primary spermatocytes derived from type B spermatogonia divide to produce haploid round spermatids by meiosis. Finally, spermatids undergo dramatic cell transformation to form mature sperm [Cheng *et al.* 2011]. The highly regulated programme of spermatogenesis involves a correspondingly highly regulated transcriptional programme, with many layers of differentiation stage-specific transcriptional controls [Bettegowda *et al.* 2010, Berkovits *et al.* 2012]. Alternative splicing (AS) generates various mRNA and protein isoforms from single genes. These isoforms differ in structure, function, localization or other properties and play critical roles in development and disease [Black 2003, Matlin *et al.* 2005, Wang *et al.* 2008]. AS in particular is known to affect up to 94% of human genes and has been proposed as a primary driver of the evolution of phenotypic complexity in mammals [Lander *et al.* 2001, Johnson *et al.* 2003]. To assess the genetic variants of testis trancriptomes between cattleyak and yak, we investigated the frequency of the different forms for AS. Overall, we identified an average of 51205 AS events (ranging from 41052 to 63220) among the cattleyak and yak samples. An average of 47364 AS events were identified in the testis transcriptome of cattleyak, which was significantly lower than those observed in yak (55045 AS events), thus suggesting that the deficiency of mRNA and protein isoforms may have contributed to the spermatogenic arrest of cattleyak. This result was consistent with our previous investigation of differentially expressed proteins between testis proteomes of cattleyak and yak, in which the number of down-regulated proteins in cattleyak (346) was much higher than those up-regulated ones (206) [Yu *et al.* 2016].

Among the four AS events, intron retention was the prevalent type of AS and occurred on average in 40% of all AS events in cattleyak and 37% in yak, while exon skipping constituted an average of 23% all AS events in cattleyak and 23% in yak (Tab. 2). Our findings are consistent with rice AS events, where intron retention occurred in 47% of all AS events in rice, while exon skipping constituted only 25% [Zhang *et al.* 2010], in contrast to human and yeast AS events, where exon skipping is the most prevalent mechanism [Wang *et al.* 2008, Sultan *et al.* 2008]. Based on the findings in rice, the human, yeast, cattleyak and yak, the occurrence of intron retention seems to differ from species to species and intron retention could not be simply concluded to be a specific or common AS feature in plants or animals.

The average occurrence of transition per sample was markedly higher than that of transversion in the testis transcriptomes of cattleyak and yak, which was in consistent with the transcriptomic mutation pattern of other tissues in mammals [Ren *et al.* 2012, Pareek *et al.* 2016, Koringa *et al.* 2013, Quinn *et al.* 2013]. The average occurrence of transition (A/G, C/T) per sample (72%) was definitely higher than that (28%) of transversion (A/C, A/T, C/G, G/T) in the testis transcriptomes of cattleyak and yak (Table S1), which could be explained by the fact that RNA editing specifically changes adenosine (A) to inosine (I),which in turn is read as guanosine (G) [Ren *et al.* 2012, Bass *et al.* 2002].

The testis transcriptomes of cattleyak exhibited a much lower incidence of SNPs (average: 172386, 124943 transitions + 47443 transversions) than those (average: 225914, 160978 transitions + 64936 transversions) of yak. Abundant spermatogenic cells in each developmental stage were identified to distribute from the basement membrane to the lumen of the seminiferous tubule in yak testes, while only a monolayer of spermatogenic cells clung to the basement membrane in cattleyak testes [Yu *et al.* 2016]. Therefore, the lower polymorphisms of cattleyak testis transcriptomes could also be attributed to the spermatogenic arrest in cattleyak, in the testes of which fewer spermatogenic cells were in differentiation compared to vigorous spermatogenic differentiation in the testes of yak. Obviously, testis transcriptomes of cattleyak comprised fewer genes with fewer mutations than those of yak.

Taken together, our work provides extensive new knowledge on the variations between the testis transcriptomes of cattleyak and yak, which brings new insights into mechanisms for male infertility of cattleyak. The lower number of AS events in the testis transcriptome of cattleyak resulted in the deficiency of mRNA/protein isoforms and their critical roles in spermatogenesis, which would lead to the spermatogenic arrest of cattleyak. The lower polymorphisms of cattleyak testis transcriptomes could also be attributed to the fact that fewer spermatogenic cells were in differentiation in cattleyak compared to vigorous spermatogenic differentiation in yak.

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**Supplementary material.** *The following online material is available for this article: Table S1 - Illumina sequencing and tag mapping of testicular transcriptome of yak (Y) and cattleyak (C). Table S2 - Novel transcripts identified from testis transcriptome of M1. Table S3 - Novel transcripts identified from testis transcriptome of M2. Table S4 - Novel transcripts identified from testis transcriptome of M3. Table S5 - Novel transcripts identified from testis transcriptome of P1. Table S6 - Novel transcripts identified from testis transcriptome of P2. Table S7 - Novel transcripts identified from testis transcriptome of P3.*

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