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Development of a PCR Assay for Early Screening of Intersex Goats

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ABSTRACT

Background: Intersex conditions are commonly observed in dioecious animals, particularly in goats, with a higher incidence in polled individuals. Understanding the phenotypic traits associated with intersex goats and developing effective screening methods are essential for early identification and genetic improvement in breeding programs.

Methods: This study involved the collection of whole blood and gonadal tissues from 11 Chuanzhong Black goats (7 normal, 4 intersex), 6 Gulin Ma goats and 1 Tianfu goat (suspected intersex). Genomic DNA was extracted from jugular venous blood for molecular analysis and gonadal tissues were examined using hematoxylin-eosin staining. A multiplex PCR assay was developed to detect specific genetic variations linked to intersex conditions.

Result: All four intersex Chuanzhong Black goats were polled (lacked horns) and exhibited abnormal external genitalia, classified as hermaphroditic, female-like, or male-like. These goats also showed signs of disordered coat condition, hair loss and dirty hindquarters. Both internal and external reproductive organs exhibited varying developmental defects. Molecular analysis revealed the absence of the Y chromosome and structural variations in the PIS region, including homozygous deletions and reverse-repeat insertions. The developed multiplex PCR assay effectively identified intersex individuals in the Chuanzhong Black, Gulin Ma and Tianfu goat breeds.

Key words: Goats, Intersex, Polled intersex syndrome (PIS).

INTRODUCTION

Horns serve as a crucial defense mechanism for wild ruminants against predators. In livestock production, horned goats are often less favored due to management challenges and the risk of injury, making the polled trait economically beneficial. The polled trait has been actively selected in goat breeding programs. However, a higher incidence of intersexuality has been observed in polled goat populations. Intersex is a developmental anomaly in which dioecious individuals exhibit monoecious characteristics due to disrupted sex differentiation or developmental abnormalities (Wang *et al.*, 2022). The incidence of intersex individuals is rare in most mammals (typically less than 1%), whereas in goats, this figure can reach as high as 10% (Bosu and Basrur, 1984; Yang *et al.*, 2021). Hormonal imbalances in intersex goats lead to urogenital tract deformities, defecation disorders and extremely high mortality (Gupta *et al.*, 2022). Therefore, early identification of intersex individuals is crucial for improving reproductive efficiency and minimizing economic losses in goat production.

Intersexuality in goats predominantly occurs in phenotypic females with a chromosomal composition of 60XX (Cheng *et al.*, 2019; He, 2017). With the exception of a few cases caused by 60XX/XY chromosomal chimerism, most cases of intersexuality are inherited as autosomal recessive traits and are closely associated with the polled trait, known as Polled Intersex Syndrome (PIS) (Bongso *et al.*, 1982; Simon *et al.*, 2022). PIS results from a structural variation on chromosome 1, initially identified as an 11.7 kb deletion (Pailhoux *et al.*, 2001) and later refined to a 10.1 kb deletion coupled with a 480 kb duplication encompassing the *KCNJ15* and *ERG* (Simon *et al.*, 2020; Aldersey *et al.*, 2020; Guo *et al.*, 2021). The deleted region contains numerous short repeat sequences and long interspersed elements, affecting the expression of at least three significant genes related to sex determination, *FOXL2*

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(Forkhead Box L2), *PISRT1* (Polled intersex syndrome regulated transcript 1) and *PFOXic* (Promoter *FOXL2* inverse complementary), which in turn affects sex differentiation in goats (Pailhoux *et al.*, 2001; Schibler *et al.*, 2000; Vaiman *et al.*, 1996). *FOXL2* silencing has been shown to induce sex reversal in XX goats (Boulanger *et al.*, 2014). Furthermore, genome-wide association studies have identified several SNPs closely linked to PIS, including three potential marker SNPs (Chr1:129789816, Chr1:129791507 and Chr1: 129791577) and suggested *MRPS22* as a candidate gene associated with infertile intersex individuals (Zhang *et al.*, 2024). These findings support the notion that mutations and structural variations at the PIS locus represent the primary cause of intersexuality in goats.

The traditional methods for detecting PIS mutations in goats include morphological observation, cell biological methods (chromosome karyotyping) and immunological methods (H-Y antigen-antibody immunoreaction). Due to varying degrees of reproductive system abnormalities in PIS goats, morphological observations alone are insufficient for accurate sex identification. The second method, which mainly utilizes the morphological and structural differences between the X and Y chromosomes to determine the composition of the sex chromosomes, is only applicable to intersex caused by chromosomal chimerism (Fábián *et al.*, 2017). The third method utilizes the H-Y antigen, a cell surface factor unique to male embryos, for sexing goat embryos. It has been found that polled intersex goats are H-Y positive (H-Y+), indicating this method's potential for early intersex goat screening (Shalev *et al.*, 1980; Wachtel, 1977). However, this method is costly and not widely accessible. In livestock, qPCR assays targeting X- and Y-specific genes have been used to determine sex ratios in bovine semen, highlighting the reliability of PCR-based sex diagnosis (Khirwat, 2024). Accordingly, some researchers have applied PCR targeting PIS mutation loci for molecular diagnosis of intersex goats (Pailhoux *et al.*, 2001). However, the existing PCR-based method failed to identify intersex individuals among Chuanzhong Black goats. We hypothesize that the reason may be the low sequence conservatism at the PIS locus among different varieties and the fact that the establishment of some of these methods were developed before the structural variations in PIS were fully characterized.

In this study, we employed morphological, histological and molecular methods to distinguish between male and female goats. PCR was used to validate the structure of PIS variation in goats, ultimately finding that only the Polled gene is present in intersexed goats. We established a molecular assay for identifying intersex goats and successfully applied it to other goat breeds. This study provides a foundation for refining goat breeding strategies, developing polled strains of Chuanzhong Black goats and exploring the molecular mechanisms underlying hermaphroditism in goats.

MATERIALS AND METHODS

The experiment was conducted from November 2022 to June 2024 at the Key Laboratory of Livestock and Poultry Multi-Omics, Ministry of Agriculture and Rural Affairs, College of Animal Science and Technology, Sichuan Agricultural University, Chengdu.

Experimental animals

This study involved 11 Chuanzhong Black goats from Jintang County, Sichuan Province, including 7 normal (4 horned: 2 males, 2 females; 3 polled females) and 4 intersex individuals, all obtained from Zhengxin Agricultural Technology Co., Ltd. To validate across breeds, 6 Gulin Ma goats suspected of intersexuality and one horned Tianfu cryptorchid buck (rarely found naturally) were included.

Intersex screening combined phenotypic, anatomical and histopathological criteria. Phenotypically, intersex goats exhibited bisexual external genitalia, dysuria and urine-stained fur. Ultrasound confirmed ovotestis or bisexual ducts and histology revealed seminiferous tubule degene-ration and ovarian cortical fibrosis, absent in normal goats.

All animals were maintained under identical conditions and fed ryegrass, broad bean hulls and concentrates twice daily. At 08:00, 5 mL of whole blood was collected via jugular venipuncture, stored at -20°C. Animals were euthanized by electrical stunning and exsanguination and gonadal tissues were fixed in 4% paraformaldehyde for HE staining and histological sectioning.

Extraction of genomic DNA from goat whole blood

Genomic DNA was extracted using a whole-blood genomic DNA extraction kit (Tiangen, Beijing, China).

PCR primer design

To effectively genotype intersex goats, we designed four pairs of specific primers using Primer Premier 5.0 software. These primers targeted: the polled locus (Polled), two structural variants of the PIS locus (PIS-1 and PIS-2) and the SRY gene on the Y chromosome. Genomic DNA was used for PCR detection of sex and PIS mutation types in goats. The primers were synthesized by Chengdu Kengke Zixi company and the primers are detailed in Table 1.

PCR amplification

(1) Reaction system

Each 10 μL reaction contained 0.8 μL diluted genomic DNA (≥ 20 ng/μL), 5 μL 2 × Taq PCR Master Mix II, 0.4 μL each forward and reverse primer and ddH, O to volume. For the multiplex PCR, 0.25 μL Polled primer and 0.15 μL PIS-1 primer were used, with other components unchanged.

(2) Reaction conditions

95°C for 5 min; 32 cycles of 95°C for 30 s, 61.4°C for annealing (1000 bp/min) and 72°C for 30 s; followed by 72°C for 5 min and storage at 4°C.

(3) Product detection

PCR products were separated on a 1.5% agarose gel and visualized using a gel imaging system. Band sizes were compared with the DNA marker to confirm target fragment amplification. Purified PCR products were sequenced by Chengdu Tsingke Zizhao Biotechnology Co., Ltd.

Paraffin-embedded tissues were sectioned and stained with HE

The collected tissues were fixed in 4% paraformaldehyde phosphate buffer (pH 7.4), paraffin embedded, sectioned for HE staining and sealed with neutral gum for histo-pathological examination.

RESULTS AND DISCUSSION

Anatomy and histopathology of the reproductive system of intersex goats

We found that these four intersex goats were all polled, though generally, the Chuanzhong black goats are horned. Compared to normal goats (Fig 1A, B, G, H, M and N), intersex goats had extremely dirty hindquarters, lusterless coats and a "bald spot" at the base of the head horns (Fig 1 C-F). We further categorized these four intersexed goats into three main groups based on their appearance: intersex No. 1 and No. 3 goats had an androgynous appearance with paired testes, a prominent vulva, labia and a bulbous protrusion of the clitoris (Fig 1C, I, O, E, K and Q). Similar to No. 1 and No. 3, intersex No. 2 goat was female in appearance but without a scrotum (Fig 1D, J and P). Intersex No.4 goat exhibited a male-like appearance (absence of vulvar structures); however, the scrotum and testes were underdeveloped, the penis was short and the urogenital opening was abnormally positioned, with a much shorter distance from the scrotum compared to a normal buck (Fig 1F, L and R).

Histological analysis revealed that all four intersex goats possessed abnormally developed uterine structures. The uterine horns were either constricted or distended, connected to the gonads and in some cases, the uterine corpus was filled with a foul-smelling fluid (Fig 3A). In some cases, the uterine corpus was found to be degenerated and thinned. The uterine cervix was fused with a shortened, malformed penis that extended externally toward the vulva, resulting in bulbous protrusions within the clitoral region (Fig 2E). Histopathological examination confirmed that the clitoral protuberance was structurally analogous to the corpus cavernosum of the penis (Fig 2F). In intersex goat No. 4, three distinct chambers were observed in the outer layer of the penis and histological examination indicated

Table 1: PCR primer information.

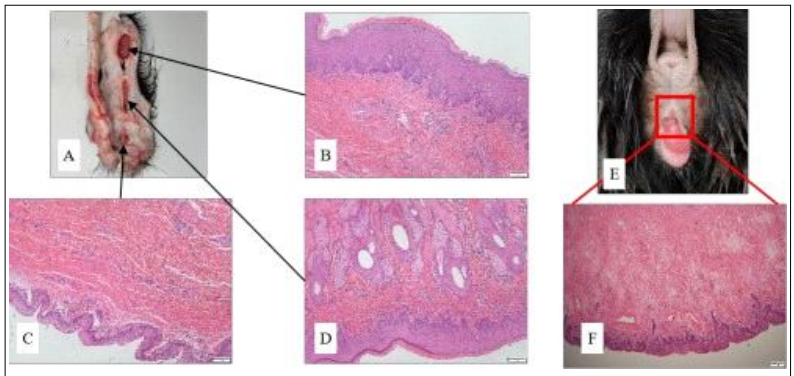
Primer	Sequence 5′ -3′	Position (goat ASR1 assembly)	Amplicon size (bp)	Tm (°C)
Polled (E G-X. *et al.*)	F: CATTTCTTCTACTAACCCATCATAA	Chr1: 150334330-306	1822	61.4
(NC_030808.1)	R: ACTGTTGGGCTCGTAAT	Chr1: 129436701-717		
PIS-1(AF404302.1)	F: ACTCATAGGCCATAGCTAAATGGT	Chr1: 129424626-649	726	61.4
	R: TGGAGAGCCCTTCCCTCTTT	Chr1: 129425351-370		
PIS-2(AF404302.1)	F: ACAGCAATACAGAAACTTTTGGAA	Chr1: 129425099-122	369	61.4
	R: TTAAATACACTTTTGGATTCGGAGT	Chr1: 129425467-443		
SRY(NW_017189563.1)	F: CTGGGATACGAGTGGAA	ChrY: 279544-560	272	61.4
	R: GACTGTGAGCGGCATAA	ChrY: 279715-731		

TS: Testicles; AN: Anus; VU: Vulva; PN: Penis.

Fig 1: Morphological observation of normal and intersex goat.

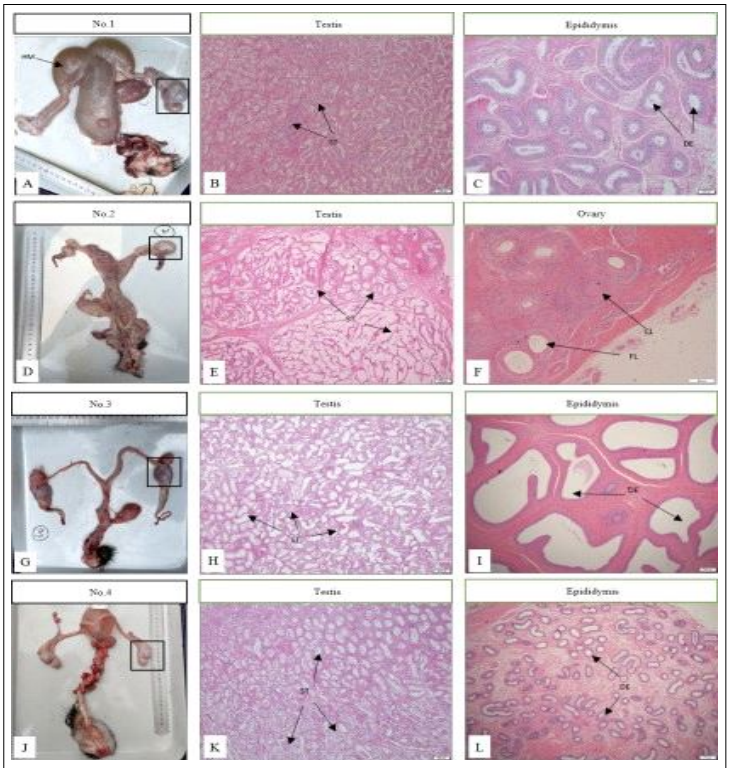
that these chambers were respectively similar in tissue structure to the prepuce and the vagina (Fig 2A-D).

Examination of gonadal tissue revealed that only intersex No. 2 had both testes and ovaries. The remaining three intersex goats had only testes and epididymides, attached bilaterally to the uterine horns, with variable sizes (Fig 3A, D, G and J). HE staining revealed that the seminiferous tubules were clearly visible as an irregular reticular structure with varying degrees of atrophy, especially in intersex No. 2 which presented the severest atrophy (Fig 3E). Severe atrophy resulted in incomplete basement membranes, leading to fusion of adjacent seminiferous tubules. The lumina exhibited vacuolation and flocculent secretions. In the testis, there was a monolayer of spermatogonia and a few supporting cells, but no other spermatogenic cells were seen and the interstitial cells were significantly proliferated, with a large number of nuclei (Fig 3B, E, H and K). Epididymal ducts in the three intersex goats exhibited incompletely developed pseudostratified columnar ciliated epithelium, with nuclear pyknosis in the lumen and irregularly arranged stereocilia. The ducts were devoid of spermatozoa; some exhibited



(A, E). Anatomical structure of gonadal tissue in intersex goat No. 4, (B, D). The vagina, (C). The foreskin, (F). Clitoral protrusion.

Fig 2: HE staining of outer penis chamber and Clitoral protrusion (20×).



The box is the tissue section sampling place; ST: seminiferous tubule; CL: Corpus luteum; FL: Ovarian follicles; DE: Ductus epididymis; HM: Hydrometra.

Fig 3: Anatomy of reproductive organs and HE staining of gonadal tissues of intersex goat (40×).

multiple circular lumens within a single epididymal duct. Erythrocytes were observed both within and around the ducts, potentially indicating a compromised blood-testis barrier (Fig 3C, I and L). In contrast, ovaries were only visible in intersex No. 2, but they were abnormally developed, with degenerated follicles and no oocytes (Fig 3F). In conclusion, anatomical and histological assessments of gonadal tissues indicated that intersex goats No. 1, 3 and 4, despite exhibiting hermap- hroditic or male-like phenotypes, possessed only testes. Intersex goat No. 2 possessed both testes and ovaries, despite its phenotypically female appearance. These findings underscore the unreliability of using external appearance alone to classify intersex types in goats.

Sex and PIS mutation in goats

To accurately detect goat sex and PIS mutations, we designed PCR primers based on the Y chromosome-specific SRY gene for sex determination. The results showed that all male Chuanzhong Black goats amplified a 272 bp band, while the 4 intersex goats, like the female goats, did not amplify this fragment, confirming that they were female (Fig 4B). We designed two pairs of PCR primers targeting the upstream breakpoint and internal breakpoint of the PIS deletion region (Fig 4A). We found that the intersex goats did not amplify the expected 369 bp and 726 bp fragments, whereas normal Chuanzhong Black goats successfully amplified these fragments (Fig 4B). Sanger sequencing and alignment revealed that the amplified products showed 99% similarity with the PIS sequence of Chuanzhong Black goats, confirming a homozygous deletion at the PIS locus.

Furthermore, using the primers targeting the down-stream breakpoint of the reverse insertion mutation at the PIS locus (Fig 4A), we amplified the 1822 bp fragment from the downstream breakpoint. The results indicated that the 4 intersex goats and 3 hornless Chuanzhong Black goats all amplified this band (Fig 4B), suggesting that they carried a reverse insertion mutation at the PIS locus. To validate the effectiveness of these primers, we applied this method to 6 intersex-suspected hornless Gulin Ma goats and the results showed a similar pattern: hornless, lacking the PIS region and SRY negative. Additionally, one Tianfu goat, identified as a male with cryptorchidism, was found to have horns and PCR results confirmed that it was SRY and PIS positive (Fig 4C).

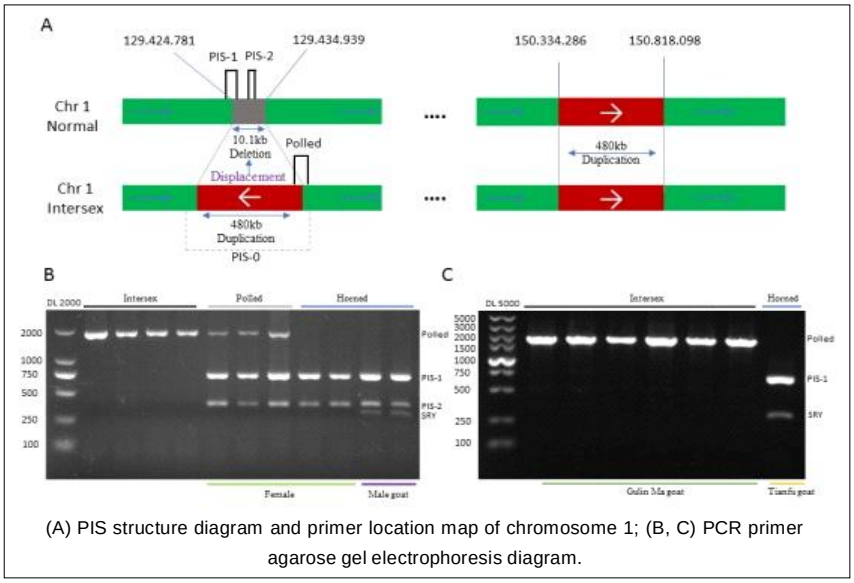
In conclusion, we optimized the conventional PCR method by utilizing three primer pairs: SRY, Polled and PIS-1, for multiplex PCR amplification. This method effectively distinguishes intersex goats (SRY-, Polled+, PIS-), normal hornless female goats (SRY-, Polled+, PIS+) and male goats (SRY+, Polled-, PIS+).

Phenotypic characteristics of intersex goats

Based on external genital morphology, the four intersex Chuanzhong Black goats in this study were initially classified as hermaphroditic, female-like, or male-like individuals. All exhibited varying degrees of developmental abnormalities in their external genitalia and were polled, consistent with previously reported phenotypic features of intersex goats (Canisso *et al.*, 2014; Quan *et al.*, 2025; Yang *et al.*, 2021; Zhang *et al.*, 2020). Interestingly, anatomical and histopathological examinations revealed discrepancies between external and internal sex characteristics: intersex No. 1 and No. 3 possessed only testes, whereas intersex No. 2 had both testes and ovaries. These findings differ from the classifications based solely on appearance, suggesting a more complex regulatory relationship between external and internal genital development in intersex goats that warrants further investigation.

Detection of PIS in goats

Effective screening and early culling of intersex goats is particularly important in production. PCR is widely used in



(A) PIS structure diagram and primer location map of chromosome 1; (B, C) PCR primer agarose gel electrophoresis diagram.

Fig 4: Detection of sex, horn and PIS variation in intersex goats.

genetic disease diagnosis due to its high specificity and reliability (Harshini, 2025; Mackay, 2004). Therefore, some studies began to use PCR to detect intersex in goats. Early studies, limited by sequencing technologies, assumed that PIS resulted solely from an 11.7 kb deletion on chromosome 1 and designed PCR primers flanking this region to genotype individuals (Pailhoux *et al.*, 2001; Pannetier *et al.*, 2005). With the advent of long-read whole-genome sequencing, a more complex PIS structural variant was identified, in which the deleted fragment was reversed and reinserted into a 480 kb repetitive region, revealing that earlier assays were insufficient (Simon *et al.*, 2020; Zhang *et al.*, 2020). Genome-wide selective scans in Chongqing goats using short-read and long-read sequencing further validated this complex PIS variant. Intersex goats consistently carried both the deletion and the reverse-repeat insertion, whereas normal goats lacked both, leading to the misclassification of PIS heterozygotes (Guang-Xin *et al.*, 2020). We designed primers for PCR amplification targeting the two mutant structures of the PIS locus and the results of amplification of the 10 intersex goats (4 Chuanzhong black goats and 6 Gulin horse goats) in this study were consistent with previously reported findings, showing two types of mutations, deletions and duplications (Guang-Xin *et al.*, 2020; Guo *et al.*, 2021). However, all polled goats had a reverse repeat insertion mutation, suggesting that the presence or absence of this repeat mutation cannot be used as a basis for distinguishing between intersex goats, but rather is closely related to the poll. This may be because previous authors selected only horned goats as controls and ignored hornless goats. Sex determination showed that all intersex goats were SRY-negative (genetically XX), consistent with previous reports that XX goats can develop male-like phenotypes (Cheng *et al*., 2019; Pannetier *et al.*, 2005). Our results both validate two mutations in intersex goats and show that their PIS reverse insertion repeat segments are strongly associated with polledness and that all normal polled goats are heterozygous for the mutation. We therefore hypothesize that the polled allele may trigger the PIS deletion, producing a normal phenotype in heterozygotes (Pp) but intersexual development in homozygous females (PP). Since PIS has not been reported in male polled goats, this mechanism appears sex-specific and may involve differential effects of the X and Y chromosomes on PIS expression (Bachtrog *et al.*, 2014; She and Yang, 2017; Veitia, 2010). This suggests that the cause of the PIS mutation occurring only in the female population in the goat might be related to the effect of the sex chromosomes and that the Y chromosome might play the role of suppressor of the PIS mutation, while the X chromosome does the reverse and the specific mechanism of action needs to be further investigated.

In this study, we validated newly identified PIS mutations in Chuanzhong Black goats and developed a reliable multiplex PCR assay for intersex screening, which was further confirmed in two additional breeds. Due to the limited number of intersex individuals, the results should be considered preliminary and larger datasets are needed to strengthen the conclusions. Overall, this work provides a practical tool for early detection of intersex goats and offers new insights relevant to polled goat breeding.

CONCLUSION

In this study, genomic DNA from three goat breeds was used to investigate mutations in the PIS region via PCR. We established a molecular assay to screen intersex goats based on phenotype and PIS mutation: both Polled and PIS were present in hornless normal goats, while only Polled was present in intersex goats. This study offers new insights into the molecular mechanisms underlying hermaphroditism in goats and contributes to advancements in goat breeding.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All operations were carried out in strict accordance with the “Code of Practice for Laboratory Animals” formulated by the Committee on Laboratory Animal Practice and Welfare Management of Sichuan Agricultural University (No. 18 of Sichuan Agricultural University [2014], No.DKY-SR220311).

Conflict of interest

The authors have read the journal’s policy and have the following competing interests: Lin Zhang is an employee of Xilingxue Agricultural Development Ltd., but there is no conflict of interest existing in this paper. The other authors have no competing interests.

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