*Animal Science Papers and Reports* vol. 29 (2011) no. 1, 19-28
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# Five novel SNPs of the bovine *LHX4* gene and their association with growth traits in native Chinese cattle breeds*

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*(Received March 25, 2010; accepted September 9, 2010)*

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**LHX4 is a LIM homeodomain transcription factor involved in pituitary ontogenesis. Some mutations of *LHX4* are associated with combined pituitary hormone deficiency that affects growth and development in animal. The objective of this study is revealing to these polymorphisms of in LHX4 gene exons 1, 2, 3 in 820 Chinese cattle and to assess possible association of polymorphisms in LHX4 gene with growth traits in NY breed. The PCR-SSCP and DNA sequencing of *LHX4* gene showed five novel synonymous mutations: (GenBank: NW_001493442.2: g.35143G >A, g.35152C>T, g.35212C>T, g.35230G>A, g.35233T>C). Genotype MM (GG-CC-CC-GG-TT) and haplotype M (G-C-C-G-T) were dominant in the four breeds, and genotype frequencies of *LHX4* in the four cattle populations agreed with Hardy-Weinberg equilibrium (*P*>0.05), although their frequencies significantly differed among the four analyzed populations (***P*<0.001). The association analysis showed that individuals with genotype MM had greater body weight than those with genotype MN (**P*<0.05) at eighteen months of age in Nanyang cattle.**

*Supported by the National 863 Program of China (No. 2008AA101010), National Natural Science Foundation of China (No. 30972080), National Key Technology R&D Program (No. 2008BADB2B03-19), Keystone Project of transfer gene in China (2009ZX08009-157B, 2008ZX08007-002 and 2009ZX08007-005B-07) and Program of National Beef Cattle Industrial Technology System (No. Nycytx-38). Basic and Foreland Technology Study Program of Henan Province (No.072300430160)..
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**KEY WORDS: growth traits / SNPs / *LHX4* gene / PCR-SSCP**

LIM-homeodomain (LIM-HD) transcription factor, LHX4 (LIM homeobox transcription factor 4) plays the essential role in pituitary development and regulates various stages of cell type specification and differentiation [Watkins *et al*. 1998, Mehta *et al*. 2008]. The important role of *LHX4* (LIM homeobox gene 4) gene had been identified by gene knockout animal models. Mice homozygous for a targeted mutation in *LHX4* suffered early postnatal death [Li *et al*. 1994]. In the single *LHX4* gene knockout animals, the development of the pituitary was arrested in the pouch stage due to apoptosis of pituitary precursor cells [Sheng *et al*. 1997, Raetzman *et al*. 2002]. In humans, studies had proved that the mutations in *LHX4* gene resulted in combined pituitary hormone deficiency (CPHD), which impaired production of GH and other anterior pituitary hormones, short stature, pubertal delay, hypoplastic anterior pituitaries [Dattani 2004, Castinetti *et al*. 2008a]. A heterozygous mutation in *LHX4* had been identified in a family affected with CPHD leading to GH, TSH, and ACTH deficiencies [Machinis *et al*. 2001]. Further study explained the mechanism: the splice site mutation (G>C) made LHX4 protein failed to bind and subsequently to activate the POU1F1 regulatory sequence [Machinis *et al*. 2005]. Up to now at least eight mutations in *LHX4* gene had been reported responsible for CPHD in humans [Machinis *et al*. 2001, Tajima *et al*. 2007, Castinetti *et al*. 2008b, Pfaeffle *et al*. 2008]. Among of them, are two single nucleotide polymorphisms (SNPs) in exon 3, which caused changes in the LHX4 protein amino acid sequence. One substitution in the LIM domain (R84C) reduced the activity of LHX4 protein [Pfaeffle *et al*. 2008]. Another mutation result in changes in the protein amino acid sequence of LHX4 (T99F). Functional studies showed that this mutation induced a complete loss of transcriptional activity of POU1F1 promoter and thus impaired the transactivation of PRL and GH promoters [Castinetti *et al*. 2008b]. Mutations in the *LHX4* gene were also showed to be associated with CPHD diseases in animal models. POU1F1, GH and PRL play critical regulation roles in milk performance, growth, reproduction, endocrine, immune and disease in animals [Vaclavicek *et al*. 2006, Lan *et al*. 2007]. Recent studies suggested that LHX4 regulated PROP1, POU1F1, ACTH, GH and PRL in *LHX3-LHX4-PROP1-POU1F1* pathway [Mullen *et al*. 2007, Mehta *et al*. 2008]. Moreover, it was recently reported that a silent mutation in exon 6 of *LHX4* gene is associated with body weight and body length in Chinese cattle [Ren *et al*. 2010]. However, there is no record of polymorphisms in the other exons of the LHX4 gene. It appears clearly essential to further research on *LHX4* gene in livestock.

The objective of this study was to analyze genetic variation of the *LHX4* gene exons 1, 2, 3, and to assess possible association of polymorphisms in *LHX4* gene with growth traits in Chinese cattle. It is expected that the results of this study will be practical for the improvement of Chinese cattle.

## Material and methods

### Animal and DNA sources

Genomic DNA samples were obtained from 820 unrelated individuals belonging to four cattle breeds: Nanyang cattle (NY, n=273), Qinchuan cattle (QC, n=308), Jiaxian cattle (JX, n=143) and Chinese Holstein (CH, n=96). All four breeds represent the main breeds of China and are reared in the provinces of Henan and Shaanxi. Records of growth traits and body sizes (body height, body length, chest girth, body weight and average daily gain) for different growth periods (birth, 6, 12, 18 and 24 months old) in 273 NY cattle were collected for statistical analysis. Genomic DNA of 820 animals were isolated from 2% heparin-treated blood samples and stored at -80 °C, following standard procedures [Sambrook and Russell 2001].

### Primer design and PCR amplification

According to the sequence of *LHX4* (GenBank accession number: NW_001493442.2), three pairs of PCR primers was designed to amplify the coding and flanking region of bovine *LHX4* gene exon 1 (*F: 5'-GTGGAATCCTGCTGGAGAAACG-3', R: 5'-GCTGTCTGGCTACCAAATTAAATCA-3'),* exon 2 *(F: 5'-AGTGCTCTGCT-GAAGGCTCAC-3', R: 5'-GGCTTAGATTAGGGCAGGGAG-3')* and exon 3 *(F: 5'-GAGGGGCTGATGAAAAGTG-3', R: 5'-TGGCAGGACAGAGGTTGG-3').* The 25 μL volume contained: 50 ng genomic DNA, 0.5 μM of each primer, 1×Buffer (including 1.5 mM $MgCl_2$), 200 μM dNTPs and 0.625 units of *Taq* DNA polymerase (Fermentas MBI). The PCR was performed using the following program: 94°C for 5 min followed by 35 cycles of 94°C for 40 s, 69°C (exon 1, exon 2) or 68°C (exon 3) annealing for 35 s, and 72°C for 30 s and a final extension at 72°C for 10 min.

### Single stranded conformation polymorphism (SSCP) and sequencing

Aliquots of 5 μL PCR products were mixed with 5 μL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanol, and 0.025% bromophenol blue), heated at 98°C for 10 min, and immediately chilled on ice. Denatured DNA was subjected to 10% PAGE (80×73×0.75 mm) in 1 × TBE buffer and constant voltage (200 V) for 2.5 h at a constant temperature of 4°C. The gel was stained with 0.1% silver nitrate [Zhang *et al*. 2007]. After polymorphism was detected, the PCR products of different electrophoresis patterns were sent to sequence in both directions in ABI PRIZM 377 DNA sequencer (Perkin-Elmer) and analyzed the sequences with BioXM software (version 2.6).

### Statistical methods

Differences in genotypic and haplotypic frequencies in the bovine *LHX4* gene exon 3 among Chinese populations were analyzed using $\chi^2$ test, which were performed by SPSS software (version 16.0). Population genetic indexes: gene heterozygosity (He), gene homozygosity (Ho), effective allele numbers (Ne) were calculated using

the PopGen software (version 3.2), and Polymorphism Information Content (PIC) was calculated by Botstein's methods [Botstein *et al*. 1980].

Statistical analysis was performed on records of growth traits in NY cattle (n=273). The growth traits of NY cattle were analyzed with the mixed linear model by the use of PROC MIXED in the SAS system (version 8.2) according to the methods of Gan *et al*. (2008). This procedure implements random effects in the statistical model and permits modeling the covariance structure of the data. The linear model was:

$$Y_{ijkl} = \mu + S_i + A_j + G_k + (AG)_{jk} + E_{ijkl}$$

where $Y_{ijkl}$ was the $l^{th}$ measurement on the $ijk^{th}$ animal, $S_i$ was the fixed effect associated with the $i^{th}$ sire, $A_j$ was the fixed effect due to the $j^{th}$ age class, $G_k$ was the fixed effect associated with the $k^{th}$ genotype, $(AG)_{jk}$ was the interaction between the $j^{th}$ age and the $k^{th}$ genotype, and $E_{ijkl}$ was random error.

Effects associated with different head or age of dam and sire were not included in the linear model, because preliminary statistical analyses indicated that these effects have no significant influence on variability of the traits in female populations. The least square means (LSMs) with standard errors and multiple range tests for two *LHX4* genotypes and traits were calculated. Because of the low frequencies of the genotypes NN (0.0073) and MH (0.0220), only one contrast was estimated-the difference between genotypes MM (n=219) and MN (n=46).

## Results and discussion

In this paper, polymorphisms in the bovine *LHX4* gene exon 1, exon 2 and exon 3 were looked for by PCR-SSCP and DNA sequencing methods. Only in exon 3 there were four unique SSCP banding patterns observed in four Chinese bovine populations (Fig. 1). In order to better understand the detailed genetic variation within the bovine *LHX4* gene, the polymorphic DNA amplification fragments were sequenced. The polymorphic DNA sequences were deposited in GenBank database

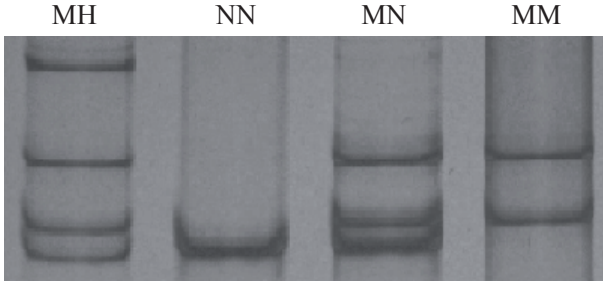


Fig. 1. The PCR-SSCP patterns of *LHX4* gene the exon 3 and its flanking region in the Chinese cattle. Four SSCP banding patterns (MM, MN, NN, MH) were observed in four Chinese bovine populations.

(under accession numbers FJ360628-FJ360632). Comparing with nucleotide sequence of the bovine *LHX4* gene (GenBank accession number NW_001493442.2) revealed five novel mutations: g.35143G>A, g.35152C>T, g.35212C>T, g.35230G>A and g.35233T>C (Fig. 2). All these mutations were synonymous, with no mutations in LHX4 protein: p.105Ala(GCG)>Ala(GCA), p.108Phe(TTC)>Phe(TTT), p.128Asp(GAC)>Asp(GAT), p.134Glu(GAG)>Glu(GAA) and p.135Asp(GAT)>Asp-(GAC). Interestingly, we found that four of these SNPs (35152 C>T, 35212 C>T, 35230 G>A, 35233 T>C) were completely linked in analyzed bovine population and thus creating a haplotype. Three haplotypes were described as: M (G-C-C-G-

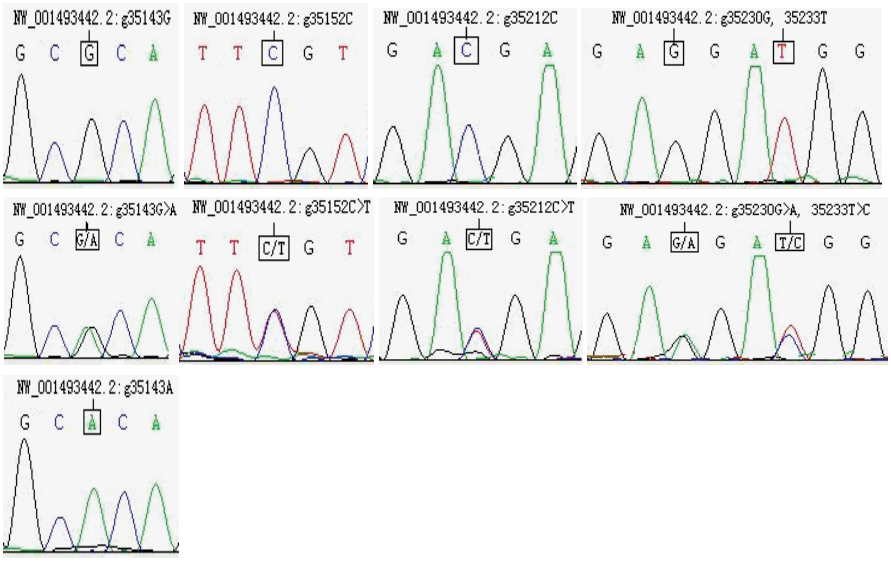


Fig. 2. The sequencing maps of five novel SNPs for exon 3 in the bovine *LHX4* gene.
a. Sequencing maps at position NW_001493442.2: g.35143 from different haplotypes of the bovine *LHX4* gene. b. Sequencing maps at position NW_001493442.2: g.35152 from different haplotypes of the bovine *LHX4* gene. c. Sequencing maps at position NW_001493442.2: g.35212 from different haplotypes of the bovine *LHX4* gene. d. Sequencing maps at position NW_001493442.2: g.35230 from different haplotypes of the bovine *LHX4* gene. e. Sequencing maps at position NW_001493442.2: g.35233 from different haplotypes of the bovine *LHX4* gene.

T), N (A-C-C-G-T) and H (G-T-T-A-C) – Figure 3. In this study, the four genotypes were described as: MM (GG-CC-CC-GG-TT), MN (G/A-CC-CC-GG-TT), NN (AA-CC-CC-GG-TT) and MH (GG-C/T-C/T-G/A-T/C), which corresponded to four polymorphic patterns found in Chinese cattle.

The frequencies of the four genotypes MM, MN, NN and MH in the four breeds were given in Table 1. Genotype MM was the dominant in the four Chinese cattle breeds. Frequencies of haplotype M were 0.8974, 0.9610, 0.8846, and 0.9792 on NY, QC, JX and CH breeds, respectively. Interestingly, there were no individual with

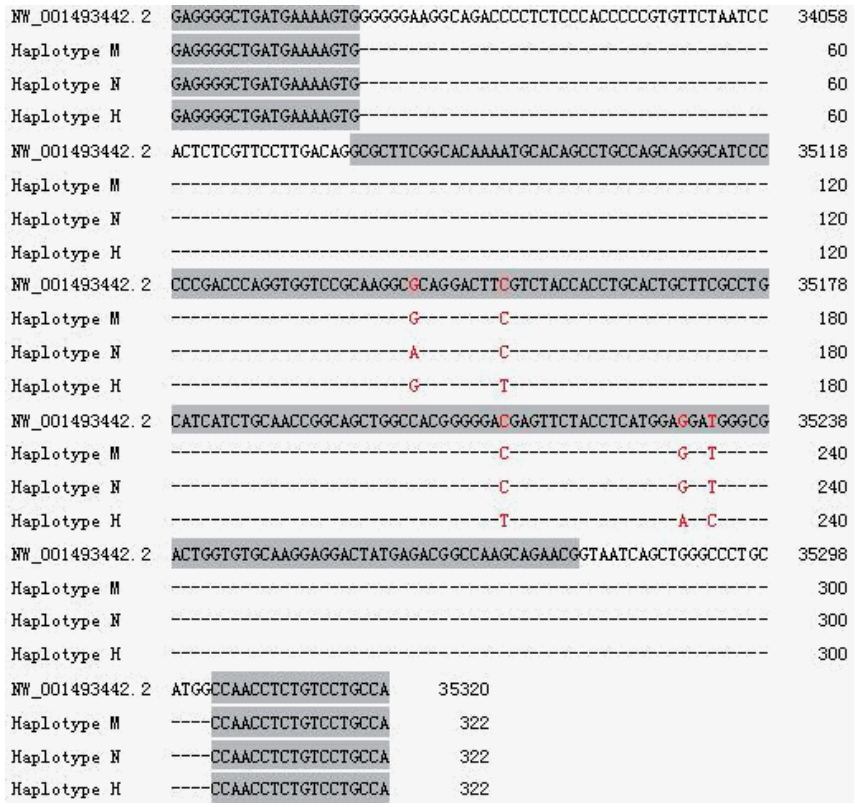


Fig. 3. SNPs *loci* for exon 3 of *LHX4* gene from different haplotypes. Haplotype M: G-C-C-G-T. Haplotype N: A-C-C-G-T. Haplotype H: G-T-T-A-C. Middle shade: *LHX4* gene exon 3. Other shades: The sites of the forward and reverse primers. Four SSCP banding patterns were described as: MM (GG-CC-CC-GG-TT), MN (G/A-CC-CC-GG-TT), NN (AA-CC-CC-GG-TT) and MH (GG-C/T-C/T-G/A-T/C).

**Table 1.** Genotypes distribution and haplotypic frequencies at the bovine *LHX4* gene exon 3 *locus*

| Breed | Observed genotypes and their frequencies | | | | | Haplotypic frequencies | | | *P*(HWE) |
|---|---|---|---|---|---|---|---|---|---|
| | MM | MN | NN | MH | total | M | N | H | |
| Nan yang (N Y) | 219<br>0.8022 | 46<br>0.1685 | 2<br>0.0073 | 6<br>0.0220 | 273 | 0.8974 | 0.0916 | 0.0110 | *P>0.05* |
| Qin chuan (Q C) | 284<br>0.9220 | 12<br>0.0390 | 0<br>0.0000 | 12<br>0.0390 | 308 | 0.9610 | 0.0195 | 0.0195 | *P>0.05* |
| Jia xian (J X) | 111<br>0.7762 | 24<br>0.1678 | 1<br>0.0070 | 7<br>0.0490 | 143 | 0.8846 | 0.0909 | 0.0245 | *P>0.05* |
| Chinese Holstein(CH) | 92<br>0.9583 | 4<br>0.0417 | 0<br>0.0000 | 0<br>0.0000 | 96 | 0.9792 | 0.0208 | 0.0000 | *P>0.05* |

M: (G-C-C-G-T) N: (A-C-C-G-T). H: (G-T-T-A-C). Genotype frequencies at the bovine *LHX4* gene exon 3 *locus*. *P* (HWE) – Hardy–Weinberg equilibrium *P* value.

genotype NN in QC and CH breeds, and no individual with genotype MH in CH breed. Moreover there were only one individual with genotype NN in JX breed and two individuals in NY breed. The genotypic frequencies of *LHX4* locus in the four breeds all agreed with Hardy-Weinberg equilibrium ($P>0.05$). Genetic index evaluated in four Chinese cattle breeds: Ho, He, Ne and PIC of *LHX4* gene are show in Table 2. The

**Table 2.** Population genetic indexes in four Chinese cattle breeds

| Breeds | Types | Ho | He | Ne | PIC |
|---|---|---|---|---|---|
| Nan yang (N Y) | meat | 0.8136 | 0.1846 | 1.2287 | 0.1725 |
| Qin chuan (Q C) | meat | 0.9242 | 0.0758 | 1.0818 | 0.0743 |
| Jia xian (J X) | meat | 0.7907 | 0.2093 | 1.2636 | 0.1947 |
| Chinese Holstein(CH) | milk and meat | 0.9590 | 0.0410 | 1.0425 | 0.0399 |

Ho – gene homozygosity; He – gene heterozygosity; Ne – effective allele numbers; PIC – Polymorphism information content.

PIC values varied from 0.0399 to 0.1947. According to the classification of PIC (PIC value < 0.25, low polymorphism; 0.25 < PIC value < 0.5, median polymorphism; and PIC value > 0.5, high polymorphism), the four breeds were all at low polymorphic level. This reflected that the genetic diversity within *LHX4* gene was not very high in the analyzed Chinese cattle. Comparing the genetic diversity of the four bovine breeds, JX cattle had the highest He and PIC, which implied that the polymorphism and genetic variation of JX breed were higher than that in other breeds. Based on $\chi^2$ test, genotypic frequencies for the various polymorphisms at *LHX4* gene were found to be significantly different among four breeds ($\chi^2$ =45.854, df =9, $^{***}P<0.001$) – Table 3. When compared with NY and JX breeds, QC and CH breeds had higher frequencies of haplotype M ($P<0.01$), which implied that this haplotype was possibly associated with milk and meat production. It also explained the *LHX4* gene played essential roles in animal development.

**Table 3.** $\chi^2$ and *P* values differences for genotypes frequencies between four Chinese breeds at bovine *LHX4* exon 3 locus.

| Breeds | Qin chuan | Nan yang | Jia xian | Chinese Holstein |
|---|---|---|---|---|
| Qin chuan | | $\chi^2$= 30.332 | $\chi^2$=25.076 | $\chi^2$=3.857 |
| Nan yang | *P*<0.001*** | | $\chi^2$=2.266 | $\chi^2$= 13.299 |
| Jia xian | *P*<0.001*** | *P*=0.519 | | $\chi^2$= 15.418 |
| Chinese Holstein | *P*=0.145 | *P*<0.01** | *P*<0.01*** | |

$\chi^2$ and *P* values haplotype differences for frequencies between two breeds were shown in the up-triangle and the down-triangle of this table, respectively (total: $\chi^2$=45.854, df=9, *P*<0.001***).

**Table 4.** Association of genotypes at the *LHX4* gene exon 3 with growth traits in NY cattle

| Growth traits | Genotypes at the *LHX4* exon 3 *locus* | | |
|---|---|---|---|
| | MM (mean±SE) | MN (mean±SE) | *P* value |
| Birth weight (kg) | 29.532±0.216 | 29.800±0.426 | 0.576 |
| Six months BW (kg) | 159.731±2.182 | 159.250±4.308 | 0.921 |
| Twelve months BW (kg) | 222.385±2.497 | 218.800±4.931 | 0.518 |
| Eighteen months BW (kg) | $300.244^{a}$±3.315 | $285.000^{b}$±6.547 | 0.040* |
| Twenty-four months BW (kg) | 369.846±4.318 | 352.500±8.527 | 0.073 |

BW – body weight.
*Values with different superscripts within the same line differ significantly at $P<0.05$ (a, b).
SE – standard error of means.

Herein, we revealed for the first time an association of the polymorphism of *LHX4* gene exon 3 with growth traits in the NY breed. Cattle with the MM genotype had greater body weight than those with genotype MN at eighteen months of age ($^{*}P<0.05$) – Table 4. The rest of the records of growth traits had no significant association ($P>0.05$) (data not show). As, the SNPs studied here were silent mutations, one may suggestion is that naturally occurring silent SNPs can lead to the synthesis of protein product with the same amino acid sequence but different structural and functional properties [Komar 2007, Sauna *et al*. 2007]. Another possible explanation is that the haplotypes are likely to be in disequilibrium with other SNPs that truly affect animal development or affect transcriptional efficiency of the *LHX4* gene [Stachowiak *et al*. 2007, Oh *et al*. 2007]. It was showed that two mutations in exon 3 (coding LIM2 domain) which affected activity of LHX4 protein had lead to CPHD in human [Pfaeffle *et al*. 2008, Castinetti *et al*. 2008]. The regulating pathway *LHX3-LHX4-PROP1-POU1F1* and the hypothalamus-pituitary-GH axis are also very important for animal development [Mehta *et al*. 2008, Vaclavicek *et al*. 2006, Mullen *et al*. 2008]. Therefore, *LHX4* gene seems to be promising candidate for a genetic marker as it plays an important role in cattle's growth traits. However, despite all the available information concerning *LHX4* in human and mouse, there is very few information known in bovine. So it appears clearly essential to further research on *LHX4* gene in livestock.

In conclusion, the present study revealed five novel SNPs in exon 3 of bovine *LHX4* gene. These polymorphisms occur as a haplotype. But the function of the completely linking silence mutations is unknown and needed further verification. The *LHX4* genotype associated significantly ($^{*}P<0.05$) with body weight at eighteen-month of age in Chinese NY cattle. Considering the haplotype N was recessive in four cattle breeds, we should reject the genotype NN in the breeding schemes of cattle.

This research will be practical for the improvement of Chinese native cattle. Furthermore, these results may be instructional for breeding selection of Chinese indigenous cattle.

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