RESEARCH ARTICLE

Polymorphisms within IGF1 and IGF1R Candidate Genes in Gaolao Cattle Population

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Abstract

IGF1 gene expresses during lactation, mediates galactopoietic effects, and regulates metabolism and growth, while IGF1R gene is related with milk components. The objectives of this study were to identify polymorphisms within IGF1 and IGF1R genes and to find association with milk traits. The blood was collected from 262 purebred Gaolao cattle from the breeding tract, and test-day milk traits were recorded. PCR-*SnaB*I analysis of the 5'UTR region of the IGF1 gene revealed only BB genotype; however; IGFG1-SSCP screening of 5'UTR region of IGF1 gene revealed polymorphism with pattern A frequency as 0.685 and of B as 0.314. IGFG2-*Tas*I PCR-RFLP analysis of 5'UTR region of IGF1 gene revealed CC genotype. The monomorphic PCR-SSCP followed by sequencing revealed 14 computational SNPs. IGFRG5-*Taq*I PCR-RFLP within exon-21 region exhibited genetic variation at the locus. The IGFRG5 screening of the same exon-21 region revealed two polymorphic SSCP patterns, which after direct sequencing revealed SNP A>G at 36th position. The findings enriched IGF1 and IGFR candidate gene variation status, and is expected to aid in selecting and breeding decisions to improve Gaolao cattle.

Key words: IGF1, IGF1R, Milk traits, Polymorphism, Zebu cattle.

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Introduction

Evaluation of genetic variations within candidate genes contributes to the molecular characterization of livestock breeds and modifications at the genetic level in the required direction to achieve sustainable progress in improving economic traits in dairy cattle. Various studies have identified candidate gene polymorphism for molecular marker development for future breeding programmes of cattle (Potu *et al*., 2022). IGF1 plays an essential physiological role in cattle's growth regulation, development, metabolism, and lactation. Many researchers have reported an association between the IGF1 genotypes and milk yield and composition (Bonakdar *et al*., 2010; Mehmannavaz *et al*., 2010; Szewczuk *et al*., 2011). As a member of the somatotropic axis, insulin-like growth factor I receptor (IGF1R) seems to be a promising candidate gene because it encodes a large portion of the putative ligand binding pocket of bovine IGF1 receptor, with milk production traits (Szewczuk, 2016). Gaolao cattle are a unique breed adapted to adverse and harsh tropical climatic conditions of the Vidarbha region of Maharashtra, consisting of some superior yielding animals. Hence, the current research was planned to identify IGF1 & IGFR gene polymorphisms and their association with milk traits.

Materials and Methods

The experiment and research plan under the Science and Engineering Research Board-Department of Science and Technology (SERB-DST) project (File No. EMR/2017/000323) was duely approved by the Institutional Animal Ethics Committee (No. NVC/IAEC/24/2019, Dt. 12/04/2019) of Nagpur Veterinary College, MAFSU, Nagpur, India.

Experimental Animals and DNA Extraction

In this study, adult purebred Gaolao cows (n=262) were identified and recorded from different villages and farms in the Wardha district. Around 05 mL of blood was collected

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from 262 unrelated Gaolao cattle, and around 15 mL of test-day milk samples were collected to generate test-day milk records. The isolation of DNA was carried out using the Phenol-Chloroform extraction protocol with some modifications in the laboratory.

PCR-RFLP and PCR-SSCP

For the current study, five primers were selected for the 5′ UTR region in the IGF-1 gene, and exon 12, and exon 21 regions of IGF1R gene (Table 1). A total of 25 µL of PCR reaction mixture volume was prepared using 1XDreamTaq Green PCR Mastermix, primers, 4% DMSO and 25-50 ng/µL template DNA. The optimized thermal cycling conditions for IGFG1 primer were 97°C for 2 min, 31 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 45 s, extension at 72°C for 60 s and final extension for 72°C for 5 min. Similarly, optimized cycling conditions for other primers were standardized at different annealing temperatures (Table 1). The PCR products obtained after the PCR amplification were checked by resolving in 1.5% agarose gel electrophoresis stained by ethidium bromide. The PCR products were subjected to restriction digestion analysis using reported restriction enzymes (Table 1). Around 5 U/µL of *Sna*BI and *Tas*I enzymes were used in the restriction digestion followed by incubation at 37°C overnight.

Sequencing, Bioinformatics and Statistical Analysis

Based on PCR-RFLP and PCR-SSCP analysis results, the selective PCR products were selected for confirmation of mutation by sequencing. The obtained DNA sequences were edited and analyzed using bioinformatics tools like BioEdit (Version-7), BLAST (Version BLASTN), Clustal omega (Version, 1.2.4) softwares. The Popgene 32 software (Version, 1.32) was used for the estimation of gene and genotypic frequencies and to test Hardy-Weinberg equilibrium. The effect of IGF1 and IGF1R genotypes on milk yield and components was tested by logistic regression model using SPSS Version 20 (IBM, USA). The model is as below;

$$GT_{ijklmno} = \mu + MY_i + F_j + S_k + L_l + P_m + e_{ijklmno}$$

Where, GT_{ijklmn} is the observation of Genotype, µ= overall mean, MY_i = random effect of milk yield, F_j= random effect of fat percent, S_k= random effect of SNF percent, L_l= random effect of lactose percent, P_m= random effect of the protein percent, $e_{ijklmno}$= residual error.

Results and Discussion

For the current study, gene polymorphisms at 5′ UTR region in the IGF1 gene and exon 12 and exon 21 region of IGF1R gene were studied in the Gaolao cattle population.

IGF1G1-*SnaBI* PCR-RFLP Genotyping

The IGF1G1 primer (Ge *et al*., 2001) was used to amplify 249 bp PCR product of the 5′UTR region of the IGF1 gene in Gaolao cattle. In the current study, RE digestion analysis using *Sna*BI enzyme revealed only 249 bp fragments, *i.e., BB* in all the 262 animals screened, indicating the monomorphic status of IGF1G1-*Sna*BI locus and fixation of *B* allele in the studied population (Fig. 1). Association study was not possible due to the monomorphic status of the locus in the Gaolao cattle; however, Wasielewska and Szatkowska (2019) studied IGF1 polymorphism in 5′UTR region and association studies on Black-and-White variety of Holstein-Friesian cows and reported frequency of *BB, AB & AA* genotype as 0.257, 0.483 and 0.259, respectively, and reported higher milk yield for *BB* genotype and lowest for *AB* genotype. Similarly, Szewczuk *et al.* (2013) screened 191 animals of Polish Holstein-Friesian using IGF1-*SnaBI* locus and reported genotypic frequencies for *BB*, *AB* and *AA* genotype as 0.183, 0.549 and 0.267, respectively. They found favourable association between *BB* genotype and milk yield, milk fat, and protein yields ($P \leq 0.01$). Polasik *et al.* (2014) reported the frequency of *BB, AB* and *AA* genotypes as 0.20, 0.62 and 0.18, respectively, and found that cows exhibiting *BB/AB* genotype were superior for milk yield.

Similar to the present study, PCR-RFLP analysis of the 5′UTR region of the IGF1 gene was carried out by Muin (2010) using *Sna*BI enzyme in 242 animals comprising three populations of Bali cattle, and reported only *BB* genotype.

Table 1: Primers, sequence, region, product size, annealing temperatures and technique used in IGF1 and IGFR gene polymorphism study in Gaolao cattle

Gene / Region	Primer Sequence	Product Size/*Tm*(˚C)	Techniques	References
IGF1G1/ 5′ UTR	F:5′ATTACAAAGCTGCCTGCCCC3′ R:5′ACCTTACCCGTATGAAAGGAATATACGT3′	249 bp/ 58˚C	IGF1-*SnaBI*/ IGFG1-SSCP	Ge *et al.,* 2001
IGF1G2/ 5′ UTR	F:5′TCATCCAGCTGAGAGATTTGAAT 3′ R:5′TGTGTGTGTGTGTGTGTGAAT 3′	146 bp/ 56˚C	IGFG2–*Tas*I/ IGFG2-SSCP	Zych *et al*., 2007
IGF1G6/ 5′UTR	F:5′GGCCAAGCAGCAGAGTAGAG3′ R:5′ GGAAACAGCTGGGGGAAC3′	623 bp/ 58˚C	Direct sequencing.	Ramesha *et al.,* 2015
IGFRG4/ Exon 12	5′ -TTCTTGCCTGTTTCAATTGTTG- 3′ 5′ -CTCGACTTGGGATCCATATTTT- 3′	164 bp/ 54^{0}C	IGFRG4-SSCP	Szewczuk, 2016
IGFRG5/ Exon 21	5′ -GCCGGTCACCATAGGTCTCG- 3′ 5′ -AGTGGGGGTTTTGGCAGAAT- 3′	163 bp/ 54^{0}C	IGFRG5-*Taq*I/ IGFRG5-SSCP	Szewczuk, 2016

*T*m, Annealing Temperature

Omer *et al*. (2018) studied IGF1-*SnaB*I polymorphism in Sudanese cattle breeds and reported higher frequency of *B* allele (0.932) as compared to *A* allele (0.068) in the Nyalawi breed. They also reported a higher frequency for *B* allele (0.921) as compared to *A* allele (0.079) in Mesairi breed of Sudanese cattle. In an IGF1 polymorphism study (Putra *et al*., 2018) using *SnaB*I enzyme in Pasundan cattle, only *BB* genotype indicated monomorphic locus status.

However, compared to our study, various other researchers have reported relatively less frequency of *BB* genotype in the studied cattle population groups. After genotyping 56 Turkish Grey and Holstein crossbred bulls for IGF1-*SnaB*I polymorphism, the absence of *BB* genotype was found, with a higher frequency of *AB* genotype 0.63 for Turkish Grey and 0.69 for Holstein crossbred cattle (Ardicli, 2018). In 282 animals of Iranian Holstein cattle (Mehmannavaz *et al*., 2010), there was less genotype frequency (0.28) for *BB* genotype, and higher breeding value for milk and fat yield in *AB* genotyped bulls. The PCR-RFLP polymorphism for the locus studied in 760 calves of Angus cattle revealed less frequency for *B* allele (36.1%) as compared to *A* allele (63.9%, Ge *et al*., 2001). In other studies, the frequency for *B* allele reported was 0.33 in 163 Montbeliarde cows without a difference between genotype and milk traits (Szewczuk, 2016). The frequency of *B* allele at the locus was 0.42 in 100 Holstein cows of Kazakistan (Beishova *et al.,* 2019). In other study, Czerniawska-Piatkowska *et al.* (2021) reported less *B* allele frequency than the current study. They screened 555 animals consisting of Jersey, Polish Holstein Friesian black and white & red and white and reported the average frequency of *B* allele as 0.47 and *A* allele as 0.53 in Jersey & Polish Holstein Friesian black and white breed. They found a significant difference between *BB* genotype and milk and protein yield, indicating the significance of *BB* genotype for milk traits. Genotyping of 135 daughter animals of Flazhok, Ombeto and Riverson bull revealed the frequency of *B* allele as 0.45 and *A* allele as 0.55 with non-significant difference (Ulyanov *et al.,* 2021). Szewczuk *et al.* (2012) did genotyping of 201 Polish Holstein cows and reported the frequency of *B* allele as 0.46 and *A* allele as 0.54; however, they did not find an effect of genotype on milk traits. In their another study (Szewczuk *et al.,* 2013) screening of 191 Polish HF cattle, they recorded 0.46 frequency for *B* allele with favourable association of *BB* genotype with milk, fat and protein yield. Thus, it can be concluded that genotype *BB* which is desirable genotype for milk traits exist prominently in Gaolao cattle.

IGF1G1- SSCP and Sequencing

The IGFG1 amplified 249 bp fragment was analyzed by PCR-SSCP analysis using 12% non-denaturing PAGE, revealing polymorphism with two SSCP patterns (Fig. 1). The computational SNP (A-T) was found at 180th position after alignment analysis between the sequence of Gaolao cattle with the reference sequence (GenBank Acc. No.: AY803779.1).

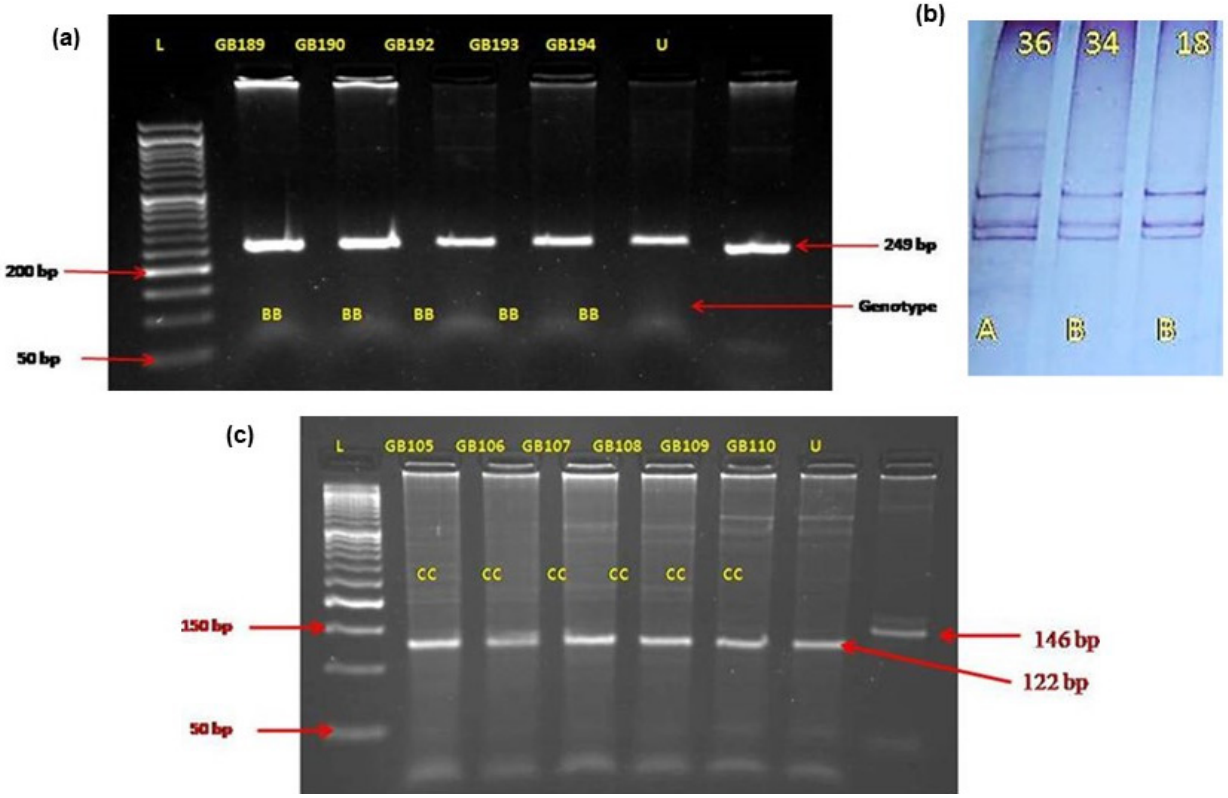

Fig. 1: IGF1 Candidate gene polymorphism analysis (a) Monomorphic PCR-RFLP at IGF1G1-*SnaB*I locus for 249 bp gene fragment of 5'UTR region resolved in 3.5% agarsoe gel electophoresis (b) Polymorphic IGF1G1-SSCP patterns resolved in 12% non-denaturing PAGE stained by silver staining (c) Monomorphic IGF1G2-*Tas*I PCR-RFLP resolved in 3.5% agarose gel electrophoresis in 146 bp of 5'UTR region in Gaolao cattle population.

Similarly, two SNPs were discovered in the 5′ UTR region of the IGF1 gene in 36 bulls of Jersey cattle (Ramesha *et al.,* 2015). Some SNPs were detected in the 5′ non-coding region of the IGF1 gene in 148 HF cows (Zych *et al.,* 2007). Screening of 5′ flanking region of the IGF1 gene in 752 Chinese Holstein cows showed single transition SNP at position g.1407T-C and relation of *TT* genotype related with milk fat and protein yield (Alim *et al.,* 2012).

IGF1G2 -*Tas*I and Sequencing

The PCR product of 146 bp size (IGF1G2 primer) was amplified as per the PCR components and thermal cycling conditions with minor variations in the laboratory (Zych *et al.,* 2007). In the present IGF1G2 -*Tas*I genotyping study, it was found that the *A* allele was fixed in the studied Gaolao cattle population (Fig. 1). Similarly the high frequency of *A* allele (0.84) was found in 148 Holstein Friesian cows (Zych *et al.,* 2007). Genotyping of 191 Polish HF black and white cattle using IGFG2-*Tas*I revealed frequency of *A* allele as 0.75 and *AC* genotype was significantly associated with milk fat and protein yield (Szewczuk *et al.,* 2013). A similar study was conducted in 658 Polish HF cattle (Szewczuk *et al.,* 2011; Zhang *et al.,* 2014), who reported frequency of *A* allele as 0.87 and reported association of *C* allele with milk yield, protein and fat yield, indicating the absence of desirable *CC* genotype in Gaolao cattle. Because of monomorphic IGF1G2-*Tas*I PCR-RFLP, the region was again screened using PCR-SSCP (IGF1G2-SSCP) tool, which revealed monomorphic status.

IGF1G6 and IGF1RG4-SSCP Sequencing

The IGF1 candidate gene of 623 bp size from 5′ UTR region was screened using the PCR-SSCP method which revealed monomorphism. The IGF1G6 amplified sequence revealed 14 computational SNPs consisting 03 transition and 11 transversion mutations. In other studies; SSCP analysis of 5′ UTR region in 216 males belonging Malnadgidda, Khillar, HF, Jersey and Murrah and Surti buffalo breeds reported two SNPs in Jersey and Murrah breeds (Ramesha *et al.,* 2015). IGFR candidate gene fragment of 164 bp from exon-12 revealed monomorphism at the IGFRG4-SSCP locus. However, the polymorphic status of exon 12 region of the IGFR gene was previously reported in 163 Montbeliarde cows which were genotyped using IGFR-*Msp*I, and reported the frequency of *A* allele as 0.16 and *G* allele as 0.84, with the association of *A* allele with milk, fat and protein yield (Szewczuk, 2016).

IGFRG5-*Taq*I PCR-RFLP Genotyping

The IGFRG5-*Taq*I (5U) analysis in 248 Gaolao cattle population in the 163 bp exon-21 region of the IGFR gene revealed two different genotypes, *i.e., AA*=145, 18 bp, *AG*=163,145 & 18 bp band sizes (Fig. 2). IGFRG5-*Taq*I locus was found polymorphic with an allele frequency for the *A* allele 0.82 and for allele *B* 0.18. The $\chi 2$ test value observed for Exon-21 region at IGFRG5-*Taq*I locus of the IGFR gene exhibited significant deviation from H-W equilibrium ($p<0.05$), indicating that the experimental Gaolao population was not in H-W equilibrium and animals differ in their genotypic distribution. The values for expected heterozygosity, effective number of alleles and Shannon's Information index indicate sufficient genetic variation at the IGFRG5-*Taq*I locus in the Gaolao cattle population (Table 2). The association study of IGFRG5-*Taq*I polymorphism with milk traits using logistic regression

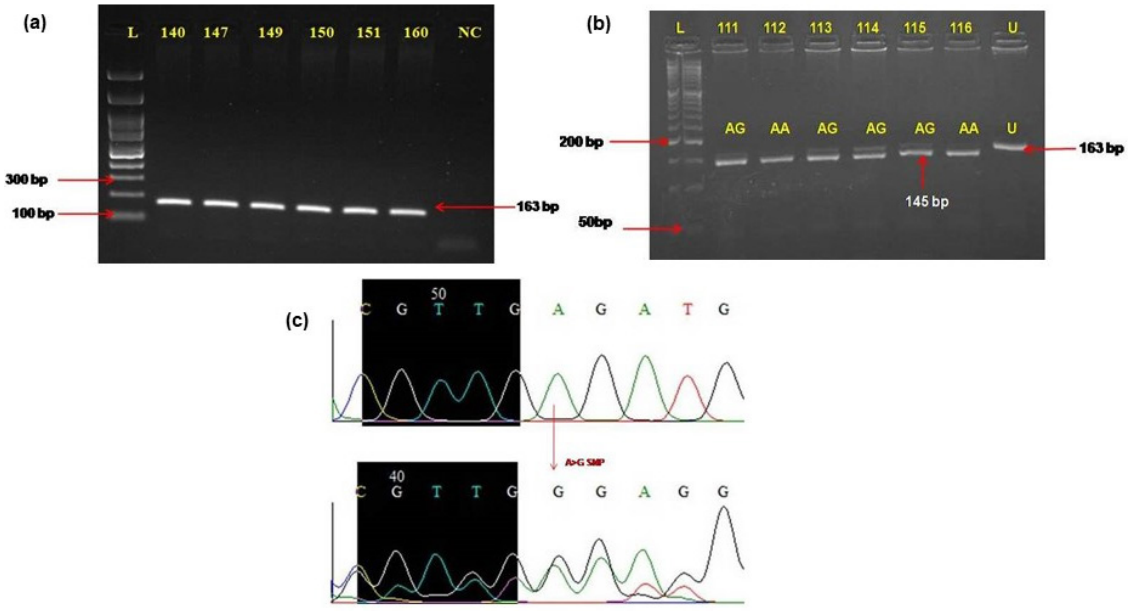

Fig. 2: IGFR Candidate gene polymorphism analysis (a) PCR amplicon off 163 bp fragment of exon-21 region gene (IGFRG5) (b) Polymorphic IGFRG5 - *TaqI* PCR-RFLP resolved in 3.5% agarose gel electrophoresis (c) SNP A>G at 36th position identified in IGFRG5 Gene sequence of Gaolao cattle.

Table 2: Genetic diversity measures at IGFRG5 -*Taq*I locus in Exon-21 region of IGFR gene in Gaolao cattle

Locus	n	Genotypes	Frequency		χ2	p	n_e	H_e	*I
			Genotypic	Allelic					
IGFRG5 -*Taq*I	248	AA (159)	0.64	0.82 (A)	11.71	0.00*	1.42	0.30	0.47
		AB (89)	0.36	0.18 (B)					

H_e = Expected Heterozygosity, N_e = Effective number of alleles, *I = Shannon's Information index, *Significant (P<0.05)

Table 3: Least Square Means & average effect of milk production traits at polymorphic IGFRG5 –*Taq*I locus in Gaolao cattle

Genotypes	n	Fat % ± SE	SNF % ± SE	Lactose% ± SE	Protein% ± SE	Milk yield ± SE
AA	106	4.51±0.39	8.75±0.05	4.56±0.04	3.17±0.02	4.67±0.16
AG	77	3.96±0.12	8.62±0.09	4.52±0.05	3.11±0.03	4.80±0.18
Total	183	4.28±0.23	8.69±0.05	4.54±0.03	3.14±0.02	4.73±0.12
p value	-	0.251	0.173	0.578	0.168	0.611

analysis revealed the non-significant difference in milk traits for both genotypes in 183 Gaolao animals. Mean values for milk production traits are presented in Table 3.

The same exon 21 region of IGFRG5 was screened using SSCP followed by sequencing, revealing two polymorphic patterns with frequency *A* =0.71 & *B* = 0.29 in Gaolao cattle with the absence of relation with milk traits. The direct amplicon sequencing representing SSCP patterns *A & B* revealed SNP *A>G* at 36th position 130 bp sequence of IGFRG5 gene fragment (Fig. 2). Two computational SNPs *G>T* at 39th position and *C>T* at 83rd position were found in 119 bp sequence of IGFRG5 gene sequence, indicating the existence of sequence variation. Thus, the region was polymorphic with two RFLP genotypes and SSCP patterns and SNPs. Similarly, the polymorphic status of exon 21 region of the IGFR gene in 163 Montbeliarde cows at IGF1R-*Taq*I, revealed frequency of *A* allele as 0.31 and *G* allele as 0.69 with the association of *A* allele with milk traits (Szewczuk, 2016).

Conclusions

The findings of the present study will surely aid in filling the information gap related to the status of IGF1 gene monomorphism at IGFG1-*SnaB*I & IGFG2-*Tas*I loci & existence of polymorphisms at IGF1RG5-*Taq*I PCR-RFLP and IGF1RG5-SSCP in Gaolao cattle breed. However, the identified IGF1RG5-*Taq*I PCR-RFLP and IGF1RG5-SSCP polymorphisms did not differ significantly with milk production traits. After validation in large sample sizes, the identified DNA sequence variations within IGF1 & IGF1R candidate genes may be helpful in selection and breeding decisions for genetic improvement of indigenous Gaolao cattle.

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