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Polymorphism of exon 2 of *DIO2* gene and its association with seasonal reproduction in sheep

JianNing HE[1,2], DongWei HUANG[1], Ran DI[1], JinXin WANG[1], MingXing CHU[1,*], QiuYue LIU[1], WenPing HU[1], XiangYu WANG[1], ZhangYuan PAN[1]

[1]Key Laboratory of Farm Animal Genetic Resources and Germplasm Innovation of Ministry of Agriculture, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, P.R. China

[2]College of Animal Science and Technology, Qingdao Agricultural University, Qingdao, P.R. China

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Abstract: *DIO2* was studied as a candidate gene for reproduction in sheep. Three pairs of primers were designed to detect single nucleotide polymorphisms (SNPs) of exon 2 of *DIO2* in nonseasonal and seasonal reproduction breeds. The polymorphisms were detected using SeqMan II and analyzed by PCR-RFLP. The sheep *DIO2* gene was cloned and its mutations were detected in five sheep breeds whose reproductive seasonality and litter sizes were different. Association analyses were performed to reveal the relationships between SNPs and reproductive seasonality as well as litter size in sheep. The results showed that a 1041-bp DNA fragment of the sheep *DIO2* gene was obtained and a part of the coding sequence of 591 bp encodes 196 amino acids, having high homology with that of other mammals. Moreover, three mutations (G318T, G482C, and C885T) were detected. The test of genotype distribution in five sheep breeds showed that there were significant differences between nonseasonal and seasonal breeds for 318 and 482 loci. However, the associations between the three polymorphisms and sheep seasonal reproduction were not very distinct. Additionally, there was no significant associations between polymorphisms and litter sizes in Small Tail Han sheep. These results may provide the basic theory for further study.

Key words: Sheep, seasonal reproduction, *DIO2* gene, polymorphism

1. Introduction

Deiodinase iodothyronine type II (DIO2), the protein encoded by this gene, belongs to the deiodinase iodothyronine family. This family includes deiodinase iodothyronine types I, II, and III (DIO1, DIO2, and DIO3, respectively), and they regulate the activity of thyroid hormone (1–3). DIO2 plays an important role in activation of thyroid hormone by conversion of the prohormone thyroxine (T4) to the active hormone triiodothyronine (T3) through the removal of an iodine atom on the outer ring (4,5). Expression of the *DIO2* gene shows different distributions in different species. In rodent species, expression is found in specialized ependymal cells, termed tanycytes, originating from the third ventricle (6). In goats and sheep, the expression of *DIO2* is widely distributed in the mediobasal hypothalamus, including the median eminence, arcuate nucleus, infundibular recess, and thyroid (7–9). Some studies showed that internally timed and spatially regulated changes in *DIO2* and *DIO3* expression may drive the cycling between breeding and nonbreeding states in long-lived seasonal species, and this may be either pars tuberalis-dependent or pars tuberalis-independent at different phases of the circannual cycle (10).

In temperate regions, the reproductive activities of mammals are controlled by changes in photoperiod. Photoperiodic information is translated into a daily cycle of melatonin secretion from the pineal gland. *DIO2* may be involved in photoperiodic control of seasonal breeding based on the melatonin signal messenger (11–14). In general, long days induced expression of *DIO2* and increased T3 content in the hypothalamus while short days suppressed *DIO2* expression and decreased T3 content, for example in the Japanese quail (15), Syrian hamster (11), and sheep (8). However, in Saanen goat, expression of *DIO2* and T3 content in the mediobasal hypothalamus was suppressed by long-day conditions (7). The *DIO2* gene controls animals' seasonal reproduction by catalyzing the conversion of prohormone T4 to bioactive T3 and controls local thyroid hormone concentration (16).

* Correspondence: mxchu@263.net

Sheep are generally animals with seasonal reproduction in temperate regions. They display regular reproductive activities (estrus and ovulation) mainly in autumn/winter with gradually shortening day length, without sexual activities in other seasons (17,18). It is well established that seasonal reproduction in sheep is regulated by photoperiod, environmental temperature, nutritional status, social interactions, lambing date, and lactation period. To date, the molecular mechanism underlying the seasonality of reproduction in sheep is not clear. However, some genes have been studied for associations between genetic polymorphisms and seasonality, including *Clock*, *TSHB*, *MTNR1A*, *Kiss1*, and *GPR54* (19–23).

To date, studies concerning the *DIO2* gene and sheep reproduction are rare. Small Tail Han sheep and Tan sheep are local breeds in China. Small Tail Han sheep display significant characteristics of year-round estrus and high prolificacy, while Tan sheep display seasonal estrus and low prolificacy. Dorper sheep display year-round estrus, while Texel sheep and Suffolk sheep display seasonal estrus (24). On the basis of its important role in reproduction, the *DIO2* gene may be considered as a candidate gene for animal reproductive traits. The objectives of the present study were firstly to detect the single nucleotide polymorphisms (SNPs) of exon 2 of the sheep *DIO2* gene in nonseasonal estrous breeds (Small Tail Han and Dorper sheep) and seasonal estrous breeds (Texel, Suffolk, and Tan sheep), and secondly to investigate the association between the sheep *DIO2* gene and prolificacy in Small Tail Han sheep in which the polymorphisms are segregating. The results may provide the basic theory for further study.

2. Materials and methods

2.1. Animals and genomic DNA isolation

Blood samples (10 mL, jugular vein, ACD anticoagulant) were collected from 191 Small Tail Han ewes (Jiaxiang Sheep Breeding Farm, Shandong, China) lambed in 2010, along with data on litter size in the first, second, or third parity, and 69 Tan ewes (Tan Sheep Breeding Farm, Ningxia, China), 61 Texel ewes (HITEK Ranch [Beijing] Ltd. Co., Beijing, China), 81 Suffolk ewes (HITEK Ranch [Beijing] Ltd. Co.), and 72 Dorper ewes (Beijing Aoxin Stud Farm Co. Ltd, Beijing, China).

Genomic DNA was extracted from whole blood using the phenol-chloroform method, dissolved in TE buffer (10 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA [pH 8.0]), and kept at –20 °C.

The 191 Small Tail Han ewes were selected at random and they were the progeny of five rams (n = 36, 38, 38, 39, 40). Because the five rams had been sold, their blood was not collected for genotyping. No selection for litter size or other fertility traits was performed in the flock in previous years. Lambing seasons consisted of 3-month groups starting with March through May as season 1 (spring, n = 45), June through August as 2 (summer, n = 47), September through November as 3 (autumn, n = 50) and December through February as 4 (winter, n = 49).

2.2. Primers and PCR amplification

According to *DIO2* sequences of sheep (GenBank: GQ468498), bovines (GenBank: NC_007308), humans (GenBank: NM_001242503), and mice (GenBank: NC_000078), a total of three pairs of PCR primers named as P1 to P3 (Table 1) were designed using Primer Premier 5. Primer P1 was designed to amplify exon 2, part of intron 1 of the sheep *DIO2* gene, and P2 and P3 were designed to detect the polymorphisms of the sheep *DIO2* gene.

PCR was carried out in a volume of 20 μL containing 0.15 μmol/L of each primer, 1X PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl [pH 8.0], 0.1% Triton X-100), 2.0 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 100 ng of ovine genomic DNA, and 0.05 U/μL Taq DNA polymerase (Promega, Madison, WI, USA), with the rest of the volume made up of ddH_2O. Amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at an appropriate temperature (Table 1) for 45 s, and extension at 72 °C for 35 s, with a final extension at 72 °C for 10 min in a Mastercycler 5333 (Eppendorf AG, Hamburg, Germany).

2.3. Cloning and sequencing

The PCR products were separated on 1.5% agarose gel, purified with Agarose Gel DNA Fragment Recovery Kit

Table 1. Primer information.

Primer name	Primers (5' to 3')	Product size (bp)	Annealing temperature (°C)
P1	F: ATTCTCTTTAGGGCCATTCATTAC R: TTCTTCTTTTCCATCTTGGGATCT	1041	55
P2	F: AAAACAGCGGATGGAAC R: ACGTTGGCATTATTGTCC	350	50
P3	F: AAAATTGCTTATCTGGGAGG R: TTGGGATCTTTTCATTCAGG	353	50

Ver. 2.0 protocols (TaKaRa, Dalian, China), and inserted into the pMD18-T vector according to the provided protocols. The recombinant plasmid was transformed into *Escherichia coli* TOP10 competent cells. At least seven positive clones were sequenced in both directions for each individual using the ABI3730 automatic sequencer (PerkinElmer Applied Biosystems, Foster City, CA, USA) by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China).

2.4. Polymorphism screening and detection

Genomic DNA from Small Tail Han sheep and Suffolk sheep was used as a template for amplification with the three pairs of primers (P1, P2, and P3) and the sequences were aligned to search for base variations.

The polymorphisms in sheep *DIO2* were analyzed by PCR-RFLP, which was performed by mixing 5 µL of PCR product, 5 U of the restriction enzymes (*Hae*III, *Bst*NI, and *Tsp*RI; New England Biolabs, Beverly, MA, USA) and 1 µL of corresponding 10X reaction buffer with incubation at 37 °C, 60 °C, and 65 °C overnight, respectively. After restriction enzyme digestion, the products were detected by 1.5%–2.0% agarose gels and genotyped using an AlphaImager 2200 and 1220 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA, USA).

2.5. Sequence analysis

After sequencing, the sequences were used to construct the contig of the *DIO2* gene and to detect the SNPs using the SeqMan II program (DNASTAR 6.0). The contig was processed through manual removal of errors, such as wrong bases and gaps. Sequence similarity alignments, molecular homology assessment, and phylogenetic analysis were performed through MegAlign (DNASTAR 6.0), BLAST (NCBI), and ClustalW2, respectively. A phylogenetic tree was constructed using MEGA 4.0 with the neighbor-joining (NJ) procedure, and support for internodes was assessed after 1000 bootstrap resampling steps.

2.6. Statistical analysis

The following fixed-effects model was employed for analysis of litter size in Small Tail Han ewes and least squares means were used for multiple comparisons in litter size among the different genotypes:

$$y_{ijklm} = m + S_i + LS_j + P_k + G_l + e_{ijklm},$$

where y_{ijklm} is the phenotypic value of the litter size, m is the population mean, S_i is the fixed effect of the ith sire (i = 1, 2, 3, 4, 5), LS_j is the fixed effect of the jth lambing season (j = 1, 2, 3, 4), P_k is the fixed effect of the kth parity (k = 1, 2, 3), G_l is the fixed effect of the lth genotype (l = 1, 2, 3), and e_{ijklm} is the random residual effect of each observation.

Analysis was performed using the general linear model procedure of SAS (Ver. 8.1) (SAS Institute Inc., Cary, NC, USA). Mean separation procedures were performed using a least significant difference test.

3. Results

3.1. Sequence analysis of sheep *DIO2* gene

Genomic DNA fragments of the sheep *DIO2* gene were successfully amplified using the P1 primer. The results showed that the amplification fragment sizes (Figure 1) were consistent with the target ones at 1041 bp (Table 1) and had a good specificity, which could be used for further analyses, such as SNP detection and PCR-RFLP.

A partial DNA fragment of the sheep *DIO2* gene of 1041 bp was obtained and submitted to GenBank (accession number: JX262388) after sequence homology analysis. It contains exon 2 and part of intron 1. The splicing of the exon and intron was consistent with the GT-AG rule. A part of the coding sequence of 591 bp encoding 196 amino acids was also obtained. This protein contains selenocysteine (Sec) residues encoded by the UGA codon, which normally signals translation termination.

Alignments of the sheep *DIO2* sequence with the corresponding regions of the goat (AB201476), cattle (AY858551), human (NM_001242503), pig (NM_001001626), mouse (NM_010050), rat (NM_031720), and chicken (NM_204114) *DIO2* gene were analyzed. The molecular evolutionary relationship was analyzed using MEGA 4.0 (NJ procedure, bootstrap replicates = 1000) based on the deduced proteins of different species and the phylogenetic tree is shown in

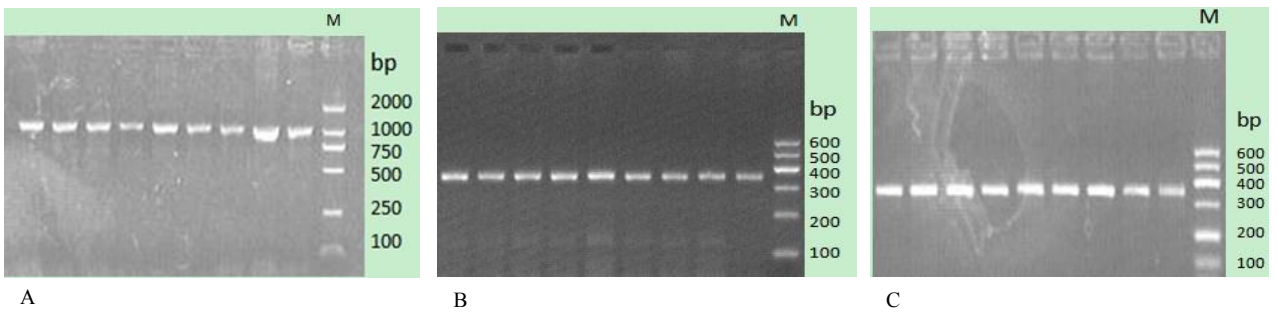

Figure 1. Amplification results of sheep *DIO2*. M: DNA Marker D2000 and DNA Marker I (Tiangen, Beijing, China). A: PCR products with primer P1 (1041 bp); B: PCR products with primer P2 (350 bp); C: PCR products with primer P3 (353 bp).

Figure 2. From the NJ trees, we found that the molecular phylogenetic relationship was congruent with species evolution and sheep *DIO2* is closely related to that of goats, cattle, pigs, and humans.

3.2. Polymorphism identification and detection

The sequences amplified with primers P1, P2, and P3 were aligned between Small Tail Han sheep and Suffolk sheep. Three mutations, G318T, G482C, and C885T, were found in exon 2 and the 3' regulatory region of the sheep *DIO2* gene. G-to-T transition at locus 318, G-to-C transition at locus 482, and C-to-T transition at locus 885 changed the recognition sites of restriction endonucleases *Hae*III, *Bst*NI, and *Tsp*RI. Two mutations (G318T, G482C) were found in exon 2 of the sheep *DIO2* gene and did not give rise to amino acid change. Mutation C885T was found in the 3' regulatory region. These three polymorphisms can be detected by PCR-RFLP using the amplification products of primers P1, P2, and P3. As shown in Figure 3 (AI), the G-to-T transition at locus 318 expressed three genotypes, GG, GT, and TT, and sequences of the homozygous genotypes are presented in Figure 3 (AII). As shown in Figure 3 (BI), the G-to-C transition at locus 482 gave rise to three genotypes, GG, GC, and CC, and sequences of the homozygous genotypes are presented in Figure 3 (BII). The C-to-T transition at locus 885 expressed three genotypes, CC, CT, and TT (Figure 3 (CI)), and sequences of the homozygous genotypes are presented in Figure 3 (CII).

3.3. Allele and genotype frequencies of *DIO2* gene in five sheep breeds

Allele and genotype frequencies of the *DIO2* gene in five sheep breeds are listed in Table 2. The results indicated that at locus 318 the G allele is the predominant allele in Small Tail Han, Texel, Suffolk, and Dorper sheep, but not in Tan sheep. Small Tail Han sheep deviated from Hardy–Weinberg equilibrium ($P < 0.05$). GG was the preponderant genotype in Small Tail Han, Texel, Suffolk, and Dorper sheep, and GT was the preponderant genotype in Tan sheep. At locus 482, the C allele is predominant allele in these five breeds. CC was the preponderant genotype in all five sheep breeds. Tan, Texel, and Suffolk sheep deviated from Hardy–Weinberg equilibrium ($P < 0.05$, $P < 0.05$, and $P < 0.001$). At locus 885, the C allele is the predominant allele in Small Tail Han, Texel, Suffolk, and Dorper sheep, but not in Tan sheep. CC was the preponderant genotype in Small Tail Han, Texel, and Dorper sheep. CT was the preponderant genotype in Tan and Suffolk sheep. Suffolk and Dorper sheep deviated from Hardy–Weinberg equilibrium ($P < 0.001$).

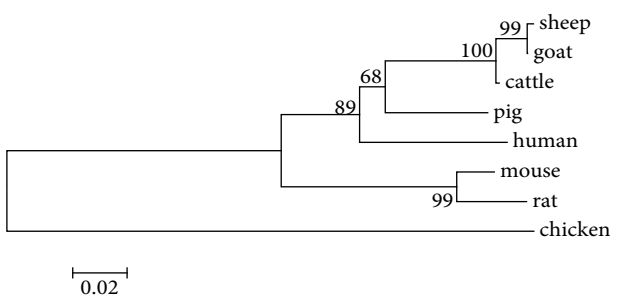

Figure 2. Phylogenetic tree of the *DIO2* gene by NJ method.

3.4. Test of difference for *DIO2* genotype distribution in different sheep breeds

The test results of differences for *DIO2* genotype distributions in five sheep breeds are summarized in Table 3. For locus 318, significant difference existed between a nonseasonal breed (Small Tail Han sheep) and seasonal breeds (Tan, Texel, and Suffolk sheep). For locus 482, there were significant differences between a nonseasonal breed (Small Tail Han sheep) and seasonal breeds (Tan, Texel, and Suffolk sheep), and between a nonseasonal breed (Dorper sheep) and seasonal breeds (Texel and Suffolk sheep). For locus 885, there were significant differences between the nonseasonal breeds (Small Tail Han and Dorper sheep) and all three seasonal breeds (Tan, Texel, and Suffolk sheep). Our results showed that for all pairs of nonseasonal breeds and seasonal breeds in this study, there were no consistent significant differences of genotype distribution in each locus. However, these three mutations may have some correlations with seasonality and need to be further investigated.

3.5. Influence of fixed effects on litter size in Small Tail Han sheep

Litter size in Small Tail Han sheep was significantly influenced by sire, lambing season, and parity (all $P < 0.05$). The least squares means and standard errors for litter size of different genotypes of *DIO2* in Small Tail Han sheep are given in Table 4. There were no significant differences of litter size among genotypes for the three loci. The results indicated that this gene may have no effect on sheep litter size.

4. Discussion

Thyroid hormones play critical roles in body growth and development, basic metabolism, and reproductive activities. Many factors affect thyroid hormone synthesis, transfer, and function, the most important being DIO2, which converts the prohormone T4 to the bioactive hormone T3. To date, there have been many studies on polymorphisms of the *DIO2* gene. The SNP Thr92Ala of the human *DIO2* gene is associated with bipolar disorder (25), type 2 diabetes (26), and hepatic glucose output (27). Guo et al. studied the association of the human *DIO2* gene (rs225014, rs225012, and rs225010) with mental retardation in iodine-deficient areas of China and found a positive association of mental retardation with two SNPs

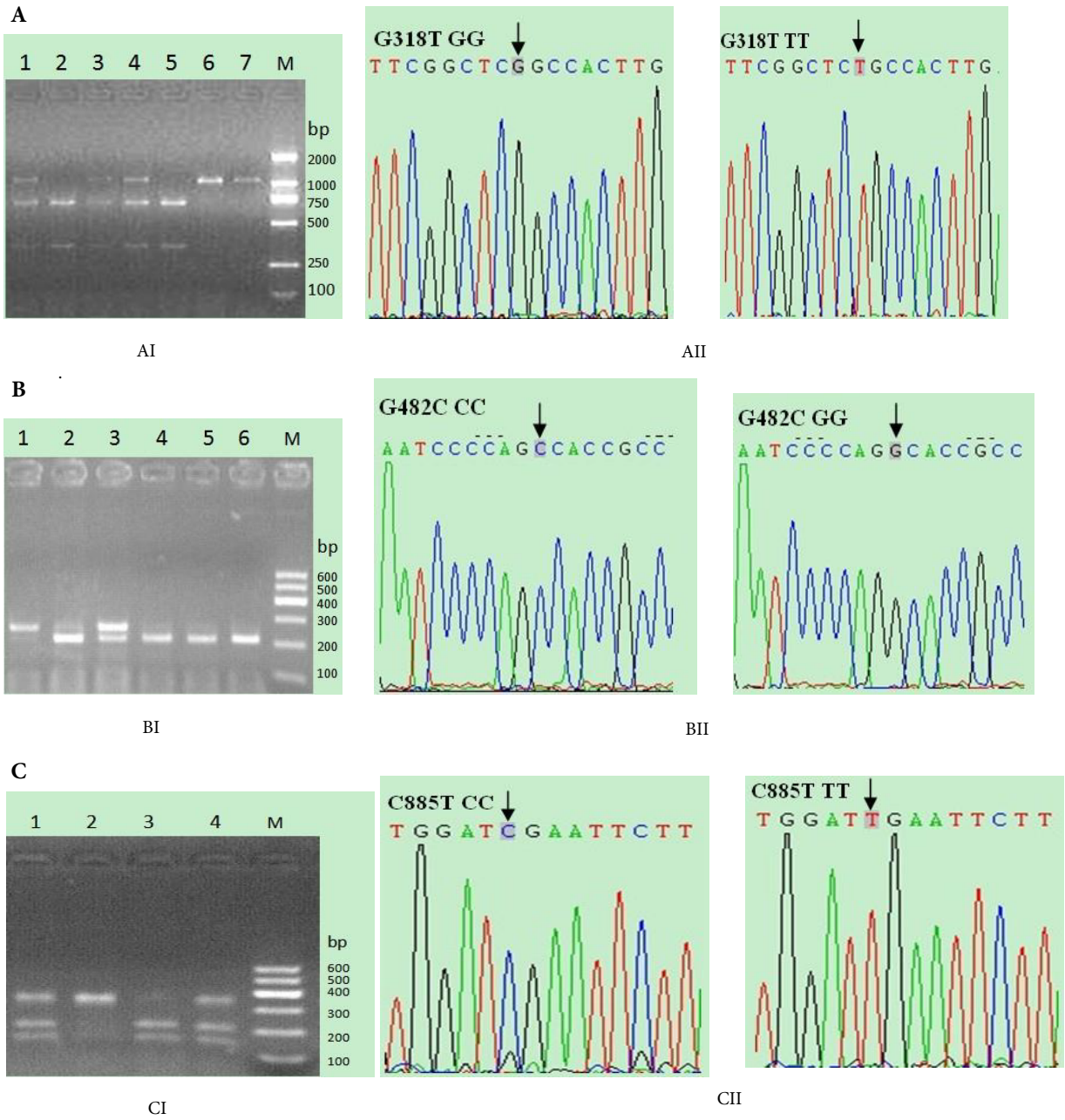

Figure 3. A: PCR-RFLP (*Hae*III) analysis of G318T in sheep *DIO2* gene and sequences of different genotypes. AI: PCR-RFLP analysis of the 318 locus in sheep *DIO2* gene. Three genotypes: GG, bands 2 and 5 (723 / 318 bp); GT, bands 1, 3, and 4 (723 / 318 / 1041 bp); TT, bands 6 and 7 (1041 bp). M: DNA Marker D2000. AII: Sequences of genotypes GG and TT of G318T in sheep *DIO2* gene. B: PCR-RFLP (*Bst*NI) analysis of G482C in sheep *DIO2* gene and sequences of different genotypes. BI: PCR-RFLP analysis of the 482 locus in sheep *DIO2* gene. Three genotypes: GG, band 1 (251 bp); GC, band 3 (205 / 251 bp); CC, bands 2, 4, 5, and 6 (205 bp). M: DNA Marker I. BII: Sequences of genotypes GG and CC of G482C in sheep *DIO2* gene. C: PCR-RFLP (*Tsp*RI) analysis of C885T in sheep *DIO2* gene and sequences of different genotypes. CI: PCR-RFLP analysis of the 885 locus in sheep *DIO2* gene. Three genotypes: CC, band 3 (136 / 217 bp); CT, bands 1 and 4 (136 / 217 / 353 bp); TT, band 2 (353 bp). M: DNA Marker I. CII: Sequences of genotypes CC and TT of C885T in sheep *DIO2* gene.

(rs225012 and rs225010) (27). Nair et al. identified 12 SNPs in 83 Pima Indians, in which rs225011 and rs225015 were modestly associated with early-onset type 2 diabetes mellitus. Thr92Ala, rs225011, rs225015, and rs6574549 were also nominally associated with hepatic glucose output, and rs6574549 was associated with fasting insulin,

Table 2. Allele and genotype frequencies of the three SNPs of *DIO2* gene in five sheep breeds.

Locus	Breed		Small Tail Han sheep	Dorper sheep	Tan sheep	Texel sheep	Suffolk sheep
G318T	Number		188	69	64	58	79
	Genotype frequency	*GG*	0.90 (170)	1 (69)	0.16 (10)	1 (58)	1 (79)
		GT	0.08 (14)	0 (0)	0.53 (34)	0 (0)	0 (0)
		TT	0.02 (4)	0 (0)	0.31 (20)	0 (0)	0 (0)
	Allele frequency	*G*	0.94	1	0.42	1	1
		T	0.06	0	0.58	0	0
	H–W test	χ^2	19.75***	--	0.51	--	--
G482C	Number		190	71	69	60	79
	Genotype frequency	*GG*	0.09 (18)	0.10 (7)	0.25 (17)	0.07 (4)	0.11 (9)
		GC	0.35 (66)	0.44 (31)	0.33 (23)	0.17 (10)	0.17 (13)
		CC	0.56 (106)	0.46 (33)	0.42 (29)	0.76 (46)	0.72 (57)
	Allele frequency	*G*	0.27	0.32	0.41	0.15	0.20
		C	0.73	0.68	0.59	0.85	0.80
	H–W test	χ^2	2.54	0.01	6.74*	7.20*	18.07***
C885T	Number		191	72	66	61	81
	Genotype frequency	*CC*	0.61 (117)	0.81 (58)	0.17 (11)	0.82 (50)	0.26 (21)
		CT	0.37 (71)	0 (0)	0.56 (37)	0.18 (11)	0.73 (59)
		TT	0.02 (3)	0.19 (14)	0.27 (18)	0 (0)	0.01 (1)
	Allele frequency	*C*	0.80	0.81	0.45	0.91	0.62
		T	0.20	0.19	0.55	0.09	0.38
	H–W test	χ^2	4.58	72***	1.18	0.60	24.62***

* P < 0.05, *** P < 0.001.

Table 3. Test of difference for the 318 / 482 loci (above diagonal) and the 885 locus (below diagonal) genotype distributions of the *DIO2* gene in five sheep breeds.

Breed	Small Tail Han sheep	Dorper sheep	Tan sheep	Texel sheep	Suffolk sheep
Small Tail Han sheep		7.10* / 1.96	132.22*** / 10.48**	5.99* / 8.58*	8.11* / 9.02*
Dorper sheep	55.53***		98.01*** / 5.58	0 / 12.88**	0 / 13.63**
Tan sheep	63.40***	69.38***		87.80** /16.47***	107.10*** / 13.74**
Texel sheep	9.16**	24.85***	56.91***		0 / 0.91
Suffolk sheep	29.18***	87.37***	22.08***	43.81***	

* P < 0.05, ** P < 0.01, *** P < 0.001.

Table 4. Least squares mean and standard error for litter size of different genotypes of the three loci in the *DIO2* gene in Small Tail Han sheep.

Locus	Genotype	Number	Litter size
G318T	GG	170	$2.32^{a} \pm 0.09$
	GT	14	$2.39^{a} \pm 0.20$
	TT	4	$2.42^{a} \pm 0.23$
G482C	GG	18	$2.25^{a} \pm 0.20$
	GC	66	$2.31^{a} \pm 0.14$
	CC	106	$2.36^{a} \pm 0.12$
C885T	CC	117	$2.34^{a} \pm 0.11$
	CT	71	$2.32^{a} \pm 0.13$
	TT	3	$2.18^{a} \pm 0.22$

Least squares means with the same superscript for the same locus have no significant difference ($P > 0.05$).

insulin action, and energy expenditure (28). In the present study, we found three SNPs in five sheep breeds, which were G318T, G482C, and C885T.

In temperate regions, the reproductive activities of mammals are controlled by photoperiod rhythm. Photoperiodic information is translated into the change of melatonin secretion. *DIO2* may be involved in photoperiodic control of seasonal breeding based on the melatonin signal messenger (11–13). Watanabe et al. found that expression of hamster *DIO2* in the mediobasal hypothalamus is induced by light, and under long-day conditions melatonin injections decreased *DIO2* expression (29). Revel et al. found that *DIO2* was highly expressed in reproductively active hamsters, whereas it was dramatically reduced in sexually inhibited ones (11). Yasuo et al. found that reciprocal changes in *DIO2* and *DIO3* expression act as gene switches of the photoperiodic molecular cascade to trigger induction of luteinizing hormone secretion in Japanese quail as a long-day breeder (30). This is different for short-day breeders, as Yasuo et al. found that expression of goat *DIO2* in the mediobasal hypothalamus of Saanen goat was suppressed by artificial long-day conditions, which is the opposite of the results for long-day breeders (7). In our study, the genotype frequency distributions of the three mutations were detected in the five selected sheep breeds. There were significant differences between a nonseasonal breed (Small Tail Han sheep) and seasonal breeds (Tan, Texel, and Suffolk sheep) for loci 318 and 482. However, no consistent significant difference was found between the nonseasonal estrous breeds and seasonal estrous breeds for each locus, indicating that the three loci may have no definite relationships with seasonal reproduction or that the effect of the three mutations on reproductive seasonality may only exist in some sheep breeds. Tests of differences in genotype distribution may be not a precise way for measuring reproductive seasonality. In the present study, we also analyzed the effects of sheep *DIO2* gene mutations on litter size. The results showed there was no significant association between genotypes and litter size for the three polymorphic loci. The reason for this is that these mutations do not cause amino acid changes, so they do not affect the function of the protein. However, these three mutations may have some correlations with seasonality and need to be further investigated.

Acknowledgments

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