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Identification of single nucleotide polymorphisms in GDF9 gene associated with litter size in Garut sheep

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ABSTRACT The growth differentiation factor 9 (GDF9) gene has been regarded as having major impacts on ovulation rate and litter size in sheep. The aim of this study was to identify the single nucleotide polymorphisms (SNPs) of the GDF9 gene and their association with litter size in Garut sheep. For this purpose, a total of 60 ewes of Garut sheep were included in this study. Based on the sheep GDF9 reference sequences (Genbank Acc. No. AF078545.2), one pair of primers (5'-CTGCTGTTTAACCTGGATCGTG-3 5'-GGAGAGCCATACCGATGTCC-3 as forward and reverse, respectively) was used for PCR amplification. The results revealed that four SNPs (g.54C>T, g.60G>A, g.304G>A, and g.333G>A) were found in Garut sheep by direct sequencing. For SNP g.54C>T, the sheep exhibited the highest frequency of allele C and genotype CC. On the other hand, SNPs g.60G>A, g.304G>A, and g.333G>A showed a higher frequency of allele G than allele A, and the GG genotype was predominant in the population. SNP g.333G>A had a significant effect on litter size ($p < 0.05$), and ewes with the GG genotype had a higher litter size than those with the GA genotype. Genotype distributions for all identified SNPs were in agreement with Hardy-Weinberg equilibrium. We highlight that SNP g.333G>A may be useful as a genetic marker for litter size in Garut sheep.

KEYWORDS Garut sheep; GDF9 gene; prolificacy; single nucleotide polymorphism (SNP)

1. Introduction

Garut, a local sheep in Indonesia, is mainly used as fighting art as well as a meat producer. Because of its important roles for local communities, its population should be increased (Heriyadi 2005). With the aim of increasing sheep populations in Indonesia and improving the reproductive traits of Garut sheep, it is necessary to detect the genetic variation of these sheep in terms of their reproductive traits. A substantial amount of genetic variation will provide the possibility for genetic improvement. In Garut sheep farming, litter size is a major concern with regards to profitability. Litter size depends on the number of ovum ovulated by ewes. To date, genetic improvement of reproductive traits in many sheep breeds in Indonesia has mainly been based on the use of conventional breeding methods. Recently, the use of molecular techniques for livestock genetic improvement has provided better gains by enabling the identification of major genes related to reproductive traits that can be further utilized as a marker-assisted selection in sheep breeding.

The prolificacy is controlled by multiple genes, which are characterized as fertility genes (Davis 2004). The members of the transforming growth factor-β (TGF-β) superfamily, including bone morphogenic protein 15 (BMP15), growth differentiation factor 9 (GDF9), and bone morphogenic protein-IB have been proved to play a crucial role in follicular growth and ovulation rate (Kasiriyan et al. 2009). Furthermore, as reported in several studies, GDF9, BMP1, BMPR1B, and leptin are considered to be major genes affecting prolificacy (Chen et al. 2005; Polley et al. 2009; Pokharel et al. 2018). Reproduction quality in sheep is determined by a number of factors, including feed quality, environmental conditions, and ovulation, the latter of which begins with folliculogenesis that involves the roles of several hormones and genes, such as GDF9 and BMP15 genes. GDF9 has been mapped to sheep chromosome 5 (Hanrahan et al. 2004). It spans approximately 5644 bp (Genbank Acc. No. AF078545.2) and contains two exons and one intron. In addition, GDF9 is a growth factor and a member of the TGF-β superfamily that is secreted by oocytes in growing ovarian follicles (Hanrahan et al. 2004). It also acts as a simulator in primary-follicle development until ovulation, and makes major contributions to the animal's prolificacy (Gilchrist et al. 2008; Goyal et al. 2017; Wang et al. 2018). In female mammalian reproduction, growth and differentiation factors play important roles during early folliculogenesis (Elvin et al. 1999). In the ovary, GDF9 is exclusively expressed, specifically in the oocytes of humans (Vitt et al.

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2000), cattle (Bodensteiner et al. 2000), mice (McGrath et al. 1995; Dube et al. 1998; Lan 2003), and sheep (Bodensteiner et al. 2000; Juengel et al. 2002).

Many researchers have shown great interest in the investigation of major genes related to ovulation rate and prolificacy in sheep. He et al. (2010) found one point mutation of the GDF9 gene in Chinese goats that is associated with ovulation rate. Many significant associations have been reported between GDF9 polymorphism and ovulation rate, prolificacy, and fertility in sheep (Hanrahan et al. 2004; Ghaffari et al. 2010; Ghaderi et al. 2010; Souza et al. 2014; Mullen and Hanrahan 2014; Khodabakhshzadeh et al. 2016; Ahmad et al. 2017). Moreover, current investigations in sheep, humans, and rodents show that the GDF9 and BMP15 genes can be regarded as important genes associated with prolificacy in mammals (McNatty et al. 2005). However, there is no report on the genetic variation of the GDF9 gene in Garut sheep. Therefore, the aim of this study was to identify the single nucleotide polymorphisms (SNPs) in Garut sheep. Identification of the GDF9 gene polymorphism can be very useful in better understanding the genetic mechanisms of prolificacy in sheep, and the results of this study are expected to be valuable as genetic markers for further studies, as well for future genetic improvement of Garut sheep.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of sixty ewes of Garut sheep raised in UPTD-BBTDK Margawati, Garut, Java, Indonesia, were used in this study. The animals were sampled based on their birth type (single, twin and, triple) with 20 samples per type. Blood samples, approximately 3 mL, were collected from the jugular vein of each ewe in EDTA vacutainer tubes and held on ice until delivery to the laboratory. Genomic DNA was extracted using the gSYNCTMDNA Extraction Kit (Geneaid, New Taipei City, Taiwan).

2.2. Primer design and DNA amplification

The primer design was carried out based on a previous report by Hartatik (2016), in oligoprimer primer3 (http://primer3.ut.ee) using Genbank sequence AF078545.2 as a template, DQ301499.1, NM_001142888.2, KT853039.1, and FJ429111.1. One pair of primers (5'-CTGCTGTTTAACCTGGATCGTG-3 and 5'-GGAGAGCCATACCGATGTCC-3 as the forward and reverse primer, respectively) was designed to amplify a 770 bp product of the GDF9 gene, which is located between 3326 and 4095 bp based on the GDF9 reference sequence (AF078545.2). PCR was performed in a 30 µL volume containing 2 µL genomic DNA, 15 µL KAPA2G Fast Ready Mix PCR Kit (Kapa Biosystems, Wilmington, United States), 1.5 µL of each primer, and 10 µL aquabidest. The PCR conditions were 94°C for 5 min and 35 cycles of 5 s at 94°C, 40 s at 59°C, 30 s at 72°C, and a final step of 5 min at 72°C (Hanrahan et al. 2004).

2.3. Sequencing and SNPs identification

Thirty microliters (30 µL) of each PCR product were sent to 1st Base Sequencing Laboratories (Selangor, Malaysia) for direct sequencing. Sequence analysis and alignment were carried out using Bioedit version 7.2.5 in order to identify polymorphisms of the GDF9 gene. The genotype of each ewe was determined based on the sequencing results. We analyzed the electropherogram of each sample, where a heterozygous genotype should appear as double peaks in the BioEdit software.

2.4. Statistical analysis

Allelic and genotypic frequencies were calculated by direct counting, and their distributions were tested for Hardy-Weinberg equilibrium with chi-square analysis using Equation 1 (Falconer et al. 1996):

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i} \quad (1)$$

where χ^2 is the chi-square, O_i is the observed frequencies, E_i is the expected frequencies and n is the number of measurements. The association between GDF9 genotypes and litter size was analyzed using analysis of variance (ANOVA) with the mathematical model shown in Equation 2:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij} \quad (2)$$

where μ is the overall mean, T_i is the genotype effect of the GDF9 gene and ε_{ij} is a random residual effect.

3. Results and discussion

3.1. PCR amplification and SNPs detection

Using a pair of specific primers, we successfully amplified a 770 bp fragment of the GDF9 gene for 59 samples, and the size of the PCR products in gel electrophoresis was the same as expected (Figure 1). In gel electrophoresis, a clear band indicated a good quality of DNA samples. In contrast, smeared bands may indicate poor sample quality, and that the DNA samples are contaminated with protein, especially during the extraction process (Yuwono 2006; Novitasari et al. 2014; Widyastuti 2017). The PCR

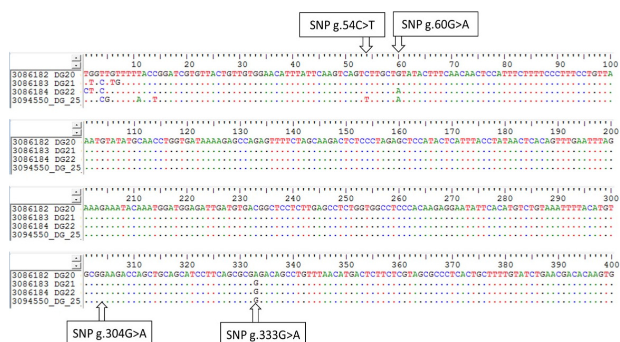

FIGURE 1 Alignment of the GDF9 gene sequences.

products of each sample were further subjected to direct sequencing.

Based on the sequencing results, the GDF9 gene sequences of 59 samples were aligned (Figure 2). A total of four SNPs (g.54C>T, g.60G>A, g.304G>A, and g.333G>A) were identified. Accordingly, we determined four types of ewes with different genotype combinations (Table 1. Type A consisted of five ewes (8%), one ewe (2%), and one ewe (1%) having a birth type of single, twin, and triple, respectively. Type B consisted of 13 ewes (22%), 17 ewes (29%), and 16 ewes (27%) having a birth type of single, twin, and triple, respectively. Type C consisted of one ewe (2%) having a single birth type. Type D consisted of one ewe (2%), one ewe (2%), and three ewes (5%) having a birth type of single, twin, and triple, respectively. Initially, Hanrahan et al. (2004) observed eight polymorphisms in the entire coding region of the sheep GDF9 gene. Later investigation by Bahrami et al. (2014) and Khodabakhshzadeh et al. (2016) also detected some point mutations (SNP) in the GDF9 gene located in exon 1 and exon 2, respectively. Four mutations detected in the present study indicated that the GDF9 gene in Garut sheep was found to be polymorphic.

The results of the allelic and genotypic frequencies and chi-square test of each SNP of the GDF9 gene are presented in Table 2. For SNP g.54C>T, Garut sheep exhibited the highest frequency of allele C and genotype CC. On the other hand, SNPs g.60G>A, g.304G>A, and g.333G>A showed a higher frequency of allele G than allele A, and the GG genotype was predominant in the population. These results agreed with the findings reported by Ghaderi et al. (2010) in Iranian sheep and Polley et al. (2009) in Garole sheep; they reported that homozygous animals for the GDF9 mutation were predominant in both populations. In this study, we also analyzed the genotype distribution of the GDF9 gene using a chi-square test (χ^2). The χ^2 test was performed to verify whether the genotype distribution of the GDF9 gene in the studied population agreed with Hardy-Weinberg equilibrium expectations. The results showed that genotype distributions of the GDF9 gene polymorphisms in Garut sheep were in accordance with Hardy-Weinberg equilibrium.

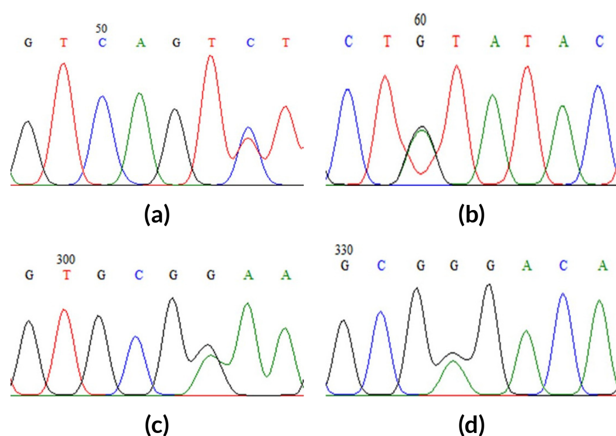

FIGURE 2 Electropherogram of the GDF9 sequences: a) SNP g.54C>T; b) SNP g.60G>A; c) SNP g.304G>A; d) SNP g.333G>A.

3.2. SNPs marker association with litter size

The GDF9 gene polymorphisms detected in the present study were associated with litter size. The results of association analysis are presented in Table 3. Among the four SNPs, only SNP g.333G>A had a significant effect on litter size ($p < 0.05$). Ewes with the GG genotype had a larger litter size than those with the GA genotype.

Varying patterns of inheritance are detected to have an association with the GDF9 gene. Several studies reported that GDF9 gene polymorphisms are associated with sterility and increased ovulation rate in sheep. Hanrahan et al. (2004) reported a number of mutations of the GDF9 and BMP15 genes in the Cambridge and F700-Belclare breeds. They concluded that an increased ovulation rate in heterozygous carriers and sterility in homozygous carriers in both breeds are associated with mutations in the GDF9 and BMP15 genes. As reported by Paz et al. (2014) and Javanmard et al. (2011), ewes with the homozygous genotype had a larger litter size than those with the heterozygous genotype. In contrast, although Arta and Rahayu (2013) detected polymorphisms in the bovine GDF9 gene, these polymorphisms are not associated with the success of artificial insemination in Peranakan Ongole cattle. Among the four SNPs identified in the present study, only SNP g.333G>A of the GDF9 gene can be used as a marker-assisted selection in Garut sheep.

The GDF9 gene is an important candidate gene for the primary ovarian insufficiency. Expressed in oocytes, its characteristics are like those of BMP15, a member of the TGF gene family. Ovulation is a complex mechanism that may differ among individuals, depending on either genetic or environmental factors. In sheep, the ewe's repro-

TABLE 1 Four types of ewes based on the identified SNPs.

Type	No. of ewes			SNP g.54C>T	SNP g.60G>A	SNP g.304G>A	SNP g.333G>A
	Single birth	Double birth	Triple birth				
A	5	1	1	CC	GA	GG	GA
B	13	17	16	CC	GG	GG	GG
C	1	0	0	CT	AA	GA	GA
D	1	1	3	CT	GA	GA	GG

TABLE 2 Allelic and genotypic frequency and chi-square test.

SNP	Total	Genotype			Allele		χ^2
g.54C>T	Observed	54.00 (CC)	5.00 (CT)	0.00 (TT)	0.96 (C)	0.04 (T)	0.12
	Expected	54.11 (CC)	4.79 (CT)	0.11 (TT)			
g.60G>A	Observed	46.00 (GG)	12.00 (GA)	1.00 (AA)	0.88 (G)	0.12 (A)	0.05
	Expected	45.83 (GG)	12.33 (GA)	0.83 (AA)			
g.304G>A	Observed	53.00 (GG)	6.00 (GA)	0.00 (AA)	0.95 (G)	0.05 (A)	0.17
	Expected	53.15 (GG)	5.69 (GA)	0.15 (AA)			
g.333G>A	Observed	51.00 (GG)	8.00 (GA)	0.00 (AA)	0.93 (G)	0.07 (A)	0.31
	Expected	51.27 (GG)	7.46 (GA)	0.27 (AA)			

$\chi^2_{0.05;2} = 5.99$

TABLE 3 Association results between genotypes of the sheep GDF9 gene and litter size.

SNP	Genotype	No. of ewes	Litter size
g.54C>T	CC	53	1.98 ± 0.82
	CT	6	2.17 ± 0.98
g.60G>A	GG	46	2.06 ± 0.80
	GA	12	1.83 ± 0.94
	AA	1	1.00
g.304G>A	GG	53	1.98 ± 0.82
	GA	6	2.17 ± 0.98
g.333G>A	GG	51	2.09 ± 0.81[a]
	GA	8	1.37 ± 0.74[b]

[a,b]Mean with different superscript within the same column differ significantly ($p < 0.05$).

ductive efficiency is commonly affected by its prolificacy trait. Prolificacy is a descriptor for the ability of ewe to produce a certain litter size in each parturition. Prolificacy is genetically inherited, and thus, genes encoding this trait must be identified for future sheep genetic improvement strategies. In sheep farming, prolificacy is an economic trait that determines profitability in sheep production. In genetic improvement strategies, a selection program is needed to improve some economic traits. Litter size is included as an important trait enhancing the productivity of sheep (Mishra 2014).

The investigation of candidate genes associated with economic traits is a crucial step in animal breeding. *Ovis aries* genome research and the detection of polymorphic sites of genes encoding economic traits are useful tasks for mapping and marker-assisted selection. The investigation of candidate genes concerned with economic traits will provide the possibility for the genetic improvement and modification of the desired traits. Molecular analysis is an important element in the utilization of genes encoding the economic traits of a given livestock species. In the sheep industry, many of the identified SNPs markers were found to be useful tools for determining the genetic basis for prolificacy traits in many breeds.

In this study, we found that SNP g.333G>A of the GDF9 gene may play an important role in the prolificacy trait, as indicated by higher levels of litter size in homozygous GG ewes compared with the heterozygous AG ewes. However, SNP g.333G>A did not change the amino acid encoded. Souza et al. (2014) detected a point mutation (c.943C>T) in the GDF9 gene of Brazilian sheep, resulting in a non-conservative amino acid change (p.Arg315Cys) in the cleavage site of the propeptide. In Belclare and Cambridge sheep, mutation in the GDF9 gene causes an amino acid substitution (S77F) that has been detected to have an impact on increased prolificacy (Hanrahan et al. 2004). Some previous studies reported that mutations in the GDF9 gene are associated with increased litter size in sheep (Davis 2004; Hanrahan et al. 2004; Liao et al. 2004)). In contrast, GDF9 gene polymorphisms detected in Shal sheep showed that the genetic basis for the twinning or multiple litter size is not associated with mutated alleles in the sheep GDF9 gene (Ghaffari et al. 2010). These reports suggest that the GDF9 gene may play an effective role in fertility and twinning in Garut sheep. However, for the future genetic improvement of Garut sheep, it is necessary to provide a suitable environment for these sheep. Furthermore, understanding the genetic basis concerned with litter size is a convenient and efficient tool in the selection program. Future genetic improvement of Garut sheep also requires further investigation of other SNPs or genes encoding the prolificacy traits, which is determined by multiple genes. According to the results of the present study, it can be highlighted that GDF9 gene polymorphism may affect twinning in Garut sheep.

4. Conclusion

It was clear that GDF9 gene polymorphisms were detected in Garut sheep. SNP g.333G>A was significantly associated with litter size. Thus, this SNP may prove to be valuable as a genetic marker for future work on the populations of Garut sheep.

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Authors' contributions

S and TH were the supervisors of this research. They fully contributed to the guidance of this research and the improvement of the final manuscript of this article. RYR carried out the laboratory work, analyzed the data, and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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