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Follicle-stimulating hormone receptor gene exploration as possible markers for prolific trait of local goat in Indonesia

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ABSTRACT

The objective of this study was to discover and identify the effect of SNP of follicle-stimulating hormone receptor (FSHR) gen on prolific traits in Kejobong (KJ) and Etawah grade (EG) doe. A total of 15 blood samples were taken from 11 KJ and 4 EG with various parity and type of birth. The FSHR gene was amplified from the DNA template by *Polymerase Chain Reaction* (PCR); the PCR products were then sequenced to determine *Single Nucleotide Polymorphisms* (SNP). Results showed that 3 SNPs were identified, and those SNPs altered amino acid sequence formed 6 haplotypes and divided the doe based on the type of birth. In conclusion, SNPs identified in this study are associated with a prolific trait that can be used as a genetic marker and haplotype 3, 4, and 5 showed the highest prolific on KJ and EG doe.

Keywords: FSHR, Genetic marker, Local goat, Prolific, SNPs

INTRODUCTION

Recently, molecular genetics led to the discovery of candidate genes with crucial effects on economic importance. As one of the economic traits, reproductive traits improvement in livestock has become of main concern, especially in small ruminants like goats, which are known to have high litter size. Kejobong goat (KJ) and Etawah Grade goat (EG) are two local goat breeds in Indonesia that are known to have highly prolific traits with litter sizes of 1.40 and 1.20, respectively (Panjono *et al*., 2012). As stated by Febriana *et al.* (2017), prolific was one of the reproductive traits which showed the ability to produce more than a kid at birth. Regulated by different fecundity genes, prolific traits related to genetic factors improve ovulation rate and litter size.

hormone (FSH) is secreted by the anterior pituitary and plays a key role in normal reproductive function (Hsueh *et al*., 1989; Gharib *et al*., 1990; Howles, 2000). In the absence of sufficient FSH, follicles fail to develop beyond the early antral stage, leading to the failure of ovulation (Simoni *et al*., 1997; Erman and Oktay, 2009). Furthermore, normal levels are necessary for forming of the placenta, and thus, conception. FSH action must be mediated by the FSH receptor (FSHR), a member of the family of G-protein

-coupled receptors expressed solely in granulose cells (Ranniki *et al*., 1995; Fan *et al*., 1998; Livshyts *et al.,* 2009).

As complex trans-membrane proteins, FSHR is characterized by seven hydrophobic helices inserted in the plasmalemma. The intracellular portion of the FSHR is coupled to a Gs protein and upon receptor activation by the hormonal interaction with the extracellular domain, the cascade of events that ultimately leads to the specific biological effects of the gonadotropin would be initiated (Simoni *et al.,* 1997; George *et al*., 2011). Due to the important roles of FSH in follicular growth and ovarian steroidogenesis in females, mutations in the FSHR gene could affect reproductive ability. Moreover, Amitha *et al.* (2019) reported that heat stress has a negative impact on expression patterns of FSHR in Malahabari goats.

So far, a large number of single nucleotide polymorphisms (SNP) have been detected in the FSHR gene, which is associated with reproductive traits (Siddiki *et al*., 2020) such as superovulation response in cows (Yang *et al*., 2010), as well as litter size in sheep (Chu *et al*., 2012; Salah *et al*., 2019) and goat (Guo *et al.,* 2013; Hatif *et al.,* 2017; Shinde *et al.,* 2019; Zi *et al*., 2020). So, the objective of this study was to discover and identify the effect of SNP of FSHR gene on prolific traits in Kejobong and Etawah grade doe.

MATERIALS AND METHODS

Sample collection, DNA isolation, Gene amplification, and Sequencing

A total of 16 blood samples were taken from 11 Kejobong doe and 5 Etawah Grade does with various parity and type of birth (Table 1). Blood samples were taken using 3 cc Spuit through *Jugular venous* that was cleaned with alcohol before blood withdrawal. The blood was then collected in vacutainers tubes with anticoagulant (EDTA). The DNA genome then was isolated from the blood sample by using gSYNC DNA mini kit (Geneaid Biotech Ltd.) according to the manufacturer's standard protocol.

FSHR gene was amplified using forward primer 5'-gtcttctgctacaccatattt-3' and reverse primer 5'-tgtccctgtgggtcacttt-3'. Gene amplification was performed by standard PCR methods, with a total volume of 50 µL comprising 25 µL KAPA2G Fast Ready Mix + Dye (Kapa Biosystems Ltd.), 1 µL forward primer, 1 µL reverse primer, 20 µL ddH2O and 3 µL DNA template. PCR was conducted by conditions: predenaturation (at 94°C for 5 min); denaturation (at 94°C for 30 sec), primer annealing (at 56°C for 30 sec), elongation (at 72°C for 30 sec), and final elongation (at 72°C for 10 min) and performed as much as 35 cycles. PCR products were then electrophoresed with 1% Agarose gel at 100 V for 20 min and visualized under a UV trans-illuminator. PCR products were then sequenced through the 1st Base DNA Sequencing Service, Singapore.

Data Analysis

FSHR gene sequence results were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 to find out single nucleotide polymorphism (SNP) and genotype within the sample (Tamura *et al.,* 2021). Clustal W was used to align the sequence (Thompson *et al*., 1994). The FSHR gene sequence was also alignment with AY765375.1 from GenBank as a comparator. The nucleotide sequence then was translated into amino acid forms to determine the effect of nucleotide mutations in the FSHR gene on amino acid sequence alteration. The phylogeny tree was analyzed using the Kimura 2-parameter model method and was built based on the Neighbor-Joining method with 1000 bootstrap replications (Kimura, 1980; Saitou and Nei, 1987; Felsenstein, 1985).

RESULTS

The results of the amplification obtained a partial amplicon of the 5'FSH-R regulatory region along 255 bp (Figure 1), which was indicated by the position of the DNA band between 200 bp and 300 bp of the marker. However, the result of amplification showed a dimer primer which requires extraction gel before it is processed for sequencing. Sequencing results showed that 1 of

Table 1. Sample Information

No.	Code	Breed	Parity	Type of Birth
1	D1	KJ	2	TW
2	D3	KJ	1	S
3	D5	KJ	1	TW
4	D10	KJ	5	TW
5	D11	KJ	2	S
6	D16	KJ	5	S
7	D24	KJ	2	TP
8	D25	KJ	4	TW
9	D26	KJ	1	TP
10	D31	KJ	3	TW
11	D34	KJ	3	TP
12	B	EG	1	TW
13	H	EG	1	TW
14	J	EG	1	S
15	T	EG	1	Q
16	V	EG	1	TP

KJ: Kejobong; EG: Etawah grade; S: single; TW: twin; TP: triplet; Q: quadruplet

Table 2. Identified SNP of FSHR Gene within Sample

No	Name	Site	Point mutation	Triplet codon alteration	Mutation form
1	SNP1	4	G4>T	GTT4>TTT Valine>Phenylalanine	Transversion Nonsynonymous Parsimony
2	SNP2	16	A16>C	ACC9>CCC Threonine>Proline	Transversion Nonsynonymous Parsimony
3	SNP3	43	A43>G	AAA16>GAA Lysine>Glutamic acid	Transition Nonsynonymous Parsimony

Table 3. Haplotype of KJ and EG Doe based on FSHR Gene.

Haplotype	Sample	Mutation Point
1	D1, D3, D10, D11, D25, D31	4g16a43g
2	D5, H	4t16a43g
3	D16, D26, T	4g16c43g
4	D24	4g16c43a
5	D34	4g16a43a
6	B, J	4t16c43a

16 samples could not be continued to the alignment stage. Alignment results of FSHR gene sequence within samples were found 3 SNPs (Figure 2) that originated from 255 bp sequence. The three SNPs were parsimony form and comprised of 1 transition mutation and 2 transversion mutations that caused amino acid sequence alteration (Table 2).

SNP 1 was found at the 4th site that undergoes a transversion mutation from Guanin - Thymine. The base mutation causes a change in the amino acid codon triplet, GTT-TTT, which was started by Valine and turned into Phenylalanine. Other mutations were transversion mutations such as SNP2 that were found at the 16th site of Adenine - Cytosine. It changed the codon triplet ACC that was translated as Threonine into CCC as Proline. Another SNP found at the 43rd site as SNP 3, the nucleotide base Adenine underwent a transition mutation to Guanin which causes

changes in the amino acid codon triplet, AAA-GAA, converting Lysine to Glutamic acid. All mutations occurred in the first sequence of the amino acid codon triplet which causes changes in non-synonymous amino acids. In addition, the mutation form which was entirely in the form of parsimony indicated that there was no specific differentiation in the FSHR gene sequence within the goat breed. The nucleotide base mutations that caused amino acid sequence alteration in this study formed 6 types of haplotypes (Table 3). Samples that had the same type of haplotype indicated that these samples had the same FSHR gene sequence.

Alignment results of the FSH-R sequence among KJ, EG, and sequences from GenBank (AY765375.1) as outgroups were found in as many as 11 SNPs (Table 4). Six SNPs originate from transition mutations, 2 SNPs originate from transversion mutations and 2 other SNPs origi-

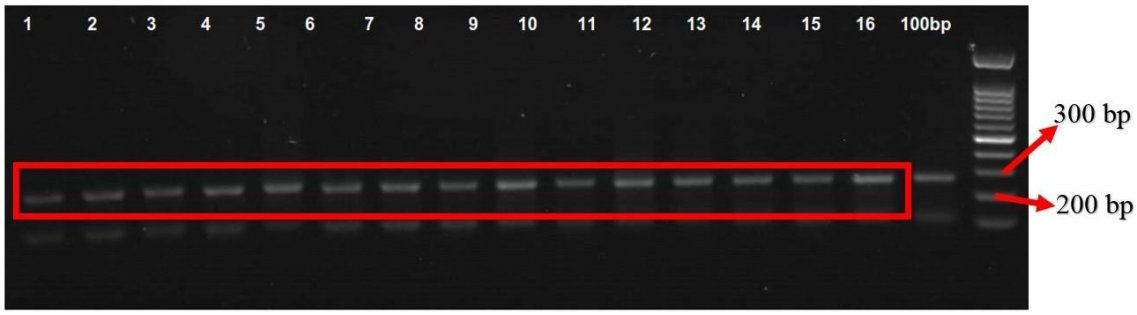

Figure 1. PCR result of FSHR gene

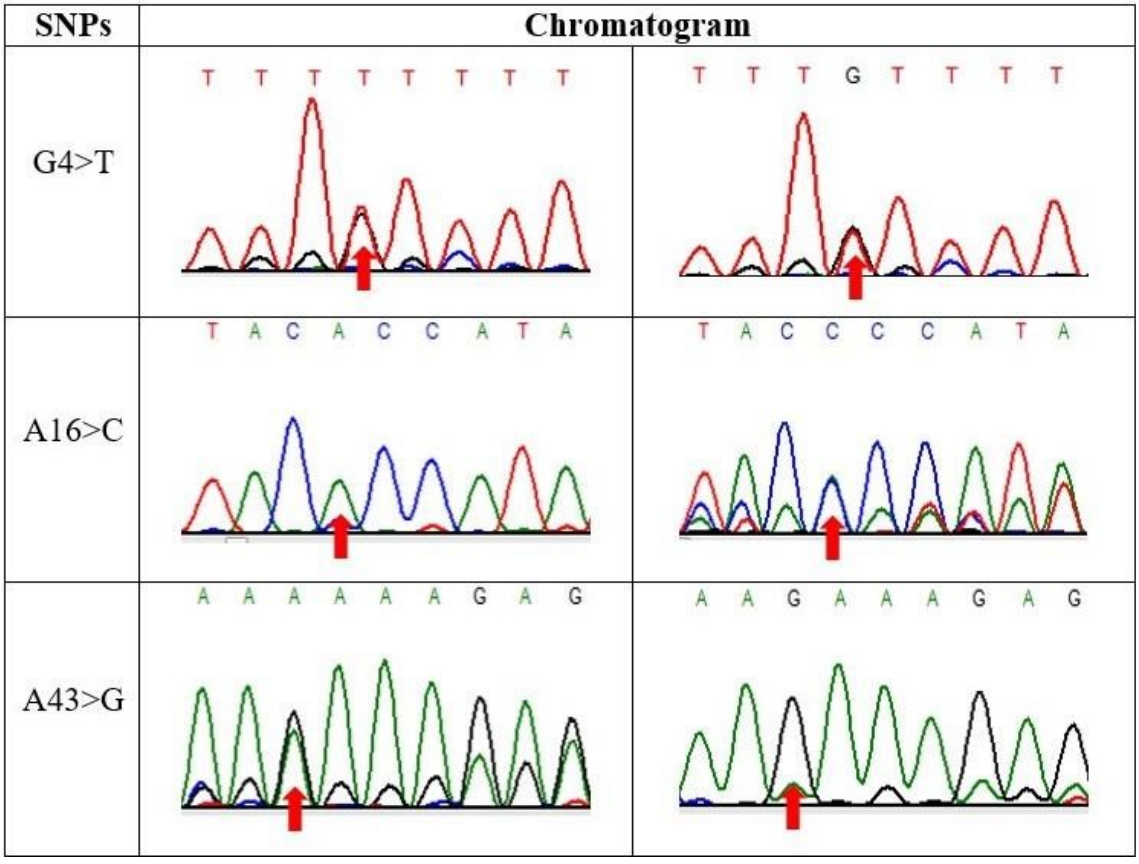

Figure 2. Chromatogram of 3 founded SNPs.

nate from insertion-deletion (indel) mutations (Figure 3). The discovery of indel mutations at sites 403rd and 417th in this study caused the deletion of Serine and the addition of Glycine. As a result of indel mutations, there was a shift in the translation of amino acids as known as frameshift mutation. It could be seen from the final sequence of AY765375.1 which consisted of Lysine (K) – Serine (S) – Aspartic acid (D) – Proline (P) – Glutamine (Q) – Threonine (T), whereas the final sequence in this study consisted of Lysine (K) – Valine (V) – Threonine (T) – Histidine (H) – Arginine (R) – Glycine (G). Sequence differences between AY765375.1 and the sample in this study may be used as genetic markers for reproductive traits, especially fecundity. However, the limited information regarding reproductive data leading difficulties to examine the linkages (effects) between sequence differences and the reproductive ability. Phylogeny tree showed that there were 2 main clusters based on the FSHR sequence (Figure 4).

DISCUSSION

FSHR, a special receptor of the FSH hormone, was located in the granulose cell membrane in the ovaries, uterus and testes. Acting to mediate the action of the hormone FSH for folliculogenesis, FSHR is a trans-membrane receptor belonging to the G protein-coupled (GPCR) receptor family. The results of this study were different from the results of research conducted by Guo *et al.* (2013), who reported that 2 SNPs were found, namely T70A and G130C in Jining Gray, Inner Mongolia Cashmere and Boer goats using the same primers as this study. The two SNPs were found to form two different genotypes, namely DD (ACAGA-CTCTT) and CC (ACTGA-CTGTT). The frequency of CC genotypes was mostly found in Jining Gray goats (46%) known as high-fecundity breeds compared

Table 4. Identified SNP of FSHR Gene in KJ and EG Doe aligned with AY765375.1

No	Code	Site	Point mutation	Triplet Codon alteration	Mutation form
1	SNP1	166	C166>T	CCT>TTT Proline > Phenylalanine	Transition Nonsynonymous Singleton
2	SNP2	167	C167>T		
3	SNP3	169	G169>T	GTC>TTT Valine > Phenylalanine	Tranversion Nonsynonymous Parsimony
4	SNP4	171	C171>T	GTC>GTT Valine	Transition Synonymous Parsimony
5	SNP5	174	C174>T	TTC>TTT Phenylalanine	Transition Synonymous Parsimony
6	SNP6	181	A181>C	ACC>CCC Threonine>Proline	Tranversion Nonsynonymous Parsimony
7	SNP7	208	G208>A	GAA>AAA Glutamic Acid>Lysine	Transition Nonsynonymous Parsimony
8	SNP8	393	A393>G	AAA>AAG Lysine	Transition Nonsynonymous Singleton
9	SNP9	403	A403> --	AGT>-GT Serine > -	Deletion Singleton
10	SNP10	417	-- 417>G	GGA>GGG - >Glycine	Insertion Singleton
11	SNP11	421	G421>A	GTC>ATC Valine>Isoleucine	Transition Nonsynonymous Singleton

to Inner Mongolia Cashmere (19%) and Boers (22%), known as low-fecundity breeds. Interestingly, the 2 SNPs's position were conserved in this study. In that position, the sequences of both goat breeds in this study were ACTGA-CTGTT which means that all samples in this study had the same CC genotype as found in the Jinning Gray goat. This showed that all samples of does in this study had the genetic potential to give birth to twin kids or even more.

The three discovered SNPs in this study caused amino acid sequence alterations in the FSHR coding gene. Those alteration were suspected of affecting the expression of mRNA and protein levels. Cui *et al.* (2009) reported that mutations found at the 4 sites of the FSHR gene not only affected the level of mRNA and protein expressed, but also affected the number of follicles, oocytes and serum FSH hormone in Yunling black and Boar goats. Even cases of infertility can be motivated by the inactivity of the function of FSHR caused by mutations in the FSHR gene (Desai *et al.,* 2011)

The phylogeny tree showed that there were 2 main clusters based on the FSHR sequence (Figure 3). The random distribution of KJ and EG in the phylogeny tree indicated no specific differences between the two breeds. The distribution of doe in phylogeny tree based on birth type showed doe that had single (S) (D3, D11, J) and twins (TW) (D25, D31, D10, D1, D5, H, and B) grouped into Cluster I. Cluster II was occupied randomly by a doe with triplet (TP) and quadruplet (Q) (D26, D24, D34, and T), except for D16 that had a single type. The random distribution of samples from the analysis of the FSHR sequence indicated that the type of birth is a polygenic traits, controlled by many genes. Interestingly, although all goats have the potential for prolificacy trait, due to different environmental influences, the genetic potential is not well expressed. So there are variations in the type of birth even in the same cluster / haplotype group. For example, D16 delivered single off-

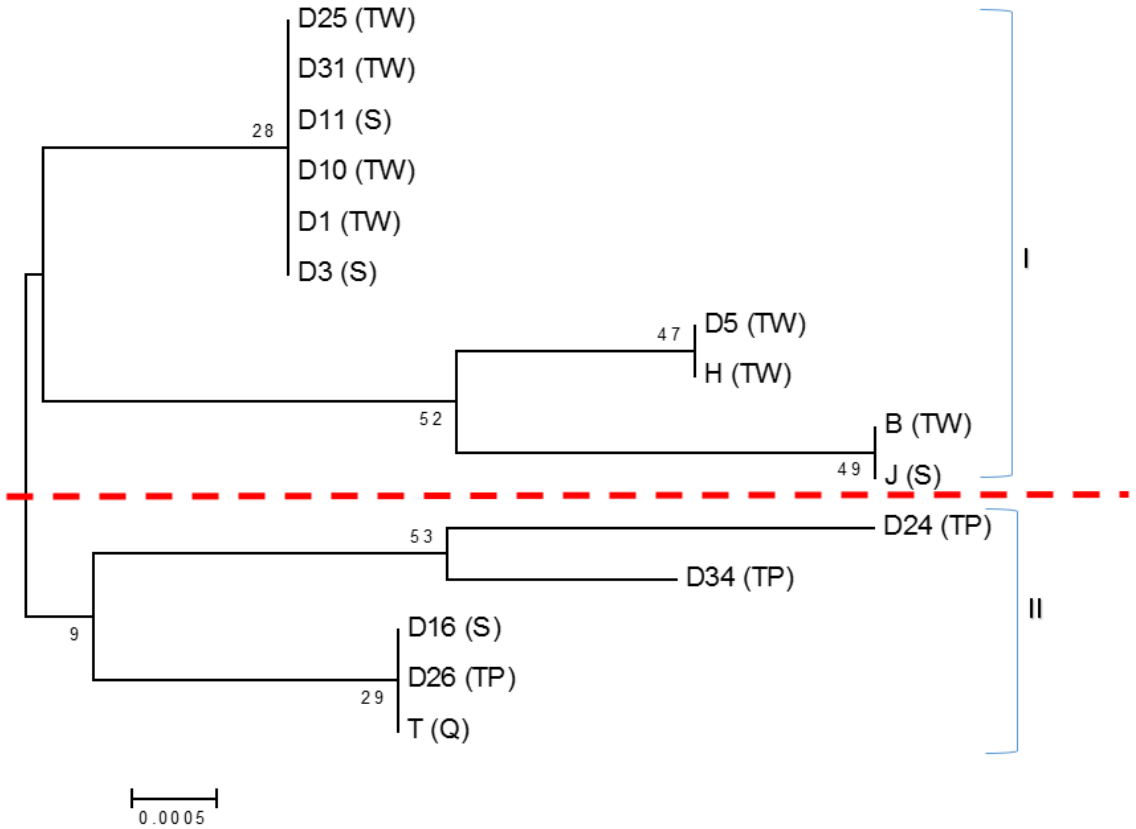

Figure 4. Phylogeny tree of doe based prolification and FSHR gene sequence

spring at parity 5. However, based on the phylogenic tree (Figure 4) it was placed in the same group of does with triplet and quadruplet. Similarly to this case, Akpa *et al.* (2011) which worked with Nigerian goat, found that twinning does started to increase at parity 2 then decrease sharply at parity 5. These are possibly influenced by the physiological maturity of the doe. Accordingly, culling the doe after parity 4 is recommended for economic profit in breeding program. Therefore, SNPs that were found in FSHR gene are possible to be used as genetic marker for prolificacy trait that well expressed up to parity 3.

CONCLUSION

SNPs identified in FSHR gene in this study are associated with prolific trait. Haplotype 3, 4, and 5 showed the highest prolific trait and can be used as genetic marker in KJ and EG. Further study is needed to confirm the effect of those SNPs on the offspring.

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CONFLICT OF INTEREST

All authors declare that there has no conflict of interest with any parties, individuals, organizations and companies in this study.

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