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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.

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Follicle Stimulating Hormone Receptor (FSHR) Gene SNPs as an Informative Markers for Prolific Trait of Local Goat in Indonesia

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Abstract

Objective of this study was to discover and identify the effect of SNP of FSHR gen on prolific traits in Kejobong (KJ) and Etawa grade (EG) doe. A total of 15 blood samples were taken from 11 KJ and 4 EG does with various parity and type of birth. 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). Result showed that 3 SNPs were identified, 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 is associated with prolific trait that can be used as 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 economic traits, reproductive traits improvement in livestock has become of main concern, especially in small ruminant like

goats, which known had high litter size. Kejobong goat (KJ) and Etawa Grade goat (EG) are two local goat breeds in Indonesia that known had high prolific trait with litter size 1.40 and 1.20, respectively (Panjono *et al*., 2012). As stated by Febriana *et al.* (2017) prolific was one of reproductive traits which showed the ability to produce more than a kid in a birth. Regulated by different fecundity genes, prolific trait related to genetic factor improving ovulation rate and litter size.

Follicle-stimulating 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 a must for the formation of the placenta and thus conception. FSH action must be mediated by 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 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 give negative impact on expression patterns of FSHR in Malahabari goat.

So far, a large number of single nucleotide polymorphism (SNP) have been detected in the FSHR gene, which are 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, objective of this study was to discover and identify the effect of SNP of FSHR gen on prolific traits in Kejobong and Etawa grade doe.

MATERIALS AND METHODS

Ethical approval

The protocol was based on the standard rule of animal treating as appointed in the Republic of Indonesia's law, number 41, 2014.

Sample collection, DNA isolation, Gene amplification and sequencing

A total of 15 blood samples were taken from 11 Kejobong doe and 4 Etawa 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 withdrawing. The blood was then collected in vacutainers tubes with anticoagulant (EDTA). DNA genome then was isolated from 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 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: pre-denaturation (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 UV trans-illuminator. PCR products were then sequenced through 1st Base DNA Sequencing Service, Singapore.

Data Analysis

FSHR gene sequence result were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 to find out single nucleotide polymorphism (SNP) and genotype within sample (Tamura *et al.*, 2021). Clustal W was used to alignment the sequence (Thompson *et al*., 1994). FSHR gene sequence was also alignment with AY765375.1 from genbank as 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 sequences 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).

RESULT

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 marker. However, the result of amplification showed dimer primer which requires extraction gel before it is processed for sequencing. Sequencing result showed that 1 of 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 tranversion mutations that caused amino acid sequences 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 started by Valine and turned into Phenylalanine. Other mutations were transversion mutations 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 Adenin underwent a transition mutation to Guanin which causes changed in the amino acid codon triplet, AAA→GAA, converting Lysine to Glutamic acid. All mutations occured in the first sequence of the amino acid codon triplet which causes changed in non-synonymus 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 goat breed. The nucleotide base mutations that caused amino acid sequences alteration in this study forming 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 as much as 11 SNPs (Table 4). Six SNPs originate from transition mutations, 2 SNPs originate from tranversion mutations and 2 other SNPs originate from insertion-deletion (indel) mutations (Figure 3). The discovery of indel mutations at sites 403rd and 417th in this study caused deletion of Serine and addition of Glycine. As a result of indel mutations there was a shift in the translation of amino acids as known as frame shift 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 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 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. Results of this study was 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 found form 2 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 to Inner Mongolia Cashmere (19%) and Boers (22%), known as low-fecundity breeds. Interestingly, the 2 SNPs position was conserved in this study. In that position the sequences of both goat breed 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 doe in this study had genetic potential to give birth twin kid or even more.

The three discovered SNPs in this study caused amino acid sequences alteration in the FSHR coding gene. Those alteration suspected 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 affecting the level of mRNA and protein expressed, but also affecting 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)

Phylogeny tree showed that there were 2 main clusters based on the FSHR sequence (Figure 3). The random distribution of KJ and EG in phylogeny tree indicated no specific differences between the two breed. 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 doe with triplet (TP) and quadraplet (Q) (D26, D24, D34, and T), except for D16 that had single type. The random distribution of samples from the analysis of the FSHR sequence indicated that the type of birth is a polygenic trait, 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 offspring at parity 5, however, based on phylogenic tree (Figure 4) it was placed in the same group of doe with triplet and quadraplet. 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 possibly influenced by

physiological maturity of the doe. Accordingly, culling the doe after parity 4 is recommended for economically profit in breeding program. Therefore, SNPs that found in FSHR gene is 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 is associated with prolific trait. Haplotype 3, 4, and 5 showed 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 to 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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282

TABLES

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: Quadraplet

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 Non synonymous Parsimony
2	SNP2	16	A16>C	ACC9>CCC Threonine>Proline	Transversion Non synonymous Parsimony
3	SNP3	43	A43>G	AAA16>GAA Lysine>Glutamic acid	Transition Non synonymous 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

294 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 Non synonymous Singleton
2	SNP2	167	C167>T		
3	SNP3	169	G169>T	GTC>TTT Valine > Phenylalanine	Tranversion Non synonymous 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 Non synonymous Parsimony
7	SNP7	208	G208>A	GAA>AAA Glutamic Acid>Lysine	Transition Non synonymous Parsimony
8	SNP8	393	A393>G	AAA>AAG Lysine	Transition Non synonymous 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 Non synonymous Singleton

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296

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FIGURES

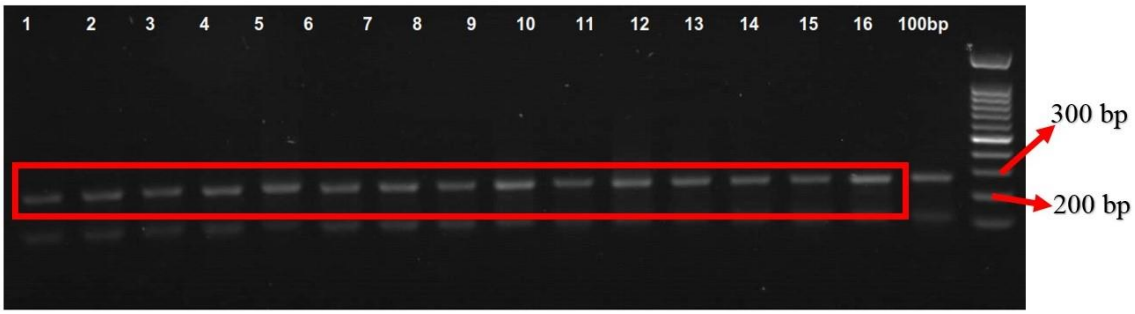


Figure 1. PCR result of FSHR gene

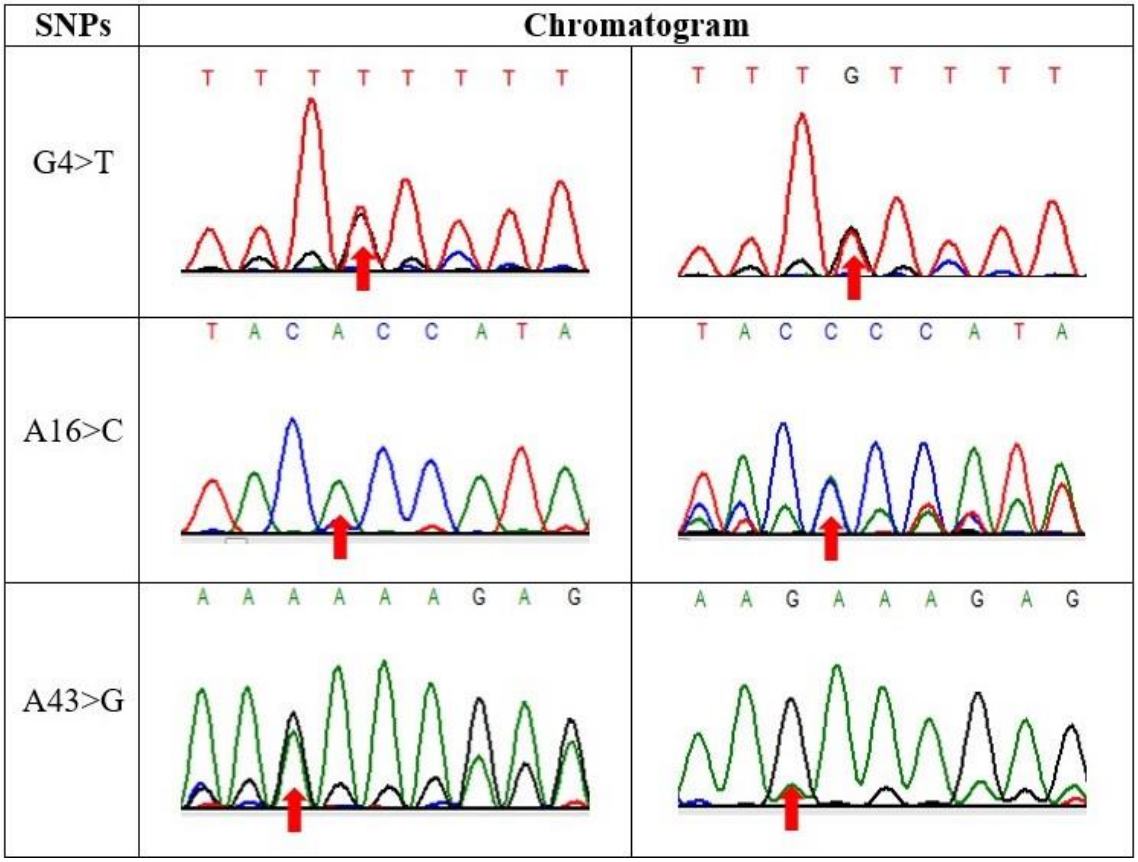
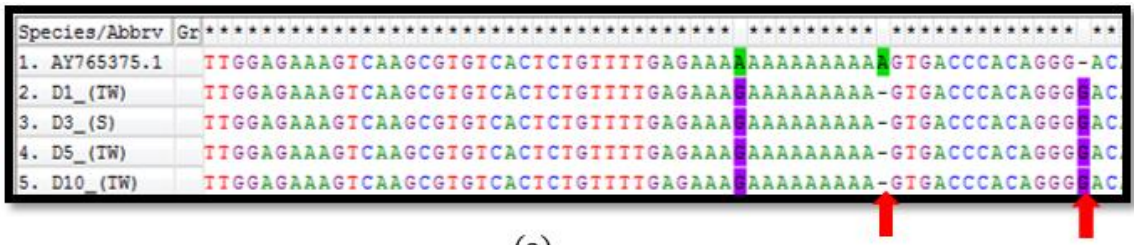
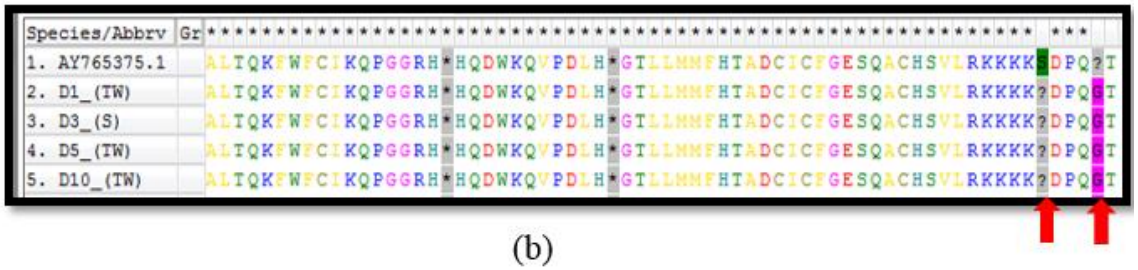


Figure 2. Chromatogram of 3 founded SNPs



(a)



(b)

(c)

Figure 3. (a) Indel mutations of nucleotide bases (b) Results of amino acid translation due to indel mutations caused removal of Serine and addition of Glycine (c) Differences result of amino acid translation due

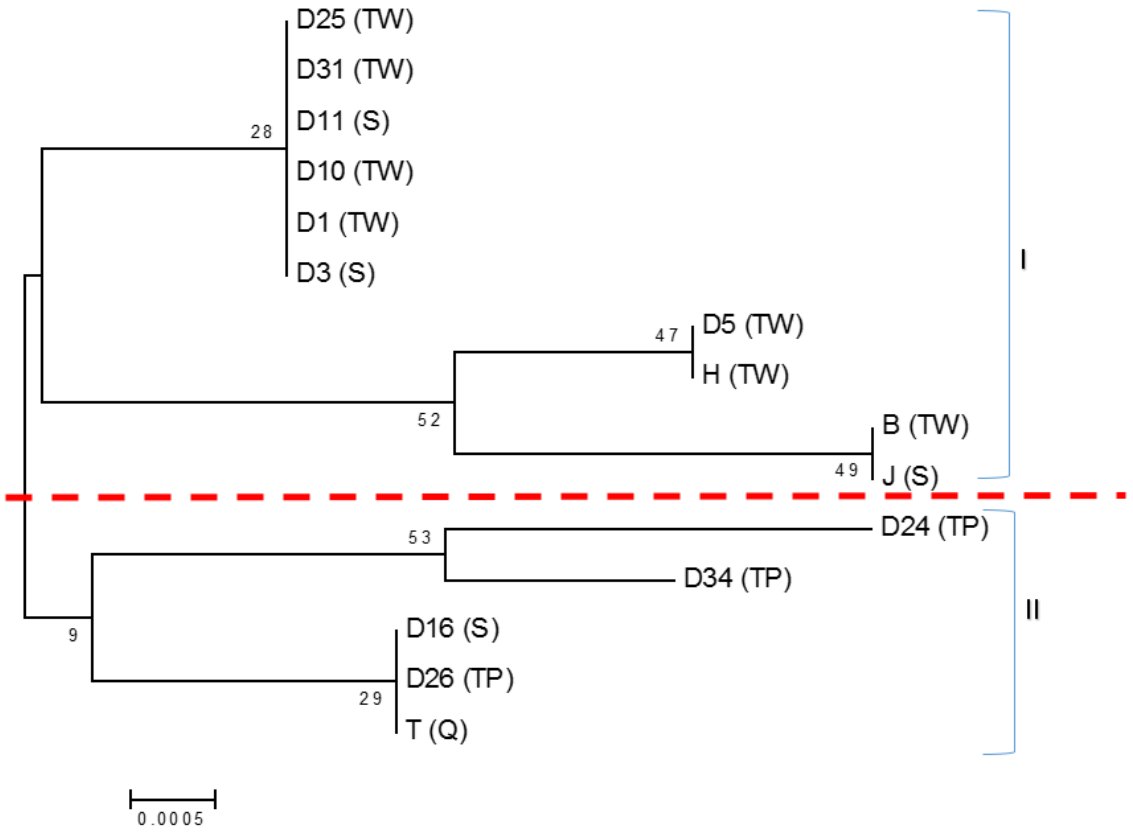


Figure 4. Phylogeny tree of doe based prolification and FSHR gene sequence

[JITAA ID 50343] Editor Decision Minor Revisison

Mr. JITAA UNDIP <jitaa.undip@gmail.com>

Mon 06/02/2023 2:50 PM

To: Enny Tantini Setiatin <ennytantinisetiatin@lecturer.undip.ac.id>

Enny Tantini Setiatin:

We have reached a decision regarding your submission to Journal of the Indonesian Tropical Animal Agriculture, "Follicle Stimulating Hormone Receptor (FSHR) Gene SNPs as an Informative Markers for Prolific Trait of Local Goat in Indonesia".
ased on an evaluation by the review panels our decision is MINOR REVISION,
Please look at the detailed comments on the platform JITAA and check your article according to the author's guidelines. The revised due date is February 13, 2023

Prof. Joelal Achmadi
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Journal of the Indonesian Tropical Animal Agriculture
http://ejournal.undip.ac.id/index.php/jitaa

General comment
Although this study employs a molecular approach, it cannot answer the study's objective.

The limited number of samples was the constraint in generating the conclusion, though it could be using citations from another study.

Specific comments:
Title
Rephrase the title as FSHR gene exploration as a possible marker.
The title does not correspond to the study's goal to discover and identify the effect of SNP.

Material and method

- Ethical approval
  Instead of using the Republic of Indonesia, the method must be approved by in-house ethical clearance or any other institution.
  The general regulation, Law 41 of 2014, cannot be approved as ethical clearance in the specific study.
- Sample: There were 15 blood samples mentioned; see table 1 for a list of 16. Please clarify and go over the manuscript again. There are 16 samples mentioned in the result section.
- What exactly was the AY765375.1? Please include an explanation. This is consistent with the outcome of Table 4. What is the purpose of using the GenBank ID as a comparator?
- Is there a link between phylogenic tree analysis and the prolific trait marker? Please explain or leave this out.
- See table 3. It mentioned the haplotype of the goat sampled. There is no information on how to generate the haplotype. Add this in the material and method
- See table 4. It would be preferable if the identified SNPs in each goat breed were listed per ID sample.

Result

- See line 92. Sequencing results showed that 1 of 16 samples could not be continued to the alignment stage; what does this mean?
- Line 97, and in accordance with the results of table 2, please check whether the mutations, such as ensemble, were recorded in the database. If this is the case, it could be added to the list.

Re: [JITAA ID 50343] Editor Decision Minor Revisison

Enny Tantini Setiatin <ennytantinisetiatin@lecturer.undip.ac.id>

Thu 09/02/2023 6:53 PM

To: Mr. JITAA UNDIP <jitaa.undip@gmail.com>

Dear Editor JITAA

Hereby, I enclosed revision of our manuscript entitle :
Follicle Stimulating Hormone Receptor (FSHR) Gene Exploration as Possible Markers for Prolific Trait of Local Goat in Indonesia .
I also have uploaded the revision via OJS.

Thank you

From: Mr. JITAA UNDIP <jitaa.undip@gmail.com>
Sent: Monday, 6 February 2023 2:50 pm
To: Enny Tantini Setiatin <ennytantinisetiatin@lecturer.undip.ac.id>
Subject: [JITAA ID 50343] Editor Decision Minor Revisison

Enny Tantini Setiatin:

We have reached a decision regarding your submission to Journal of the Indonesian Tropical Animal Agriculture, "Follicle Stimulating Hormone Receptor (FSHR) Gene SNPs as an Informative Markers for Prolific Trait of Local Goat in Indonesia".
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Please look at the detailed comments on the platform JITAA and check your article according to the author's guidelines. The revised due date is February 13, 2023

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AUTHOR'S RESPONSE

No	Section	Reviewer Comment	Author's response
1	Title	Rephrase the title as FSHR gene exploration as a possible marker. The title does not correspond to the study's goal to discover and identify the effect of SNP.	Has been revised.
2	Materials and Methods	Ethical approval: Instead of using the Republic of Indonesia, the method must be approved by in-house ethical clearance or any other institution. The general regulation, Law 41 of 2014, cannot be approved as ethical clearance in the specific study.	Has been removed
3		Sample: There were 15 blood samples mentioned; see table 1 for a list of 16. Please clarify and go over the manuscript again. There are 16 samples mentioned in the result section.	Has been revised. Clarification : This study was using 16 samples
4		What exactly was the AY765375.1? Please include an explanation. This is consistent with the outcome of Table 4. What is the purpose of using the GenBank ID as a comparator?	Clarification: AY765375.1 (as comparator from Genbank) used to identify the specific characteristics the sequences of the samples in this study, so that the comparison is not only by inter-population but also using outgroup from genbank (AY765375.1)
5		Is there a link between phylogenic tree analysis and the prolific trait marker? Please explain or leave this out.	Clarification: The phylogenetic tree in this study is used to describe the distribution of parents (sample) based on the type of birth, so that it can be supporting data for confirmation between genetic sequences and the ability of the parents seen from the type of birth
6		See table 3. It mentioned the haplotype of the goat sampled. There is no information on how to generate the haplotype. Add this in the material and method	Clarification: The haplotype were generated by aligning one sample with another to find SNP, and SNPs collection in one sample will

			become haplotype (in another way it similar to SNP pattern).
7		See table 4. It would be preferable if the identified SNPs in each goat breed were listed per ID sample.	Clarification : SNP within sample already listed in table 2.
8	Result	See line 92. Sequencing results showed that 1 of 16 samples could not be continued to the alignment stage; what does this mean?	Clarification : It mean 1 sample is damage while sequencing process. So data analysis was conducted using 15 samples.
9		Line 97, and in accordance with the results of table 2, please check whether the mutations, such as ensemble, were recorded in the database. If this is the case, it could be added to the list.	Clarification : Those mutations were novelty in our study, so it were not recorded in the database yet.

Running head: SNPs of FSHR gene as genetic marker for prolific trait on goat

Follicle Stimulating Hormone Receptor (FSHR) Gene Exploration as Possible Markers for Prolific Trait of Local Goat in Indonesia

Abstract

Objective of this study was to discover and identify the effect of SNP of FSHR gen on prolific traits in Kejobong (KJ) and Etawa grade (EG) doe. A total of 15 blood samples were taken from 11 KJ and 4 EG does with various parity and type of birth. 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). Result showed that 3 SNPs were identified, 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 is associated with prolific trait that can be used as 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 economic traits, reproductive traits improvement in livestock has become of main concern, especially in small ruminant like goats, which known had high litter size. Kejobong goat (KJ) and Etawa Grade goat (EG) are two local goat breeds in Indonesia that known had high prolific trait with litter size 1.40 and 1.20, respectively (Panjono *et al*., 2012). As stated by Febriana *et al.* (2017)

prolific was one of reproductive traits which showed the ability to produce more than a kid in a birth. Regulated by different fecundity genes, prolific trait related to genetic factor improving ovulation rate and litter size.

Follicle-stimulating 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 a must for the formation of the placenta and thus conception. FSH action must be mediated by 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 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 give negative impact on expression patterns of FSHR in Malahabari goat.

So far, a large number of single nucleotide polymorphism (SNP) have been detected in the FSHR gene, which are 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, objective of this study was to discover and identify the effect of SNP of FSHR gen on prolific traits in Kejobong and Etawa 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 Etawa 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 withdrawing. The blood was then collected in vacutainers tubes with anticoagulant (EDTA). DNA genome then was isolated from 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 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: pre-denaturation (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 UV trans-illuminator. PCR products were then sequenced through 1st Base DNA Sequencing Service, Singapore.

Data Analysis

FSHR gene sequence result were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 to find out single nucleotide polymorphism (SNP) and genotype within sample (Tamura *et al.*, 2021). Clustal W was used to alignment the sequence (Thompson *et al*., 1994). FSHR gene sequence was also alignment with AY765375.1 from genbank as 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 sequences 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).

RESULT

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 marker. However, the result of amplification showed dimer primer which requires extraction gel before it is processed for sequencing. Sequencing result showed that 1 of 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 tranversion mutations that caused amino acid sequences alteration (Table 2).

SNP 1 was found at the 4^{th} site that undergoes a transversion mutation from Guanin → Thymine. The base mutation causes a change in the amino acid codon triplet, GTT→TTT, which started by Valine and turned into Phenylalanine. Other mutations

were transversion mutations 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 Adenin underwent a transition mutation to Guanin which causes changed in the amino acid codon triplet, AAA→GAA, converting Lysine to Glutamic acid. All mutations occured in the first sequence of the amino acid codon triplet which causes changed in non-synonymus 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 goat breed. The nucleotide base mutations that caused amino acid sequences alteration in this study forming 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 as much as 11 SNPs (Table 4). Six SNPs originate from transition mutations, 2 SNPs originate from tranversion mutations and 2 other SNPs originate from insertion-deletion (indel) mutations (Figure 3). The discovery of indel mutations at sites 403rd and 417th in this study caused deletion of Serine and addition of Glycine. As a result of indel mutations there was a shift in the translation of amino acids as known as frame shift 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 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 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. Results of this study was 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 found form 2 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 to Inner Mongolia Cashmere (19%) and Boers (22%), known as low-fecundity breeds. Interestingly, the 2 SNPs position was conserved in this study. In that position the sequences of both goat breed 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 doe in this study had genetic potential to give birth twin kid or even more.

The three discovered SNPs in this study caused amino acid sequences alteration in the FSHR coding gene. Those alteration suspected 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 affecting the level of mRNA and protein expressed, but also affecting 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)

Phylogeny tree showed that there were 2 main clusters based on the FSHR sequence (Figure 3). The random distribution of KJ and EG in phylogeny tree indicated no specific differences between the two breed. 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 doe with triplet (TP) and quadraplet (Q) (D26, D24, D34, and T), except for D16 that had single type. The random distribution of samples from the analysis of the FSHR sequence indicated that the type of birth is a polygenic trait, 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 offspring at parity 5, however, based on phylogenic tree (Figure 4) it was placed in the same group of doe with triplet and quadraplet. 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 possibly influenced by physiological maturity of the doe. Accordingly, culling the doe after parity 4 is recommended for economically profit in breeding program. Therefore, SNPs that found in FSHR gene is 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 is associated with prolific trait. Haplotype 3, 4, and 5 showed 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 to the offspring.

ACKNOWLEDGEMENT

This study was funded by *Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jendral Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi dan Pendidikan Tinggi* with contract number 101-101/UN7.P4.3/PP/2019.

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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274

TABLES

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: Quadraplet

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 Non synonymous Parsimony
2	SNP2	16	A16>C	ACC9>CCC Threonine>Proline	Transversion Non synonymous Parsimony
3	SNP3	43	A43>G	AAA16>GAA Lysine>Glutamic acid	Transition Non synonymous 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

286 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 Non synonymous Singleton
2	SNP2	167	C167>T		
3	SNP3	169	G169>T	GTC>TTT Valine > Phenylalanine	Tranversion Non synonymous 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 Non synonymous Parsimony
7	SNP7	208	G208>A	GAA>AAA Glutamic Acid>Lysine	Transition Non synonymous Parsimony
8	SNP8	393	A393>G	AAA>AAG Lysine	Transition Non synonymous 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 Non synonymous Singleton

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FIGURES

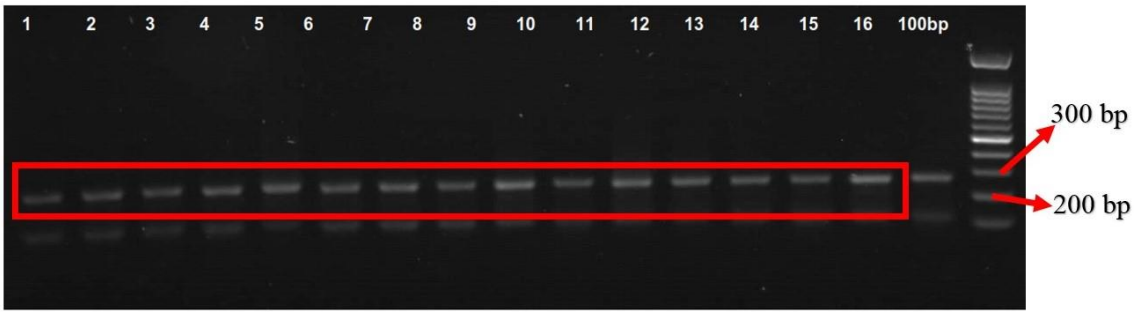


Figure 1. PCR result of FSHR gene

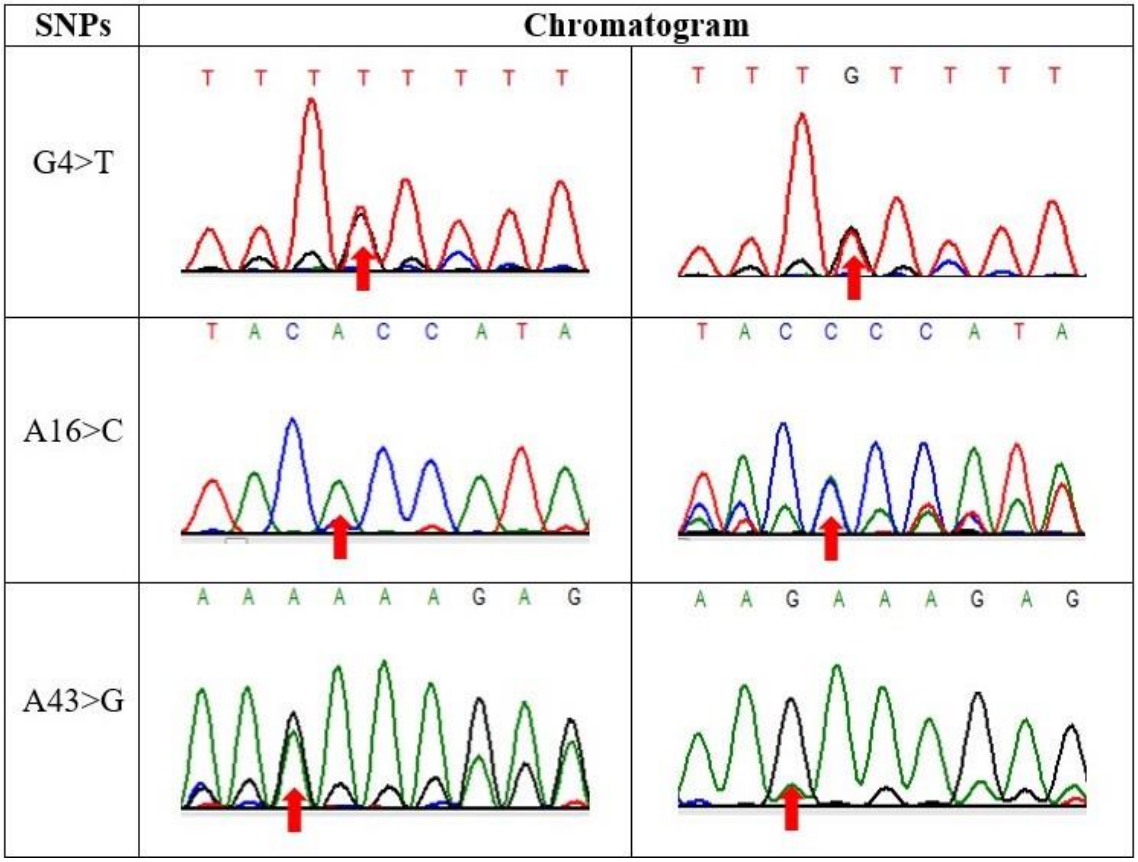
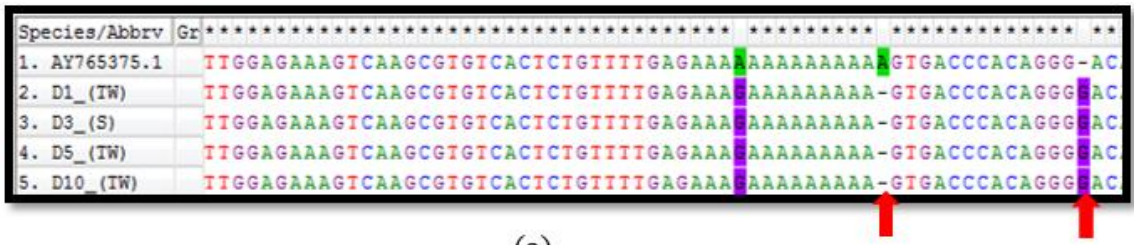
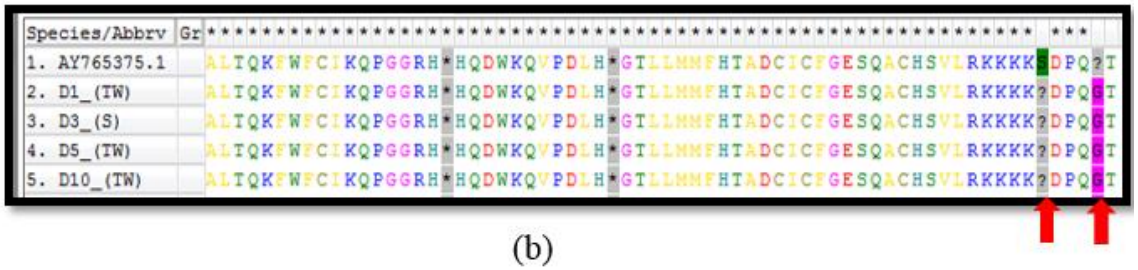


Figure 2. Chromatogram of 3 founded SNPs



(a)



(b)

(c)

Figure 3. (a) Indel mutations of nucleotide bases (b) Results of amino acid translation due to indel mutations caused removal of Serine and addition of Glycine (c) Differences result of amino acid translation due

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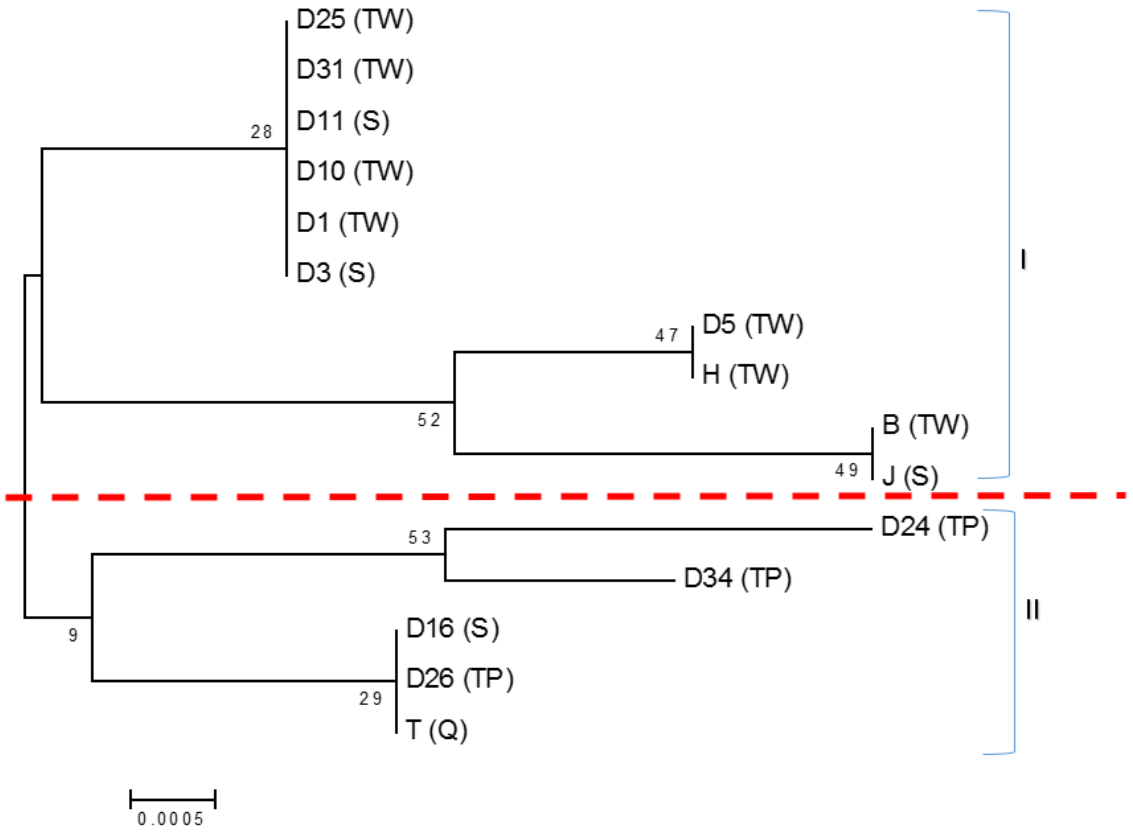

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306 Figure 4. Phylogeny tree of doe based prolification and FSHR gene sequence

[JITAA ID 50343] Editor Decision Accept

Mr. JITAA UNDIP <jitaa.undip@gmail.com>

Tue 14/02/2023 8:18 PM

To: Enny Tantini Setiatin <ennytantinisetiatin@lecturer.undip.ac.id>

Dear Enny Tantini Setiatin,

We have reached a decision regarding your submission to Journal of the Indonesian Tropical Animal Agriculture, "Follicle Stimulating Hormone Receptor (FSHR) Gene SNPs as an Informative Markers for Prolific Trait of Local Goat in Indonesia".

We are pleased to inform you that your paper has been ACCEPTED for publication.
Please add the authorship information to the article.
Please send a clean and clear last version of your article for forwarding to the editorial step.

Thank you for submitting your work to JITAA. We hope you consider us again for future submissions.
Thank you for your contribution.

Best regards,

Prof. Joelal Achmadi, Ph.D.
Editor-in-Chief
(http://ejournal.undip.ac.id/index.php/jitaa)

Journal of the Indonesian Tropical Animal Agriculture
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[JITAA ID 50343] Proof sheet and Charge of Publication

Mr. JITAA UNDIP <jitaa.undip@gmail.com>

Tue 28/02/2023 3:49 PM

To: Enny Tantini Setiatin <ennytantinisetiatin@lecturer.undip.ac.id>

Dear Dr. Enny Tantini Setiatin,

We are sending the proof sheet of your article, "Follicle Stimulating Hormone Receptor (FSHR) Gene SNPs as an Informative Markers for Prolific Trait of Local Goat in Indonesia" to be corrected.
Please check and send the information of the part of the proof sheet corrected in a separate sheet (page...., column...., row...., written....., correction .....).

We are also sending the letter regarding the charge for publication. Please complete your proof and payment steps by February 28, 2023, to be included in the next issue

Best regards,

Prof. Joelal Achmadi, Ph.D.
Editor-in-Chief
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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, 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 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 high 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 a birth. Regulated by different fecundity genes, prolific traits related to genetic factors improve ovulation rate and litter size.

Follicle-stimulating 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 a must for the formation 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 marker. However, the result of amplification showed dimer primer which requires extraction gel before it is processed for

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

sequencing. Sequencing results showed that 1 of 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

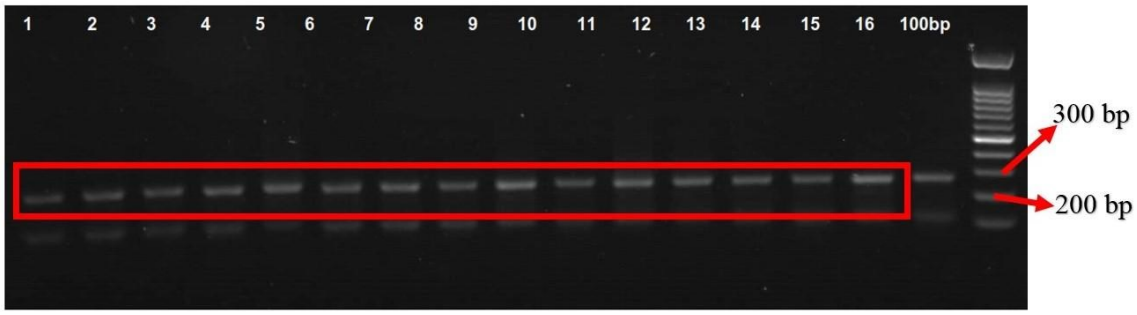

Figure 1. PCR result of FSHR gene

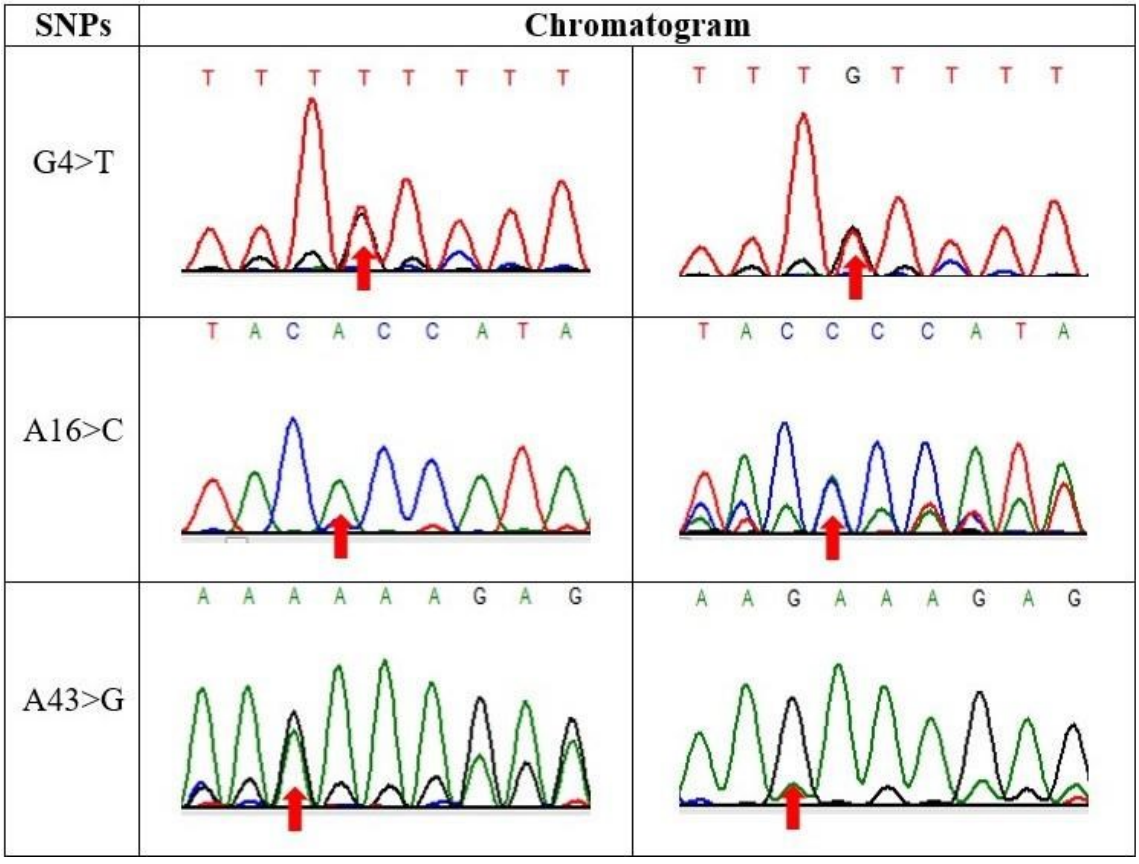

Figure 2. Chromatogram of 3 founded SNPs.

transversion mutations and 2 other SNPs originate 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. Results of this study was 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 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

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

as high-fecundity breeds compared to Inner Mongolia Cashmere (19%) and Boers (22%), known as low-fecundity breeds. Interestingly, the 2 SNPs position was 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 genetic potential to give birth twin kids or even more.

The three discovered SNPs in this study caused amino acid sequences alteration in the FSHR coding gene. Those alteration were suspected 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 affecting the level of mRNA and protein expressed, but also affecting 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)

Phylogeny tree showed that there were 2 main clusters based on the FSHR sequence (Figure 3). The random distribution of KJ and EG in 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 doe with triplet (TP) and quadruplet (Q) (D26, D24, D34, and T), except for D16 that had single type. The random distribution of samples from the analysis of the FSHR sequence indicated that the type of birth is a polygenic trait, 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 exam-

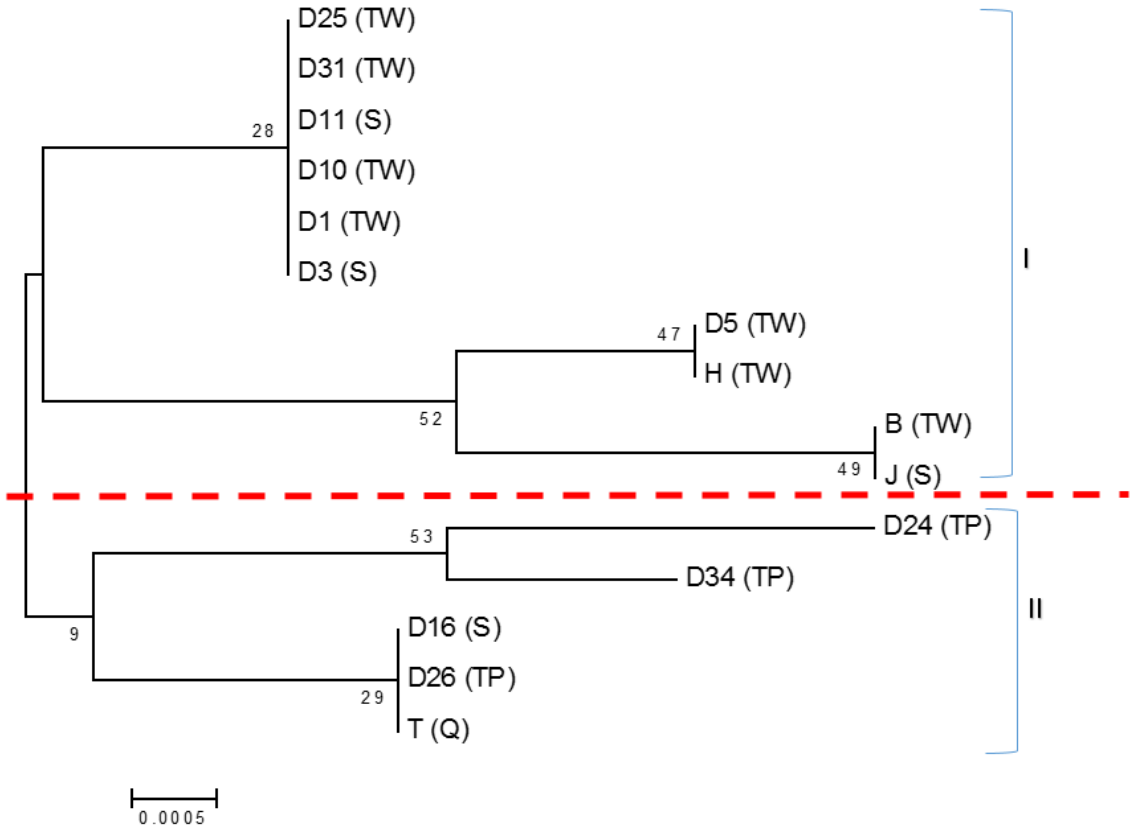

Figure 4. Phylogeny tree of doe based prolification and FSHR gene sequence

ple, D16 delivered single offspring at parity 5, however, based on 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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Page	Column	Row	Written	Correction
1 (abstract)	1	1	follicle stimulating	follicle-stimulating
1 (abstract)	1	6	amino acid sequence, formed	amino acid sequence formed
1 (abstract)	1	8	as genetic	as a genetic
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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-

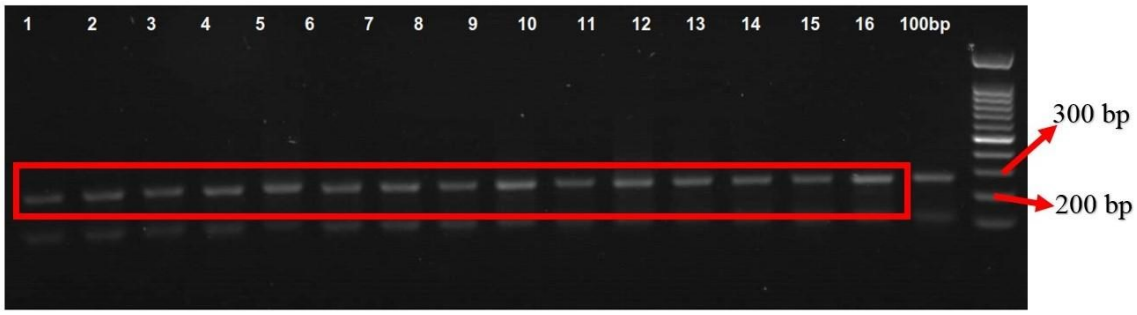

Figure 1. PCR result of FSHR gene

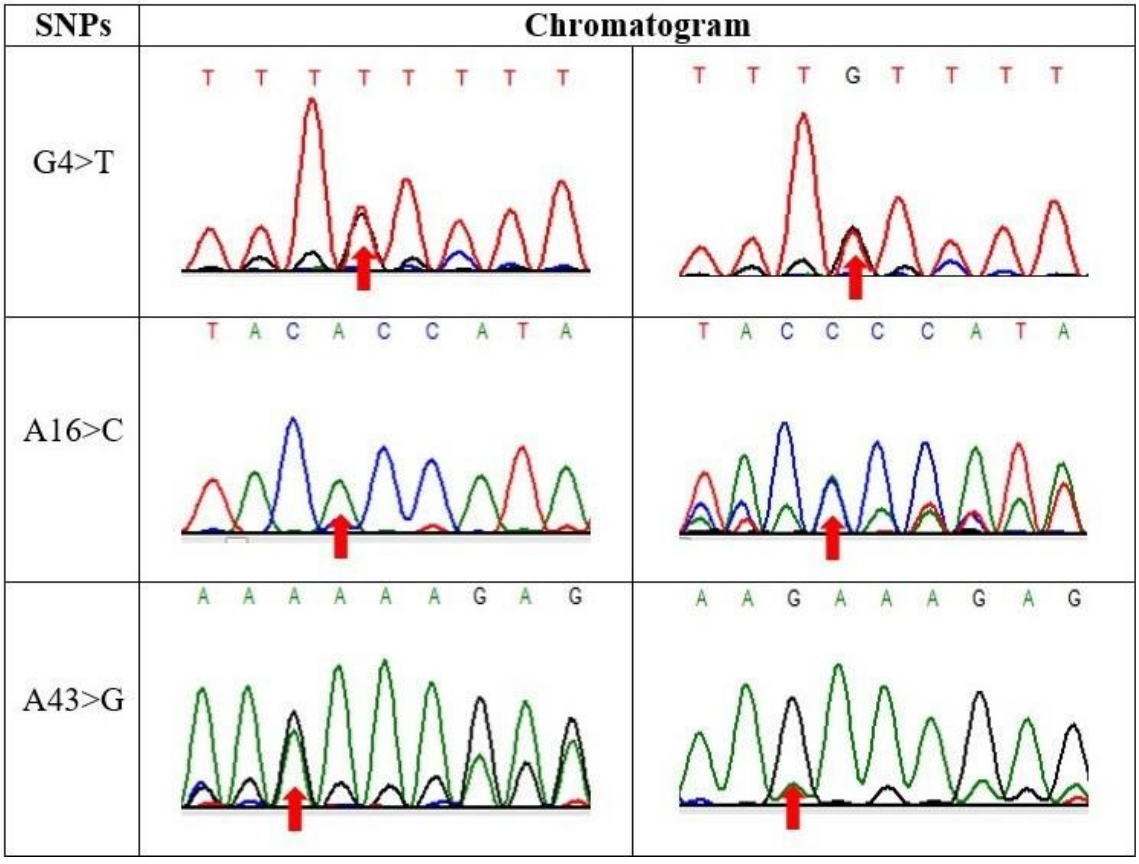

Figure 2. Chromatogram of 3 founded SNPs.

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-

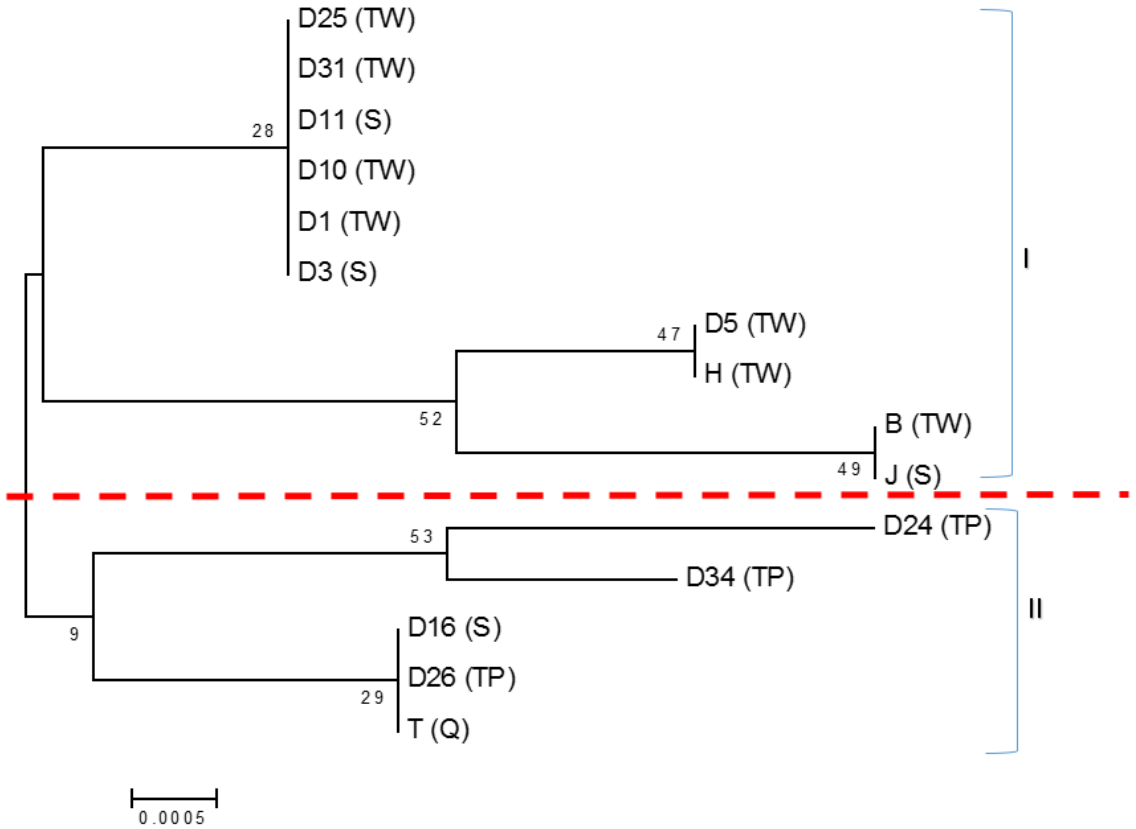

Figure 4. Phylogeny tree of doe based prolification and FSHR gene sequence

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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