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PHYLOGENETIC STUDY AND ASSOCIATION BETWEEN PROMINENT GENOTYPE AND HAPLOTYPE OF KISS1 GENE CORRELATED WITH FSH PLASMA LEVEL IN INDONESIAN NATIVE GOAT BREEDS

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ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi struktur populasi dan ekspresi gen KISS1 yang berkaitan dengan sifat reproduksi menggunakan analisis level Follicle Stimulating Hormone (FSH) dan sekuensing DNA gen KISS. Sejumlah 23 ekor induk yang terdiri dari kambing Kacang (n=7), Kejobong (n=8) and Senduro (n=8) diidentifikasi genotipenya menggunakan metode sekuensing DNA, 16 ekor diantaranya diberi perlakuan sinkronisasi estrus untuk diamati level hormon FSH menggunakan metode ELISA. *Software* MEGA X digunakan untuk menganalisa sekuens DNA, sedangkan General Linier Model (GLM) dari SAS *software* untuk menganalisa hormon FSH. Pohon filogeni memperlihatkan kesamaan yang tinggi antara kambing lokal

Indonesia dengan spesies lain yang menunjukkan gen KISS1 konservatif. Analisis hormon FSH menunjukan hasil signifikan yang lebih tinggi antara Kacang dibandingkan Senduro (P = 0.002),), litter size (LS) 3 dibandingkan LS 1 (P = 0.0175), selanjutnya haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A menunjukkan hormon hasil hormon yang lebih tinggi (P = 0.0027; P<0.0001) dan terkait dengan LS yang tinggi (3.0±0.18). Waktu pengambilan sampel dan paritas tidak memberikan perbedaan yang signifikan terhadap hormon FSH. Penelitian ini menunjukkan bahwa haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A mempunyai asosiasi dengan sifat reproduksi.

Kata kunci : FSH, gen KISS1, haplotipe, kambing lokal Indonesia, pohon filogeni

ABSTRACT

The aim of the current research was to analyze the population structure and expression of KISS1 gene associated with reproductive traits through follicle stimulating hormone (FSH) level and DNA sequencing analysis based on KISS1 gene. Genotypes of 23 goat does consist of Kacang goat (n=7), Kejobong goat (n=8) and Senduro goat (n=8) goats were investigated using DNA sequencing, 16 out of 23 samples were synchronized to examine their FSH level using ELISA method. The data were analyzed using MEGA X software for DNA sequences and General Linier Model (GLM) for FSH plasma level. The phylogenetic tree showed the highly homology between Indonesian native goats with other species showing a gene conservatism. A higher FSH plasma levels were obtained from KC than SD (P = 0.002), litter size (LS) 3 than LS 1 (P = 0.0175), further TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A (P = 0.0027; P<0.0001) and are associated with high LS (3.0±0.18). Neither sample collection times

nor parities have different significantly. The current trial indicated that TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A were correlated with reproduvtive traits.

Keywords : FSH, haplotype, Indonesian native goat, KISS1 gene, phylogenetic tree

INTRODUCTION

Goats, unlike other livestock species, are adaptable animals that can survive in tropical, mountainous, and desert environments. Goats have spread widely due to their adaptability to a variety of environments and nutrition availability, small size, prolific, useful productivity for humans, and non-competitiveness with human food, and they contribute significantly, particularly in rural areas (Aziz, 2010;Guerrero *et al.*, 2019).

In Indonesia, there are more than 19 million goats, with eight goat breeds officially confessed. In Indonesia, goat population has increased over the last five years. (Ministry of Agriculture, 2020). This condition could indicate that goats could be an alternative source of red meat. According to Aziz (2010), Indonesia is one of the 10 largest lamb production country in the world. This situation might represents the Indonesian preference on goat meat because most of goats were reared and consumed locally. Enhancing reproductive traits could be a way to increase the number of goat population.

Indigenous goat breeds are well adapted to agro-ecological conditions, helping to ensure goat farming's long-term viability in rural and harsh environments (Stella, 2018). Goats are traditionally bred in Indonesia for meat and dual-purpose production. In this study, three indigenous goat breeds were used. The Kacang (KC), Kejobong (KJ), and Senduro (SD) goat breeds were known for their reproductive traits. KC has a significant high litter size even when reared in a harsh environment and can be raised as a meat type;

KJ, the does is prolific, has a short kidding interval, and can be raised as a meat type (Sodiq and Haryanto, 2007), while the litter size (LS) in SD is 1.83 ± 0.69 and perform as dual purpose (meat and dairy) type (Ciptadi *et al.*, 2019). KJ is solely located in Central Java, whereas SD goats start to spread massively in Indonesia following KC. KC is also known as Indonesian native goats (Batubara *et al.,* 2006). Half of Indonesian goat population is existed in Java, therefore a study based on goat population in Java was expected to represent the entire goat population in Indonesia, particularly in term of specific reproductive traits.

So far, the genetic structure of important economic traits has been identified, but the number of causative genes in goats has been lower than in sheep and cattle (Amills *et al.*, 2017). The phenotypic variations of goats were shaped by a various of artificial or natural factors such as migration of human, environmental changes and influences of socioeconomic. Further, the genomic variability of goats were constructed mostly by breeding orientation and artificial selection during domestication (Wang *et al.*, 2016). Principally, the sustainable selection and advancement of a novel traits in an environmental shifting needs the genetic diversity (Mandal *et al.,* 2020).

Reproduction is a critical function for the survival of the species, thus this function is governed by complex regulatory signals. The hypothalamic-pituitary-gonadal (HPG) axis regulates reproductive activity by modulating the secretion of inhibitory factors and pituitary gonadotropins by the hypothalamus. Gonadotropin activity in the gonad is mediated by peripheral blood circulation (Nagamalleswari *et al.*, 2004). The HPG axis is divided into three regulatory signals: 1) hypothalamus: gonadotropin-releasing hormone (GnRH); 2) pituitary: gonadotropin, luteinizing hormone (LH) and follicle stimulating hormone (FSH); and 3) gonads: sex steroid and peptides (Pinilla *et al.*, 2012).

Follicle Stimulating Hormone (FSH) is a pituitary gonadotropin which have essential task in reproduction. The main roles of FSH in female are maturation and development in antral follicles, encourage the antrum formation in secondary follicles and organize a response for ovulation when the LH surge (Mahdavi and Dashab, 2017).

The present studies was undertaken to analyze the DNA sequences, appraising evolutionary distances and the population structure of the phylogenetic tree based on KISS1 gene sequences of three Indonesian indigenous goat breeds compare to other species sequences. Therefore, as KISS1 gene plays an important role on reproduction, this study was carried out to explore the relative expression of KISS1 gene through FSH plasma analysis from different goat breeds, litter size, haplotypes and genotypes to describe its relationship with litter size at kidding.

MATERIALS AND METHODS

Ethical Clearance

The protocol of the current research was under the standart rule of animal treatment as designated in the Republic of Indonesia's law, that is, number 41, 2014.

2.1. Animals and samples collection

A total of 23 heads of goat does from three Indonesian indigenous goat breeds, namely KC (n=7), KJ (n=8) and SD (n=8) were utilized in the present study. The goats were healthy, unrelated and were not pregnant. They were selected randomly based on LS, age, multiparous (2^{nd} to 5^{th} parities) and have phenotypic characteristic of each breeds. These breeds represent different regions and altitudes, KC in Grobogan regency,

KJ in Purbalingga regency, both are in Central Java while SD is from Lumajang regency East Java (Fig.1). The Grobogan, Purbalingga and Lumajang were located at 100 m, 113 m and 500 m height above mean sea level (AMSL) respectively. The goats were kept by the farmer under the homogenous environment.

2.2. Genomic DNA extraction

A total of 3 ml of blood samples were collected via the jugular vein in to sterile vacuum tubes with anticoagulant (EDTA). The blood samples were shifted to the laboratory using coolbox and freezed at -20°C until the genomic DNA extraction. Thus, GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) was applied to extract the genomic DNA from the whole blood correspond the manufacturer's guidance. The evaluation of DNA quality was set up by electrophoresis using agarose gel (1%) and Nanodrop spectrophotometer Uvidoc HD6 (UVItec Ltd., Cambridge, UK*)*.

A clear single band on agarose (1%) electrophoresis and the optical density (OD) 260/280 ratio ranged between 1.7 and 2.0 (according to the kit protocol) verifying a good quality of DNA extraction.

2.3. PCR amplification

A 1061bp fragment of intron 2 KISS1 gene was amplified with a pair of primer (F: 5'-GCTCAGTGGCCAGGAATCTG-3'; R: 5'-GCTCATAGCAGGGCCTCAAA-3'). The primers were designed using the sequence of KISS1 gene of *Capra hircus* breed Jining Grey (GenBank Accession Number GU142847.1) and Primer3 Plus software. Polymerase Chain Reaction (PCR) was perform in 50 µl volume containing 4 µl DNA extraction (20-30 ng/ µl), 1 µl for each primer (10 pmol/ µl), 19 µl ddH2O and 25 µl of MyTaq Red Mix Bioline (1st BASE) on MJ Mini Personal Thermal Cycler (Bio-Rad, USA). PCR cycling program contain of pre-denaturation at 95°C for 5 min, followed by

35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72 °C for 30 s, and post extension at 72 °C for 7 min. The amplicon was performed by electrophoresis in 2 % agarose gel (Invitrogen, Life Technologies Co, CA) at 100V for 30 min.

2.4. DNA sequencing and analysis.

The amplicon of 23 goats reflecting three indigenous Indonesian goat breeds were sequenced both forward and reverse direction using commercial service (1st BASE). The goats were selected based on breeds, litter size, parity, age and goats which treated with estrus synchronization. The goat sequences were categorized into four group, which are LS 1(KC1a, KC1b, KJ1a, KJ1b, SD1a and SD1b), LS 2 (KJ1a, KJ1b, KJ1c, KJ1a, KJ1b, SD1a, SD1b, SD 1c and SD1d), LS 3 (KC3a, KC3b, KJ3a, KJ3b, KJ3c and SD3a), LS 4 (KJ4a) an LS 5 (SD5a). Alignment of multiple-sequence were performed by software MEGA X 10.1 version to explore the variation of single nucleotide polymorphisms (SNPs).

2.5. Estrus synchronization, blood samples and hormonal assay (ELISA)

Five goat does for each KC and KJ and six SD goat does with different LS were treated with 0.30 mg medroxyprogesterone acetate (MAP) intravaginal sponges for 14 days. The blood samples were collected five times (0, 3, 6, 9 and 12 hours) after the sponge removal. A total 3 ml of blood samples were collected in plain and sterile vacutainer tubes. Then, the blood sample were centrifuged (3000 rpm/5 min) to obtain serum and stored at -20°C in ependorf tubes until assayed for FSH profile. FSH hormone levels was estimated using Goat Follicle Stimulating Hormone Kit (Bioassay Technology Laboratory Cat. No. E0006Go Shanghai, Cina) and counting using microplate reader (ZENIX-320, USA). The stand art curve range 0.05 mlU/ml – 15 mlU/ml and the

sensivity 0.028 mlU/ml. The intra-assay coefficient of variance (CV) and the inter-assay CV less than 8% and 10% respectively. The ELISA was performed as per kit guidance.

2.6. Statistical analysis

2.6.1 Population Structure

The data were analyzed MEGA X software to acquire the singleton variable, parsimony sites, genetic distance within and between goat breed and to form phylogenetic tree. The neighbour-joining method was used to build the phylogenetic tree. Different sequences of KISS1 gene from other vary species were acquired from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) for phylogenetic analysis. The distance between sequence pairs were represented by the length of each pair of branches. The scale under the tree is indicating the nucleotide substitution number. The DnaSP software were used to calculate haplotype diversity, number of haplotype, number of mutation, Fst and Tajima's D. The Arlequin software were utilized to obtain haplotype shared and haplotype frequencies.

Basic Local Alignment Search Tool (BLAST) were used to detect the homology sequences in diverse breeds or species. Six different KISS1 sequences from different species/breed have been selected from the GenBank with accession number listed below (Tabel 1).

2.6.2 Follicle Stimulating Hormone (FSH) Level

The data were analyzed using General Linier Model (GLM) of SAS Software. Multiple comparison of the means were analyzed using Tukey's post hoc. Fixed model used for FSH :

$$y_{ij} = \mu + a_i + b_j + c_k + d_l + e_{ijkl}$$

where y_{ij} is the performance of trait measured for each samples, μ is the overall mean, a_i is the fixed effect associated with *i*th genotype (i = 1,2,3), b_j is the fixed effect associated with *j*th breed (j = 1,2,3), c_k is the fixed effect associated with *k*th collection time, d_l is the fixed of associated with *l*th litter size (LS) and e_{ij} is a random error of each observation. When the $P < 0.05$ it was verify significant statistically.

RESULTS AND DISCUSSION

Nucleotide sequence identity and phylogenetic tree of KISS1 gene

The OD ratio 260/280 ranged from 1.7 to 2.0, indicating that extracted DNA was not contaminated and in good quality. Psifidi *et al.* (2015) confirming that the standart of OD ratio 260/280 is ≥ 1.8, depend on the extraction kit used. A higher ratio number showed higher purity of DNA extract. Therefore, the extracted DNA could be sequenced both forward and reverse directions immediately in this study.

BLAST from NCBI were used to find the degree of similarity between choosen sequences. Three species/breed that have the highest similarity are Jining grey goats from China, *Ovis aries* and *Capra hircus* for 99.69%, 99.47% and 97.66% respectively (Table 1). The closely related sequences could be indicated from the similarity at nucleotide level. The DNA sequences similarity interprets that the function and structure of regulatory elements or protein products of gene expression is similar (Mahdavi and Dashab, 2017) and high conservatism gene in species (Zheng *et al.*, 2018).

Homology of KISS1 with other species ranged between *Homo sapiens* (78.74%) to *Capra hircus* Jining Grey breed (99.69%). Zheng *et al.*, (2018) found the similar result in previous research on Jintang Black goat (JTG). The similarity between KC, KJ, SD and

JTG is 99.02%. This output denoted that KISS1 gene is conserve in many species because of its significant role in reproduction.

The sequences analysis could be performed by aligning the genes sequences with specific role to determine the evolutionary correlation between unrecognized sequences and approved sequences (GenBank) to construct a phylogenetic tree, branching and discrepancy between species (Mahdavi and Dashab, 2017). The analysis of nucleotide sequences using MEGA X software between the indigenous goats represented 18 variable sites which include five singleton sites, 13 parsimony sites and 14 haplotypes.

Diversity in entire population 1.18. Meanwhile, the mean distance is 1.39 that calculated from all DNA sequences which shows the average of entire sequence pairs and the amount of base change at each site. The distance within group is calculated by the average number of base change between all sequences within the group. The disparity was enumerated to be 0.0046, 0.0047 and 0.0051 in KC, KJ and SD respectively, while the distance between group are shown in Table 2. The previous experiment found that the genetic distance based on BMP15 gene was counted to be 0.4 in cows, 1.0 in pig, 4.1 in sheep and 2.1 in goats (Mahdavi and Dashab, 2017). This condition indicated that nucleotide substitutions in goat species of BMP15 gene higher than KISS1 gene caused by evolution correlated with gene expression mechanisms, thus this condition showed that KISS1 gene more conserve than BMP15 gene.

The common haplotype in three Indonesian goat breeds is CCATAGCGGGGCAT (H1) and the haplotype frequencies are 0.143, 0.5 and 0.125 for KC, KJ and SD respectively. In addition, overall haplotype CCATAGCGGGGCAT (H1) frequency in the entire population is 26.1% and the haplotype diversity is 0.913.

The average pairwise fixation index (Fst) value is 0.021 (KC and KJ) and 0.082 (KC and SD). This value is lower than previous values resulted in South East Asian. Barker *et al.,* (2001) reported that Fst value between south-east Asian goat is 0.14. In contrary, the Fst value between KJ and SD is 0.195. This data showed that genetic structure differentiation between KJ and SD is higher than KC to KJ and SD, thus indicated that KC is an ancestor for KJ and SD. In accordance, Lestari *et al.* (2018) reported that KJ is a crossbred of KC goat and Etawah Grade (EG). Further research needed to investigate the phylogenetic relationship between KC and SD.

The nucleotide frequency of thymine/uracil, cytosine, adenine and guanine is 23.3%, 27.7%, 23.5% and 25.5% respectively. Nucleotides substitution rate between species/group were estimated using Tamura-Nei model in MEGA X software (Table 3). These results denote the opportunity for replacement of each nucleotide with another one. The distance was estimated using the amount of bases and pair comparison method. The distance between *Homo sapiens* and *Ovis aries* were the maximum (6.393), while the closest distance was between Indonesian goats and Jining grey goats. This data could be confirmed with the phylogenetic tree, where *Homo sapiens* and *Ovis aries* found in different branch. Furthermore, Indonesian native goats and Jining grey goat located in the same node.

Adaptation is in reaction to selection of production methods and connected with local environmental situation. The alpine (*Capra ibex*) ibex and Spanish (*Capra pyrenaica*) have shown that both species were introgressed with domestic goat based on major histocompatibility complex (MHC) genes (Stella *et al.*, 2018). Phylogenetic tree of domestic and wild goat species based on Y-chromosome, nuclear marker or mitochondrial are discrepant (Amills *et al.*, 2017). The incongruous between nucleus and

mtDNA phylogenies were caused by interspecific hybridization, rather than lineage sorting or paralogy (Ropiquet and Hassanin, 2006).

BLAST was used to identify similarity between sequences. Other homolog species were used to aligned the nucleotide sequences of KISS1 gene to illustrate the phylogenetic tree. The nucleotide sequence of Indonesian goat breed was identical with Jining grey goats, *Ovis aries* and *Bos indicus* for 99.69%, 99.20% and 88.98% respectively (Fig. 2). This data also confirm the Fst value above (Table 2). The similarity between goats, sheeps and cattle which are ruminants, shows that KISS1 gene may have equivalent function in ruminants.

The phylogenetic tree shows two main clades of the phylogenetic relationship of all sequences. The last nodes of the phylogenetic tree denotes the current sequences of samples used, while the internal nodes pointed as suspect ancestor sequences. The nearest genetic relationship is between Indonesian native goats and Jining grey goat because it located in the same node. The other branch in the same clade with Indonesian goat breeds are *Capra hircus* and *Ovis aries*. Hereinafter the next clade consist of *Bos indicus*, *Homo sapiens* and *Sus scrofa*. The phylogenetic tree denoted a similarity and distance between species based on KISS1 gene. The phylogeny tree from prior research (Zheng *et al.*, 2018) showed similar clustering among various species which acquired in the in this study even the accession numbers of NCBI used are different.

KISS1 gene expression and FSH plasma level

An estrus synchronization was used in the current research using progestagen intravaginal sponge. In accordance with Wildeus (2000) reported the previous research in different breeds such as Cashmere, Alpine, Boer, Saanen and Nubian revealed that the

effectiveness of estrus synchronization using intravaginal sponges might represent a significant differences led by distinct species, breed, treatment management and mating system. The utilization of pregnant mare serum gonadotropin (PMSG) and progestagen sponges for estrus synchronization has resulted a satisfactory outcome. Internal appliances conceiving different kind of progestagen, implanted in female reproduction tract during 12-14 days were used widely (Bitaraf *et al.*, 2007).

The intravaginal sponge were implanted for 14 days in this research. The long term progestagen intravaginal treatment (12-14 days) gave better result than short term (5-7 days) but not differ significantly, whether on oestrus intensity, oestrus response, onset of oestrus, concentration of progesterone serum at 21 days after artificial insemination (AI), length of oestrus, gestation period, kidding and fecundity rate different significantly (Ngangi *et al.*, 2002;Kor *et al.*, 2011). On the other hand, intravaginal progestagen sponges used in estrus synchronization on ewes could improve ovulation time and estrus expression, on the other hand shorten duration of oestrus (Mahmoud and Senosy, 2019).

The basal concentration of progesterone hormone is reached six hours after the sponge taken out from female reproduction tract (Ngangi *et al.*, 2002). The first three observations (0, 3 and 6 hours) were in luteal phase while other observations (9 and 12 hours) were in the earlier follicular phase. This might explain that the FSH plasma level increase slightly during the collection time (Table 5). In sheep, KISS1 expression in the sheep preoptic area (POA) is greater just previous to the late follicular phase GnRH/LH surge than luteal phase (Smith *et al.*, 2013). For future research, longer duration need to be considered to evaluate the FSH plasma level to reach significant result.

KISS1 gene produce kisspeptin (Kp). This peptide were performed through their receptor, G-protein-coupled receptor (GPR54). Kp have arised as important regulators

of neurons that remain in the basal forebrain and yield gonadotropin releasing hormone (GnRH). Cells in the upstream of GnRH neurons synthesized KISS1 functionally (Knoll *et al.,* 2013). KISS1 stimulates GnRH neuron activity and KISS1 expression and the release is regulated by circulating gonadal hormones (Smith, 2013). Kp has been known as key neuroendocrine gate keeper of reproduction and maintenance of adult reproduction recently (Millar *et al.,* 2010). Sequences of KISS1 gene have revealed a polymorphism related to reproductive traits. KISS1 gene might be a significant candidate gene on reproductive traits in goat (Cao *et al.,* 2010; An *et al.,* 2013; El-Tarabany *et al.,* 2017; Sahoo *et al.,* 2019).

Kp arranges the construction of preantal follicles negatively by leading the production of anti mullerian hormone (AMH) and degrade the sensitivity to FSH by prevent the induction of FSHR expression through sympathetic activators, thus lowering the recruitment of primary follicles (Panidis *et al.,* 2006;Cao *et al.,* 2019). The sympathetic nerve activity might adjust the intra ovarian Kp system and the peptide needed for appropriate coordinated ovarian function both from neural or ovarian origin (Zheng *et al.,* 2018). Furthermore, the serum levels of Kp are in contrary correlation with FSH, but have a positive correlation with testosterone, LH and dehydroepiandrosterone (DHEA) (Gorkem *et al.,* 2018).

As mentioned before, fourteen haplotypes were obtained in present research. The gene flow, bottleneck and drift events, the distribution of nucleotide polymorphisms could formed by demographic history of breed (Nordborg and Tavare, 2002), therefore estimating haplotype variations is very informative to appraise the effects of the migrations, selection or admixture in goat populations (Criscione *et al.,* 2019)

357 The statistical analysis showed that haplotype affected FSH level significantly (Table
358 4). The TCAATGCGCAACGT haplotype (H9) goats had superior FSH plasma level
359 compare to other haplotypes. The preliminary experiment revealed that
360 TCAATGCGCAACGT haplotype (H9) of KISS1 gene also had high LS (3 ± 0^{b}).
361 Moreover, the rest haplotype denoted a vary LS, not in linier with the FSH plasma level.
362 This condition might be caused by the different goat breeds used to form the haplotype
363 analysis. Nackley *et al.,* (2006) suggested the significance of haplotypes over SNPs for
364 genetic variations analysis. In agreement with this result, another research using IGF1
365 bond with IGF1 receptors (IGF1R) and IGF1R haplotypes are correlated with puberty age
366 in Brahman heifers (Fortes *et al.,* 2013); the haplotypes of FSHβ3-c had a superior effect
367 for the semen quality (Nikbin *et al.,* 2018); the casein complex haplotypes correlated with
368 milk quality traits (Inostroza *et al.,* 2020). These phenotypes were related to reproductive
369 traits. To date, there is no published journal concerning the haplotype effect to FSH
370 plasma level. Therefore, our inference should be verified with further study.
371 Table 5 shows the data of FSH based on goat breeds, sample collection time, litter
372 size, parity and genotype. The discrepancies between breeds are significant, KC and KJ
373 have a higher FSH concentration than SD. KC and KJ goats were collected from
374 Grobogan and Purbalingga regency which represented lowland area (0 – 200 m), further
375 SD goat was collected from Lumajang regency which reflected high land (500 m). In
376 accordance, a breed type have a significant effect to fresh and post-thaw semen traits
377 (Nikbin *et al.,* 2018). Both long term artificial and natural selection enforced by animal
378 husbandry and environmental change resulted different goat breeds in China. The
379 multigenic traits such as prominent cold and disease resistance, strong rough fodder

resistance, adaptiveness to stressful environment and high prolificacy reflect distinct natural gene pool (Liu *et al.*, 2019).

Further, the present investigation did not find any correlation between parity and FSH plasma level (table 5). This finding is in accordance in Damascus and Zaribi goats. The TT genotype at T121A in the intron 1 of KISS1 gene had significant higher LS than TA genotype. Moreover, the difference on estradiol$_{17\beta}$ and progesterone level caused by parity is not significant (El-Tarabany *et al.,* 2017).

The data from our previous research found that there are three obtrusive novel single nucleotide polymorphisms (SNPs) correlated with reproductive traits in three Indonesian native goat breeds. The SNPs are g.2425C>G, g.2436A>G and at g.2459G>A. The previous research found a SNP in FSHB gene promoter region within one of the conserved hormone-response element (HREs) were associated with divergent in serum FSH level in men (Grigorova *et al.,* 2008). Herewith we report for the first time polymorphisms of KISS1 gene in goats indicating a significant correlation with FSH level (Table 5).

The recent research showed that goat breed influences the FSH level significantly, wherein SD goat have lower FSH plasma level. This finding is in accordance with previous research. Another study in human found that higher body mass index (BMI) had lower kisspeptin significantly. Metastin/kisspeptin levels were correlated with all indices of insulin resistance significantly and reversely, thus it can be concluded that a significant decrease in plasma metastin levels is correlated with insulin resistance. (Panidis *et al.*, 2006;Chen *et al.,* 2010). The LH levels were correlated with plasma metastin levels positively. In fact, average body weight for KC, KJ and SD goats are 24.7 kg, 27.87 kg

403 and 48.50 kg (Batubara *et al.,* 2006;Sodiq and Haryanto, 2007;Ministry of Agriculture,
404 2014).

405 The mechanism of major decrease in KISS1 expression could lead a compensatory
406 increase in the expression of its receptor (GPR54), causing a circumstances of
407 sensitization to kisspeptin effects. Therefore, an enhancement of LH responses to Kp
408 suggesting that substitution of endogenous KISS1 levels is adequate to activate GnRH-
409 LH axis fully in spite of prejudicial metabolic conditions (Castellano *et al.,* 2005).

410 Adipose is a necessary endocrine tissue that influence reproduction through leptin
411 primarily (Kawwass *et al.,* 2015; Symonds *et al.,* 2016). Leptin acts through the GPR54
412 which is found on kisspeptin neurons in hypothalamus (Tena-Sempere, M[a]. 2006; Tena-
413 Sempere, M[b]. 2006). Kisspeptin binds to GnRH neurons and provoke GnRH release
414 (Roseweir and Millar, 2009). The leptin-kisspeptin-GnRH neuron pathway present the
415 endocrine argument for the critical body weight hypothesis, which body weight relate
416 to puberty in female (Keisler *et al.,* 1999). Thus, earlier result suggest that higher BMI
417 caused lower LH surge, in turned FSH plasma level also decrease, but this finding needed
418 further research.

419 Nowadays, the effect of Kp on FSH secretion is less information. The response of
420 KISS1 to FSH release emerge less sensitive than LH considerably. The pathway
421 organized centrally through modulation GnRH system, moreover it conducted
422 independently with other neuroendocrine regulators of gonadotropic axis such as
423 excitatory amino acids (EEAs), nitric oxide (NO) and leptin (Navarro, 2005). The positive
424 reaction of leptin in GnRH is mediated by proopiomelanocortins (POMC, precursor of α-
425 MSH), galanin-like peptide (GALP) and kisspeptin neuropeptides (Gottsch *et al.,* 2004;
426 Crown *et al.,* 2007;Quennell *et al.,* 2009). Kp is detect in the growing follicle at theca

427 cells and begins to arise in the basal cells of granular layer in rodent and human
428 (Castellano *et al.,* 2006). FSH is not under control entirely by GnRH (Charlton,
429 1983;Phillips, 2005), but the major stimulus for FSH is GnRH (Mason *et al.,* 1986).

430 In our prior study, the obtrusive genotype of KISS1 gene intron 2 that correlated
431 with higher LS, particularly average LS at the first and third parity in Indonesian native
432 goat breeds which are CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA
433 genotype at g.2459G>A (unpublished data). The data above (Table 5) showed that the
434 genotypes at g.2425C>G and g.2436A>G had the same FSH plasma level (P=0.22 and
435 P=0.34 respectively). On the other hand, the AA genotype at g.2459G>A has a superior
436 FSH level than GG genotype.

437 Preliminary study revealed that CC genotype at g.2425C>G, AA genotype at
438 g.2436A>G and GA genotype at g.2459G>A had significant greater litter size (2.58, 2.58
439 and 3.0 respectively). Furthermore, neither the CC genotype at g.2425C>G nor the AA
440 genotype at g.2436A>G have significantly different FSH levels. On the other hand, GA
441 genotype at g.2459G>A reveals a higher LS (3.0±0.18) than AA genotype which have a
442 lower LS (2.0±0.21). Thus, it can be concluded that GA genotype at g.2459G>A is the
443 most prominent genotype correlated with reproductive traits in Indonesian native goats.
444

445 **CONCLUSION**

446 The phylogeny tree reveal a high closeness between Indonesian goats and Chinese
447 goat. DNA sequences of both goat breeds are similar and the equal nodes indicates the
448 same function on both breeds and tightness along the evolutionary timescale. Capra hircus
449 and Ovis aries were also found in the same clade as the Indonesian goat breed.

Nonetheless, this finding revealed that the KISS1 gene plays a significant role in reproductive traits in a variety of species.

The identification of a polymorphisms or SNPs in KISS1 gene intron 1 paves the way to determine the effect of FSH level on goat litter size. Breed, LS, and haplotype are other factors that influence goat FSH levels. The superior haplotype and genotype of KISS1 gene is TCAATGCGCAACGT haplotype and GA genotype at g.2459G>A that correlated with high LS. These aspects could be considered in further breeding selection program for economically significant reproductive traits in goats. The current trial indicated that superior haplotype and genotype correlated with superior LS and FSH plasma level.

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Table 1. KISS1 gene sequences of different species from the GenBank used in developing the phylogenetic tree

Species	Accession number	Similarity
Jining Grey	GU. 142847.1	99.69%
Ovis aries	KP835797.1	99.47
Capra hircus	KR065750.1	97.66
Bos indicus	XM_019976949.1	87.91
Sus scrofa	AB466320.1	81.14
Homo sapiens	NG_032151.1	67.38

Table 2. The mean genetic distance between Indonesian goat breeds using the number of base pair in KISS1 gene

Goat	KC	KJ	SD
KC		0.021	0.082
KJ	0.0047		0.195
SD	0.0053	0.0061	

Note : the value above the diagonal are Fst and genetic distance value are under diagonal

Table 3. The mean distance between species using the number of base pair in KISS1 gene

Species	Jining grey	KC	KJ	SD	*Capra hircus*	*Ovis aries*	*Bos indicus*	*Homo sapiens*	*Sus scrofa*
Jining grey									
KC	0.003								
KJ	0.004	0.005							
SD	0.003	0.005	0.006						
Capra hircus	2.261	2.294	2.282	2.293					
Ovis aries	4.325	4.312	4.285	4.296	2.701				
Homo sapiens	3.427	3.342	3.404	3.387	3.049	6.393			
Bos indicus	3.850	3.843	3.896	3.819	2.781	4.259	2.840		
Sus scrofa	4.411	4.437	4.467	4.455	3.950	4.606	3.106	4.529	

Table 4. Means ± SE of FSH (mIU/ml) on haplotype (P<0.0001)

Haplotype Variations		FSH
H9	TCAATGCGCAACGT	10.65 ± 1.27^{a}
H4	TTATTGCACAACGT	8.99 ± 0.54^{b}
H2	CCATAGCGCAACGT	4.77 ± 0.49^{c}
H8	TCATAGCGGGGCGT	2.72 ± 0.14^{d}
H10	TTATTGCGCAGTGT	1.97 ± 0.08^{de}

H1	CCATAGCGGGGCAT	1.76 ± 0.14^{de}
H6	TTATTGCACAACGT	1.54 ± 0.06^{de}
H3	TCCTTGCGGGGTAT	1.49 ± 0.08^{de}
H7	TCAATGCGCAACGT	1.48 ± 0.12^{de}
H13	TTATTCTGCAATGA	1.30 ± 0.19^{e}
H14	TTATTCTGCAATGA	1.21 ± 0.09^{f}
H11	TTATTGCACAGTGT	0.67 ± 0.05^{g}
H12	TTAATCCGCAATGT	0.66 ± 0.05^{h}

641

642 Table 5. Means ± SE of FSH (mIU/ml) based on goat breeds, sample collection time,
643 litter size, parity and genotype
644

Specification	P Value	Category	Means ± SE
Breed	P = 0.002	KC	3.88 ± 0.63^{a}
		KJ	3,73 ± 0.75^{a}
		SD	1.49 ± 0.19^{b}
Sample collection time	P = 0.9361	0 hours	2.48 ± 0.59
		3 hours	2.88 ± 0.74
		6 hours	2.89 ± 0.73
		9 hours	2.97 ± 0.74
		12 hours	3.45 ± 0.99
Litter size	P = 0.0175	1 kid	1.28 ± 0.15^{b}
		2 kids	2.61 ± 0.47^{ab}
		3 kids	4.21 ± 0.78^{a}
		5 kids	3.77 ± 0.32^{a}
Parity	P = 0.0352	1st parity	3.77 ± 0.32
		2nd parity	2.27 ± 0.34
		3rd parity	4.10 ± 0.79
SNP g.2425 C>G	P = 0.2226	CC	3.27 ± 0.44
		CG	2.10 ± 0.21
		GG	1.76 ± 0.13
SNP g.2436 A>G	P = 0.3447	AA	3.22 ± 0.48
		AG	2.66 ± 0.27
		GG	1.76 ± 0.14
SNP g.2459 G>A	P = 0.0027	AA	4.01 ± 0.96^{a}
		GA	3.89 ± 0.68^{ab}
		GG	1.65 ± 0.11^{b}

645 Note : Values with different superscripts in the same column differ significantly at P<0.05

646

Figure 1. Distribution of sampling area in Java island, Indonesia

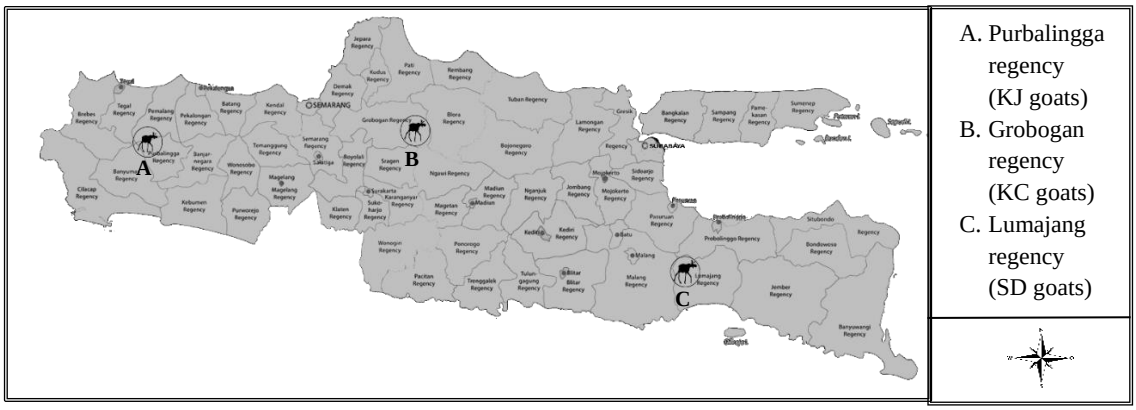
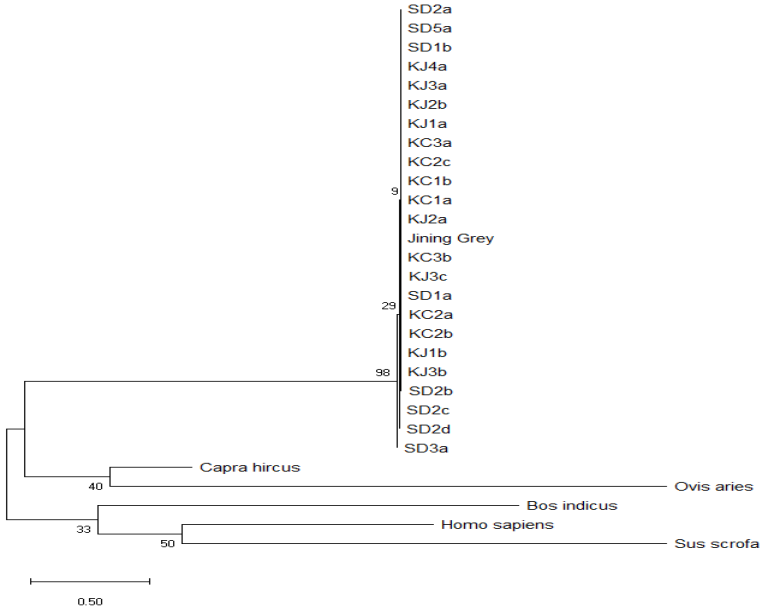


Figure 2. Phylogenetic tree of KISS1 gene of different species



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PHYLOGENETIC STUDY AND ASSOCIATION BETWEEN PROMINENT GENOTYPE AND HAPLOTYPE OF KISS1 GENE CORRELATED WITH FSH PLASMA LEVEL IN INDONESIAN NATIVE GOAT BREEDS

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ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi struktur populasi dan ekspresi gen KISS1 yang berkaitan dengan sifat reproduksi menggunakan analisis level Follicle Stimulating Hormone (FSH) dan sekuensing DNA gen KISS. Sejumlah 23 ekor induk yang terdiri dari kambing Kacang (n=7), Kejobong (n=8) and Senduro (n=8) diidentifikasi genotipenya menggunakan metode sekuensing DNA, 16 ekor diantaranya diberi perlakuan sinkronisasi estrus untuk diamati level hormon FSH menggunakan metode ELISA. *Software* MEGA X digunakan untuk menganalisa sekuens DNA, sedangkan General Linier Model (GLM) dari SAS *software* untuk menganalisa hormon FSH. Pohon filogeni memperlihatkan kesamaan yang tinggi antara kambing lokal

Indonesia dengan spesies lain yang menunjukkan gen KISS1 konservatif. Analisis hormon FSH menunjukan hasil signifikan yang lebih tinggi antara Kacang dibandingkan Senduro (P = 0.002),), litter size (LS) 3 dibandingkan LS 1 (P = 0.0175), selanjutnya haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A menunjukkan hormon hasil hormon yang lebih tinggi (P = 0.0027; P<0.0001) dan terkait dengan LS yang tinggi (3.0±0.18). Waktu pengambilan sampel dan paritas tidak memberikan perbedaan yang signifikan terhadap hormon FSH. Penelitian ini menunjukkan bahwa haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A mempunyai asosiasi dengan sifat reproduksi.

Kata kunci : FSH, gen KISS1, haplotipe, kambing lokal Indonesia, pohon filogeni

ABSTRACT

The aim of the current research was to analyze the population structure and expression of KISS1 gene associated with reproductive traits through follicle stimulating hormone (FSH) level and DNA sequencing analysis based on KISS1 gene. Genotypes of 23 goat does consist of Kacang goat (n=7), Kejobong goat (n=8) and Senduro goat (n=8) goats were investigated using DNA sequencing, 16 out of 23 samples were synchronized to examine their FSH level using ELISA method. The data were analyzed using MEGA X software for DNA sequences and General Linier Model (GLM) for FSH plasma level. The phylogenetic tree showed the highly homology between Indonesian native goats with other species showing a gene conservatism. A higher FSH plasma levels were obtained from KC than SD (P = 0.002), litter size (LS) 3 than LS 1 (P = 0.0175), further TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A (P = 0.0027; P<0.0001) and are associated with high LS (3.0±0.18). Neither sample collection times

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nor parities have different significantly. The current trial indicated that TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A were correlated with reproduvtive traits.

Keywords : FSH, haplotype, Indonesian native goat, KISS1 gene, phylogenetic tree

INTRODUCTION

Goats, unlike other livestock species, are adaptable animals that can survive in tropical, mountainous, and desert environments. Goats have spread widely due to their adaptability to a variety of environments and nutrition availability, small size, prolific, useful productivity for humans, and non-competitiveness with human food, and they contribute significantly, particularly in rural areas (Aziz, 2010;Guerrero *et al.*, 2019).

In Indonesia, there are more than 19 million goats, with eight goat breeds officially confessed. In Indonesia, goat population has increased over the last five years (Ministry of Agriculture, 2020). This condition could indicate that goats could be an alternative source of red meat. According to Aziz (2010), Indonesia is one of the 10 largest lamb production country in the world. This situation might represents the Indonesian preference on goat meat because most of goats were reared and consumed locally. Enhancing reproductive traits could be a way to increase the number of goat population.

Indigenous goat breeds are well adapted to agro-ecological conditions, helping to ensure goat farming's long-term viability in rural and harsh environments (Stella, 2018). Goats are traditionally bred in Indonesia for meat and dual-purpose production. In this study, three indigenous goat breeds were used. ~~The~~ Kacang (KC), Kejobong (KJ), and Senduro (SD) goat breeds were known for their reproductive traits. KC has a significant high litter size even when reared in a harsh environment and can be raised as a meat type;

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KJ, the does is prolific, has a short kidding interval, and can be raised as a meat type (Sodiq and Haryanto, 2007), while the litter size (LS) in SD is 1.83 ± 0.69 and perform as dual purpose (meat and dairy) type (Ciptadi *et al.*, 2019). KJ is solely located in Central Java, whereas SD goats start to spread massively in Indonesia following KC. KC is also known as Indonesian native goats (Batubara *et al.*, 2006). Half of Indonesian goat population is existed in Java, therefore a study based on goat population in Java was expected to represent the entire goat population in Indonesia, particularly in term of specific reproductive traits.

So far, the genetic structure of important economic traits has been identified, but the number of causative genes in goats has been lower than in sheep and cattle (Amills *et al.*, 2017). The phenotypic variations of goats were shaped by a various of artificial or natural factors such as migration of human, environmental changes and influences of socioeconomic. Further, the genomic variability of goats were constructed mostly by breeding orientation and artificial selection during domestication (Wang *et al.*, 2016). Principally, the sustainable selection and advancement of a novel traits in an environmental shifting needs the genetic diversity (Mandal *et al.*, 2020).

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Reproduction is a critical function for the survival of the species, thus this function is governed by complex regulatory signals. The hypothalamic-pituitary-gonadal (HPG) axis regulates reproductive activity by modulating the secretion of inhibitory factors and pituitary gonadotropins by the hypothalamus. Gonadotropin activity in the gonad is mediated by peripheral blood circulation (Nagamalleswari *et al.*, 2004). The HPG axis is divided into three regulatory signals: 1) hypothalamus: gonadotropin-releasing hormone (GnRH); 2) pituitary: gonadotropin, luteinizing hormone (LH) and follicle stimulating hormone (FSH); and 3) gonads: sex steroid and peptides (Pinilla *et al.*, 2012).

Follicle Stimulating Hormone (FSH) is a pituitary gonadotropin which have essential task in reproduction. The main roles of FSH in female are maturation and development in antral follicles, encourage the antrum formation in secondary follicles and organize a response for ovulation when the LH surge (Mahdavi and Dashab, 2017).

The present studies was undertaken to analyze the DNA sequences, appraising evolutionary distances and the population structure of the phylogenetic tree based on KISS1 gene sequences of three Indonesian indigenous goat breeds compare to other species sequences. Therefore, as KISS1 gene plays an important role on reproduction, this study was carried out to explore the relative expression of KISS1 gene through FSH plasma analysis from different goat breeds, litter size, haplotypes and genotypes to describe its relationship with litter size at kidding.

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MATERIALS AND METHODS

Ethical Clearance

The protocol of the current research was under the standart rule of animal treatment as designated in the Republic of Indonesia's law, that is, number 41, 2014.

2.1. Animals and samples collection

A total of 23 heads of goat does from three Indonesian indigenous goat breeds, namely KC (n=7), KJ (n=8) and SD (n=8) were utilized in the present study. The goats were healthy, unrelated and were not pregnant. They were selected randomly based on LS, age, multiparous (2^{nd} to 5^{th} parities) and have phenotypic characteristic of each breeds. These breeds represent different regions and altitudes, KC in Grobogan regency,

KJ in Purbalingga regency, both are in Central Java while SD is from Lumajang regency East Java (Fig.1). The Grobogan, Purbalingga and Lumajang were located at 100 m, 113 m and 500 m height above mean sea level (AMSL) respectively. The goats were kept by the farmer under the homogenous environment.

2.2. Genomic DNA extraction

A total of 3 ml of blood samples were collected via the jugular vein in to sterile vacuum tubes with anticoagulant (EDTA). The blood samples were shifted to the laboratory using coolbox and freezed at -20°C until the genomic DNA extraction. Thus, GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) was applied to extract the genomic DNA from the whole blood correspond the manufacturer's guidance. The evaluation of DNA quality was set up by electrophoresis using agarose gel (1%) and Nanodrop spectrophotometer Uvidoc HD6 (UVItec Ltd., Cambridge, UK).

A clear single band on agarose (1%) electrophoresis and the optical density (OD) 260/280 ratio ranged between 1.7 and 2.0 (according to the kit protocol) verifying a good quality of DNA extraction.

2.3. PCR amplification

A 1061bp fragment of intron 2 KISS1 gene was amplified with a pair of primer (F: 5'-GCTCAGTGGCCAGGAATCTG-3'; R: 5'-GCTCATAGCAGGGCCTCAAA-3'). The primers were designed using the sequence of KISS1 gene of *Capra hircus* breed Jining Grey (GenBank Accession Number GU142847.1) and Primer3 Plus software. Polymerase Chain Reaction (PCR) was perform in 50 µl volume containing 4 µl DNA extraction (20-30 ng/ µl), 1 µl for each primer (10 pmol/ µl), 19 µl ddH2O and 25 µl of MyTaq Red Mix Bioline (1st BASE) on MJ Mini Personal Thermal Cycler (Bio-Rad, USA). PCR cycling program contain of pre-denaturation at 95°C for 5 min, followed by

35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72 °C for 30 s, and post extension at 72 °C for 7 min. The amplicon was performed by electrophoresis in 2 % agarose gel (Invitrogen, Life Technologies Co, CA) at 100V for 30 min.

2.4. DNA sequencing and analysis.

The amplicon of 23 goats reflecting three indigenous Indonesian goat breeds were sequenced both forward and reverse direction using commercial service (1st BASE). The goats were selected based on breeds, litter size, parity, age and goats which treated with estrus synchronization. The goat sequences were categorized into four group, which are LS 1(KC1a, KC1b, KJ1a, KJ1b, SD1a and SD1b), LS 2 (KJ1a, KJ1b, KJ1c, KJ1a, KJ1b, SD1a, SD1b, SD 1c and SD1d), LS 3 (KC3a, KC3b, KJ3a, KJ3b, KJ3c and SD3a), LS 4 (KJ4a) an LS 5 (SD5a). Alignment of multiple-sequence were performed by software MEGA X 10.1 version to explore the variation of single nucleotide polymorphisms (SNPs).

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2.5. Estrus synchronization, blood samples and hormonal assay (ELISA)

Five goat does for each KC and KJ and six SD goat does with different LS were treated with 0.30 mg medroxyprogesterone acetate (MAP) intravaginal sponges for 14 days. The blood samples were collected five times (0, 3, 6, 9 and 12 hours) after the sponge removal. A total 3 ml of blood samples were collected in plain and sterile vacutainer tubes. Then, the blood sample were centrifuged (3000 rpm/5 min) to obtain serum and stored at -20°C in ependorf tubes until assayed for FSH profile. FSH hormone levels was estimated using Goat Follicle Stimulating Hormone Kit (Bioassay Technology Laboratory Cat. No. E0006Go Shanghai, Cina) and counting using microplate reader (ZENIX-320, USA). The stand art curve range 0.05 mlU/ml – 15 mlU/ml and the

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sensivity 0.028 mlU/ml. The intra-assay coefficient of variance (CV) and the inter-assay CV less than 8% and 10% respectively. The ELISA was performed as per kit guidance.

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2.6. Statistical analysis

2.6.1 Population Structure

The data were analyzed MEGA X software to acquire the singleton variable, parsimony sites, genetic distance within and between goat breed and to form phylogenetic tree. The neighbour-joining method was used to build the phylogenetic tree. Different sequences of KISS1 gene from other vary species were acquired from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) for phylogenetic analysis. The distance between sequence pairs were represented by the length of each pair of branches. The scale under the tree is indicating the nucleotide substitution number. The DnaSP software were used to calculate haplotype diversity, number of haplotype, number of mutation, Fst and Tajima's D. The Arlequin software were utilized to obtain haplotype shared and haplotype frequencies.

Basic Local Alignment Search Tool (BLAST) were used to detect the homology sequences in diverse breeds or species. Six different KISS1 sequences from different species/breed have been selected from the GenBank with accession number listed below (Tabel 1).

2.6.2 Follicle Stimulating Hormone (FSH) Level

The data were analyzed using General Linier Model (GLM) of SAS Software. Multiple comparison of the means were analyzed using Tukey's post hoc. Fixed model used for FSH :

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$$y_{ij} = \mu + a_i + b_j + c_k + d_l + e_{ijkl}$$

where y_{ij} is the performance of trait measured for each samples, μ is the overall mean, a_i is the fixed effect associated with *i*th genotype (i = 1,2,3), b_j is the fixed effect associated with *j*th breed (j = 1,2,3), c_k is the fixed effect associated with *k*th collection time, d_l is the fixed of ~~associated with~~ *l*th litter size (LS) and e_{ij} is a random error of each observation. When the P <0.05 it was verify significant statistically.

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RESULTS AND DISCUSSION

Nucleotide sequence identity and phylogenetic tree of KISS1 gene

The OD ratio 260/280 ranged from 1.7 to 2.0, indicating that extracted DNA was not contaminated and in good quality. Psifidi *et al.* (2015) confirming that the standart of OD ratio 260/280 is ≥ 1.8, depend on the extraction kit used. A higher ratio number showed higher purity of DNA extract. Therefore, the extracted DNA could be sequenced both forward and reverse directions immediately in this study.

BLAST from NCBI were used to find the degree of similarity between choosen sequences. Three species/breed that have the highest similarity are Jining grey goats from China, *Ovis aries* and *Capra hircus* for 99.69%, 99.47% and 97.66% respectively (Table 1). The closely related sequences could be indicated from the similarity at nucleotide level. The DNA sequences similarity interprets that the function and structure of regulatory elements or protein products of gene expression is similar (Mahdavi and Dashab, 2017) and high conservatism gene in species (Zheng *et al.*, 2018).

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Homology of KISS1 with other species ranged between *Homo sapiens* (78.74%) to *Capra hircus* Jining Grey breed (99.69%). Zheng *et al.*, (2018) found the similar result in previous research on Jintang Black goat (JTG). The similarity between KC, KJ, SD and

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JTG is 99.02%. This output denoted that KISS1 gene is conserve in many species because of its significant role in reproduction.

The sequences analysis could be performed by aligning the genes sequences with specific role to determine the evolutionary correlation between unrecognized sequences and approved sequences (GenBank) to construct a phylogenetic tree, branching and discrepancy between species (Mahdavi and Dashab, 2017). The analysis of nucleotide sequences using MEGA X software between the indigenous goats represented 18 variable sites which include five singleton sites, 13 parsimony sites and 14 haplotypes.

Diversity in entire population 1.18. Meanwhile, the mean distance is 1.39 that calculated from all DNA sequences which shows the average of entire sequence pairs and the amount of base change at each site. The distance within group is calculated by the average number of base change between all sequences within the group. The disparity was enumerated to be 0.0046, 0.0047 and 0.0051 in KC, KJ and SD respectively, while the distance between group are shown in Table 2. The previous experiment found that the genetic distance based on BMP15 gene was counted to be 0.4 in cows, 1.0 in pig, 4.1 in sheep and 2.1 in goats (Mahdavi and Dashab, 2017). This condition indicated that nucleotide substitutions in goat species of BMP15 gene higher than KISS1 gene caused by evolution correlated with gene expression mechanisms, thus this condition showed that KISS1 gene more conserve than BMP15 gene.

The common haplotype in three Indonesian goat breeds is CCATAGCGGGGCAT (H1) and the haplotype frequencies are 0.143, 0.5 and 0.125 for KC, KJ and SD respectively. In addition, overall haplotype CCATAGCGGGGCAT (H1) frequency in the entire population is 26.1% and the haplotype diversity is 0.913.

The average pairwise fixation index (Fst) value is 0.021 (KC and KJ) and 0.082 (KC and SD). This value is lower than previous values resulted in South East Asian. Barker *et al.*, (2001) reported that Fst value between south-east Asian goat is 0.14. In contrary, the Fst value between KJ and SD is 0.195. This data showed that genetic structure differentiation between KJ and SD is higher than KC to KJ and SD, thus indicated that KC is an ancestor for KJ and SD. In accordance, Lestari *et al.* (2018) reported that KJ is a crossbred of KC goat and Etawah Grade (EG). Further research needed to investigate the phylogenetic relationship between KC and SD.

The nucleotide frequency of thymine/uracil, cytosine, adenine and guanine is 23.3%, 27.7%, 23.5% and 25.5% respectively. Nucleotides substitution rate between species/group were estimated using Tamura-Nei model in MEGA X software (Table 3). These results denote the opportunity for replacement of each nucleotide with another one. The distance was estimated using the amount of bases and pair comparison method. The distance between *Homo sapiens* and *Ovis aries* were the maximum (6.393), while the closest distance was between Indonesian goats and Jining grey goats. This data could be confirmed with the phylogenetic tree, where *Homo sapiens* and *Ovis aries* found in different branch. Furthermore, Indonesian native goats and Jining grey goat located in the same node.

Adaptation is in reaction to selection of production methods and connected with local environmental situation. The alpine (*Capra ibex*) ibex and Spanish (*Capra pyrenaica*) have shown that both species were introgressed with domestic goat based on major histocompatibility complex (MHC) genes (Stella *et al.*, 2018). Phylogenetic tree of domestic and wild goat species based on Y-chromosome, nuclear marker or mitochondrial are discrepant (Amills *et al.*, 2017). The incongruous between nucleus and

mtDNA phylogenies were caused by interspecific hybridization, rather than lineage sorting or paralogy (Ropiquet and Hassanin, 2006).

BLAST was used to identify similarity between sequences. Other homolog species were used to aligned the nucleotide sequences of KISS1 gene to illustrate the phylogenetic tree. The nucleotide sequence of Indonesian goat breed was identical with Jining grey goats, *Ovis aries* and *Bos indicus* for 99.69%, 99.20% and 88.98% respectively (Fig. 2). This data also confirm the Fst value ~~above~~ (Table 2). The similarity between goats, sheeps and cattle which are ruminants, shows that KISS1 gene may have equivalent function in ruminants.

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The phylogenetic tree shows two main clades of the phylogenetic relationship of all sequences. The last nodes of the phylogenetic tree denotes the current sequences of samples used, while the internal nodes pointed as suspect ancestor sequences. The nearest genetic relationship is between Indonesian native goats and Jining grey goat because it located in the same node. The other branch in the same clade with Indonesian goat breeds are *Capra hircus* and *Ovis aries*. Hereinafter the next clade consist of *Bos indicus*, *Homo sapiens* and *Sus scrofa*. The phylogenetic tree denoted a similarity and distance between species based on KISS1 gene. The phylogeny tree from prior research (Zheng *et al.*, 2018) showed similar clustering among various species which acquired in the in this study even the accession numbers of NCBI used are different.

KISS1 gene expression and FSH plasma level

An estrus synchronization was used in the current research using progestagen intravaginal sponge. In accordance with Wildeus (2000) reported the previous research in different breeds such as Cashmere, Alpine, Boer, Saanen and Nubian revealed that the

effectiveness of estrus synchronization using intravaginal sponges might represent a significant differences led by distinct species, breed, treatment management and mating system. The utilization of pregnant mare serum gonadotropin (PMSG) and progestagen sponges for estrus synchronization has resulted a satisfactory outcome. Internal appliances conceiving different kind of progestagen, implanted in female reproduction tract during 12-14 days were used widely (Bitaraf *et al.*, 2007).

The intravaginal sponge were implanted for 14 days in this research. The long term progestagen intravaginal treatment (12-14 days) gave better result than short term (5-7 days) but not differ significantly, whether on oestrus intensity, oestrus response, onset of oestrus, concentration of progesterone serum at 21 days after artificial insemination (AI), length of oestrus, gestation period, kidding and fecundity rate different significantly (Ngangi *et al.*, 2002;Kor *et al.*, 2011). On the other hand, intravaginal progestagen sponges used in estrus synchronization on ewes could improve ovulation time and estrus expression, on the other hand shorten duration of oestrus (Mahmoud and Senosy, 2019).

The basal concentration of progesterone hormone is reached six hours after the sponge taken out from female reproduction tract (Ngangi *et al.*, 2002). The first three observations (0, 3 and 6 hours) were in luteal phase while other observations (9 and 12 hours) were in the earlier follicular phase. This might explain that the FSH plasma level increase slightly during the collection time (Table 5). In sheep, KISS1 expression in the sheep preoptic area (POA) is greater just previous to the late follicular phase GnRH/LH surge than luteal phase (Smith *et al.*, 2013). For future research, longer duration need to be considered to evaluate the FSH plasma level to reach significant result.

KISS1 gene produce kisspeptin (Kp). This peptide were performed through their receptor, G-protein-coupled receptor (GPR54). Kp have arised as important regulators

of neurons that remain in the basal forebrain and yield gonadotropin releasing hormone (GnRH). Cells in the upstream of GnRH neurons synthesized KISS1 functionally (Knoll *et al.,* 2013). KISS1 stimulates GnRH neuron activity and KISS1 expression and the release is regulated by circulating gonadal hormones (Smith, 2013). Kp has been known as key neuroendocrine gate keeper of reproduction and maintenance of adult reproduction recently (Millar *et al.,* 2010). Sequences of KISS1 gene have revealed a polymorphism related to reproductive traits. KISS1 gene might be a significant candidate gene on reproductive traits in goat (Cao *et al.,* 2010; An *et al.,* 2013; El-Tarabany *et al.,* 2017; Sahoo *et al.,* 2019).

Kp arranges the construction of preantal follicles negatively by leading the production of anti mullerian hormone (AMH) and degrade the sensitivity to FSH by prevent the induction of FSHR expression through sympathetic activators, thus lowering the recruitment of primary follicles (Panidis *et al.,* 2006;Cao *et al.,* 2019). The sympathetic nerve activity might adjust the intra ovarian Kp system and the peptide needed for appropriate coordinated ovarian function both from neural or ovarian origin (Zheng *et al.,* 2018). Furthermore, the serum levels of Kp are in contrary correlation with FSH, but have a positive correlation with testosterone, LH and dehydroepiandrosterone (DHEA) (Gorkem *et al.,* 2018).

As mentioned before, fourteen haplotypes were obtained in present research. The gene flow, bottleneck and drift events, the distribution of nucleotide polymorphisms could formed by demographic history of breed (Nordborg and Tavare, 2002), therefore estimating haplotype variations is very informative to appraise the effects of the migrations, selection or admixture in goat populations (Criscione *et al.,* 2019)

The statistical analysis showed that haplotype affected FSH level significantly (Table 4). The TCAATGCGCAACGT haplotype (H9) goats had superior FSH plasma level compare to other haplotypes. The preliminary experiment revealed that TCAATGCGCAACGT haplotype (H9) of KISS1 gene also had high LS (3 ± 0^{b}). Moreover, the rest haplotype denoted a vary LS, not in linier with the FSH plasma level. This condition might be caused by the different goat breeds used to form the haplotype analysis. Nackley *et al.,* (2006) suggested the significance of haplotypes over SNPs for genetic variations analysis. In agreement with this result, another research using IGF1 bond with IGF1 receptors (IGF1R) and IGF1R haplotypes are correlated with puberty age in Brahman heifers (Fortes *et al.,* 2013); the haplotypes of FSHβ3-c had a superior effect for the semen quality (Nikbin *et al.,* 2018); the casein complex haplotypes correlated with milk quality traits (Inostroza *et al.,* 2020). These phenotypes were related to reproductive traits. To date, there is no published journal concerning the haplotype effect to FSH plasma level. Therefore, our inference should be verified with further study.

Table 5 shows the data of FSH based on goat breeds, sample collection time, litter size, parity and genotype. The discrepancies between breeds are significant, KC and KJ have a higher FSH concentration than SD. KC and KJ goats were collected from Grobogan and Purbalingga regency which represented lowland area (0 – 200 m), further SD goat was collected from Lumajang regency which reflected high land (500 m). In accordance, a breed type have a significant effect to fresh and post-thaw semen traits (Nikbin *et al.,* 2018). Both long term artificial and natural selection enforced by animal husbandry and environmental change resulted different goat breeds in China. The multigenic traits such as prominent cold and disease resistance, strong rough fodder

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resistance, adaptiveness to stressful environment and high prolificacy reflect distinct natural gene pool (Liu *et al.*, 2019).

Further, the present investigation did not find any correlation between parity and FSH plasma level (table 5). This finding is in accordance in Damascus and Zaribi goats. The TT genotype at T121A in the intron 1 of KISS1 gene had significant higher LS than TA genotype. Moreover, the difference on estradiol$_{17\beta}$ and progesterone level caused by parity is not significant (El-Tarabany *et al.*, 2017).

The data from our previous research found that there are three obtrusive novel single nucleotide polymorphisms (SNPs) correlated with reproductive traits in three Indonesian native goat breeds. The SNPs are g.2425C>G, g.2436A>G and at g.2459G>A. The previous research found a SNP in FSHB gene promoter region within one of the conserved hormone-response element (HREs) were associated with divergent in serum FSH level in men (Grigorova *et al.*, 2008). Herewith we report for the first time polymorphisms of KISS1 gene in goats indicating a significant correlation with FSH level (Table 5).

The recent research showed that goat breed influences the FSH level significantly, wherein SD goat have lower FSH plasma level. This finding is in accordance with previous research. Another study in human found that higher body mass index (BMI) had lower kisspeptin significantly. Metastin/kisspeptin levels were correlated with all indices of insulin resistance significantly and reversely, thus it can be concluded that a significant decrease in plasma metastin levels is correlated with insulin resistance. (Panidis *et al.*, 2006;Chen *et al.*, 2010). The LH levels were correlated with plasma metastin levels positively. In fact, average body weight for KC, KJ and SD goats are 24.7 kg, 27.87 kg

and 48.50 kg (Batubara *et al.*, 2006;Sodiq and Haryanto, 2007;Ministry of Agriculture, 2014).

The mechanism of major decrease in KISS1 expression could lead a compensatory increase in the expression of its receptor (GPR54), causing a circumstances of sensitization to kisspeptin effects. Therefore, an enhancement of LH responses to Kp suggesting that substitution of endogenous KISS1 levels is adequate to activate GnRH-LH axis fully in spite of prejudicial metabolic conditions (Castellano *et al.*, 2005).

Adipose is a necessary endocrine tissue that influence reproduction through leptin primarily (Kawwass *et al.*, 2015; Symonds *et al.*, 2016). Leptin acts through the GPR54 which is found on kisspeptin neurons in hypothalamus (Tena-Sempere, M[a]. 2006; Tena-Sempere, M[b]. 2006). Kisspeptin binds to GnRH neurons and provoke GnRH release (Roseweir and Millar, 2009). The leptin-kisspeptin-GnRH neuron pathway present the endocrine argument for the critical body weight hypothesis, which body weight relate to puberty in female (Keisler *et al.*, 1999). Thus, earlier result suggest that higher BMI caused lower LH surge, in turned FSH plasma level also decrease, but this finding needed further research.

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Nowadays, the effect of Kp on FSH secretion is less information. The response of KISS1 to FSH release emerge less sensitive than LH considerably. The pathway organized centrally through modulation GnRH system, moreover it conducted independently with other neuroendocrine regulators of gonadotropic axis such as excitatory amino acids (EEAs), nitric oxide (NO) and leptin (Navarro, 2005). The positive reaction of leptin in GnRH is mediated by proopiomelanocortins (POMC, precursor of α-MSH), galanin-like peptide (GALP) and kisspeptin neuropeptides (Gottsch *et al.*, 2004; Crown *et al.*, 2007;Quennell *et al.*, 2009). Kp is detect in the growing follicle at theca

cells and begins to arise in the basal cells of granular layer in rodent and human (Castellano *et al.*, 2006). FSH is not under control entirely by GnRH (Charlton, 1983;Phillips, 2005), but the major stimulus for FSH is GnRH (Mason *et al.*, 1986).

In our prior study, the obtrusive genotype of KISS1 gene intron 2 that correlated with higher LS, particularly average LS at the first and third parity in Indonesian native goat breeds which are CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA genotype at g.2459G>A (unpublished data). The data above (Table 5) showed that the genotypes at g.2425C>G and g.2436A>G had the same FSH plasma level (P=0.22 and P=0.34 respectively). On the other hand, the AA genotype at g.2459G>A has a superior FSH level than GG genotype.

Preliminary study revealed that CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA genotype at g.2459G>A had significant greater litter size (2.58, 2.58 and 3.0 respectively). Furthermore, neither the CC genotype at g.2425C>G nor the AA genotype at g.2436A>G have significantly different FSH levels. On the other hand, GA genotype at g.2459G>A reveals a higher LS (3.0±0.18) than AA genotype which have a lower LS (2.0±0.21). Thus, it can be concluded that GA genotype at g.2459G>A is the most prominent genotype correlated with reproductive traits in Indonesian native goats.

CONCLUSION

The phylogeny tree reveal a high closeness between Indonesian goats and Chinese goat. DNA sequences of both goat breeds are similar and the equal nodes indicates the same function on both breeds and tightness along the evolutionary timescale. Capra hircus and Ovis aries were also found in the same clade as the Indonesian goat breed.

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Nonetheless, this finding revealed that the KISS1 gene plays a significant role in reproductive traits in a variety of species.

The identification of a polymorphisms or SNPs in KISS1 gene intron 1 paves the way to determine the effect of FSH level on goat litter size. Breed, LS, and haplotype are other factors that influence goat FSH levels. The superior haplotype and genotype of KISS1 gene is TCAATGCGCAACGT haplotype and GA genotype at g.2459G>A that correlated with high LS. These aspects could be considered in further breeding selection program for economically significant reproductive traits in goats. The current trial indicated that superior haplotype and genotype correlated with superior LS and FSH plasma level.

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Table 1. KISS1 gene sequences of different species from the GenBank used in developing the phylogenetic tree

Species	Accession number	Similarity
Jining Grey	GU. 142847.1	99.69%
Ovis aries	KP835797.1	99.47
Capra hircus	KR065750.1	97.66
Bos indicus	XM_019976949.1	87.91
Sus scrofa	AB466320.1	81.14
Homo sapiens	NG_032151.1	67.38

Table 2. The mean genetic distance between Indonesian goat breeds using the number of base pair in KISS1 gene

Goat	KC	KJ	SD
KC		0.021	0.082
KJ	0.0047		0.195
SD	0.0053	0.0061	

Note : the value above the diagonal are Fst and genetic distance value are under diagonal

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Table 3. The mean distance between species using the number of base pair in KISS1 gene

Species	Jining grey	KC	KJ	SD	*Capra hircus*	*Ovis aries*	*Bos indicus*	*Homo sapiens*	*Sus scrofa*
Jining grey									
KC	0.003								
KJ	0.004	0.005							
SD	0.003	0.005	0.006						
Capra hircus	2.261	2.294	2.282	2.293					
Ovis aries	4.325	4.312	4.285	4.296	2.701				
Homo sapiens	3.427	3.342	3.404	3.387	3.049	6.393			
Bos indicus	3.850	3.843	3.896	3.819	2.781	4.259	2.840		
Sus scrofa	4.411	4.437	4.467	4.455	3.950	4.606	3.106	4.529	

Table 4. Means ± SE of FSH (mIU/ml) on haplotype (P<0.0001)

Haplotype Variations		FSH
H9	TCAATGCGCAACGT	10.65 ± 1.27^{a}
H4	TTATTGCACAACGT	8.99 ± 0.54^{b}
H2	CCATAGCGCAACGT	4.77 ± 0.49^{c}
H8	TCATAGCGGGGCGT	2.72 ± 0.14^{d}
H10	TTATTGCGCAGTGT	1.97 ± 0.08^{de}

H1	CCATAGCGGGGCAT	1.76 ± 0.14^{de}
H6	TTATTGCACAACGT	1.54 ± 0.06^{de}
H3	TCCTTGCGGGGTAT	1.49 ± 0.08^{de}
H7	TCAATGCGCAACGT	1.48 ± 0.12^{de}
H13	TTATTCTGCAATGA	1.30 ± 0.19^{e}
H14	TTATTCTGCAATGA	1.21 ± 0.09^{f}
H11	TTATTGCACAGTGT	0.67 ± 0.05^{g}
H12	TTAATCCGCAATGT	0.66 ± 0.05^{h}

Table 5. Means ± SE of FSH (mIU/ml) based on goat breeds, sample collection time, litter size, parity and genotype

Specification	P Value	Category	Means ± SE
Breed	P = 0.002	KC	3.88 ± 0.63^{a}
		KJ	3,73 ± 0.75^{a}
		SD	1.49 ± 0.19^{b}
Sample collection time	P = 0.9361	0 hours	2.48 ± 0.59
		3 hours	2.88 ± 0.74
		6 hours	2.89 ± 0.73
		9 hours	2.97 ± 0.74
		12 hours	3.45 ± 0.99
Litter size	P = 0.0175	1 kid	1.28 ± 0.15^{b}
		2 kids	2.61 ± 0.47^{ab}
		3 kids	4.21 ± 0.78^{a}
		5 kids	3.77 ± 0.32^{a}
Parity	P = 0.0352	1st parity	3.77 ± 0.32
		2nd parity	2.27 ± 0.34
		3rd parity	4.10 ± 0.79
SNP g.2425 C>G	P = 0.2226	CC	3.27 ± 0.44
		CG	2.10 ± 0.21
		GG	1.76 ± 0.13
SNP g.2436 A>G	P = 0.3447	AA	3.22 ± 0.48
		AG	2.66 ± 0.27
		GG	1.76 ± 0.14
SNP g.2459 G>A	P = 0.0027	AA	4.01 ± 0.96^{a}
		GA	3.89 ± 0.68^{ab}
		GG	1.65 ± 0.11^{b}

Note : Values with different superscripts in the same column differ significantly at P<0.05

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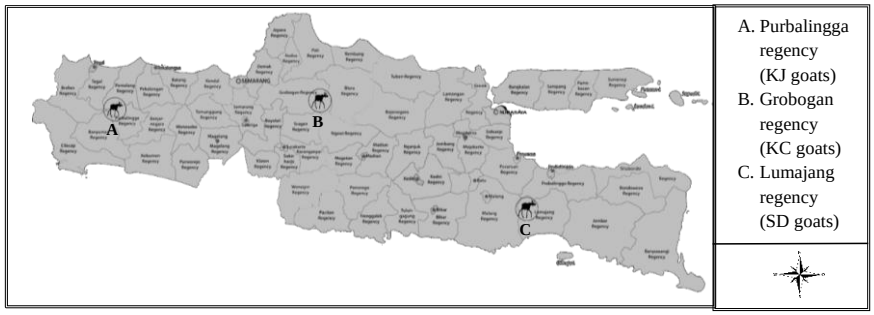
647 Figure 1. Distribution of sampling area in Java island, Indonesia



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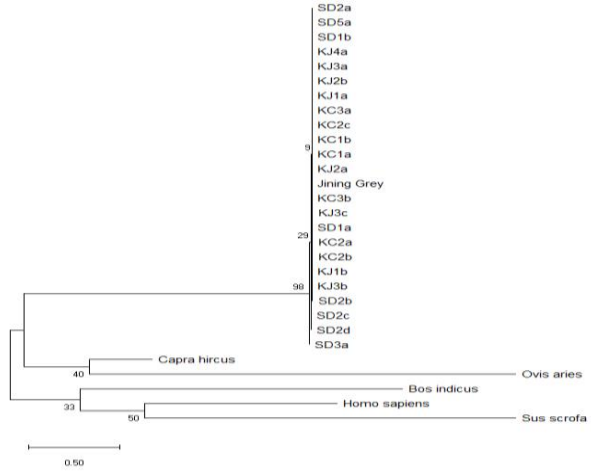
650 Figure 2. Phylogenetic tree of KISS1 gene of different species



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Article Type: FULL-LENGTH ORIGINAL SCIENTIFIC PAPER

Running Title: Phylogenetic tree and FSH level based on KISS1 gene in goat (Febriana *et al.*)

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PHYLOGENETIC STUDY AND ASSOCIATION BETWEEN PROMINENT GENOTYPE AND HAPLOTYPE OF KISS1 GENE WITH FSH LEVEL IN INDONESIAN NATIVE GOAT BREEDS

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PHYLOGENETIC STUDY AND ASSOCIATION BETWEEN PROMINENT GENOTYPE AND HAPLOTYPE OF KISS1 GENE WITH FSH LEVEL IN INDONESIAN NATIVE GOAT BREEDS

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ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi struktur populasi dan ekspresi gen KISS1 yang berkaitan dengan sifat reproduksi menggunakan analisis level Follicle Stimulating Hormone (FSH) dan sekuensing DNA gen KISS1. Sejumlah 23 ekor induk yang terdiri dari kambing Kacang (n=7), Kejobong (n=8) and Senduro (n=8) diidentifikasi genotipenya menggunakan metode sekuensing DNA, 16 ekor diantaranya diberi perlakuan sinkronisasi estrus untuk diamati level hormon FSH menggunakan metode ELISA. *Software* MEGA X digunakan untuk menganalisa sekuens DNA, sedangkan General Linier Model (GLM) dari SAS *software* untuk menganalisa hormon FSH. Pohon filogeni memperlihatkan kesamaan yang tinggi antara kambing lokal

Indonesia dengan spesies lain yang menunjukkan bahwa gen KISS1 konservatif. Analisis hormon FSH menunjukan hasil yang berbeda secara signifikan antara kambing Kacang dan Kejobong dibandingkan Senduro (P = 0.002), litter size (LS) 3 dibandingkan LS 1 (P = 0.0175), selanjutnya haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A menunjukkan hormon FSH yang lebih tinggi dibandingkan haplotipe dan genotipe yang lain (P = 0.0027; P<0.0001) dan terkait dengan LS yang tinggi (3.0±0.18). Waktu pengambilan sampel dan paritas tidak memberikan perbedaan yang signifikan terhadap hormon FSH. Penelitian ini menunjukkan bahwa haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A mempunyai asosiasi dengan sifat reproduksi.

Kata kunci : FSH, gen KISS1, haplotipe, kambing lokal Indonesia, pohon filogeni

ABSTRACT

The aim of the current research was to analyze the population structure and expression of KISS1 gene associated with reproductive traits through Follicle Stimulating Hormone (FSH) level and DNA sequencing analysis based on KISS1 gene. Genotypes of 23 goat does consist of Kacang goats (n=7), Kejobong goats (n=8) and Senduro goats (n=8) were investigated using DNA sequencing, 16 out of 23 samples were synchronized to examine their FSH level using ELISA method. The data were analyzed using MEGA X software for DNA sequences and General Linier Model (GLM) for FSH plasma level. The phylogenetic tree showed the high homology between Indonesian native goats with other species showing a gene conservatism. A significantly higher FSH plasma levels were obtained from Kacang and Kejobong than Senduro goat (P = 0.002), litter size (LS) 3 than LS 1 (P = 0.0175), further TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A have a higher FSH plasma than other haplotypes and genotypes (P = 0.0027;

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highly homologenous

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P<0.0001) and are associated with high LS (3.0±0.18). Neither sample collection times nor parities have different significantly. The current trial indicated that TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A were correlated with reproductive traits.

Keywords : FSH, haplotype, Indonesian native goat, KISS1 gene, phylogenetic tree

INTRODUCTION

Goats, unlike other livestock species, are adaptable animals that can survive in tropical, mountainous, and desert environments. Goats have spread widely due to their adaptability to a variety of environments and nutrition availability, small size, prolific, useful productivity for humans, and non-competitiveness with human food, and they contribute significantly, particularly in rural areas (Aziz, 2010;Guerrero *et al.*, 2019).

In Indonesia, there are more than 19 million goats, with eight goat breeds officially confessed. In Indonesia, goat population has increased over the last five years (Ministry of Agriculture, 2020). This condition could indicate that goats could be an alternative source of red meat. According to Aziz (2010), Indonesia is one of the 10 largest lamb production country in the world. This situation might represent the Indonesian preference on goat meat because most of goats were reared and consumed locally. Enhancing reproductive traits could be a way to increase the number of goat population.

Indigenous goat breeds are well adapted to agro-ecological conditions, helping to ensure goat farming's long-term viability in rural and harsh environments (Stella, 2018). Goats are traditionally bred in Indonesia for meat and dual-purpose production. In this study, three indigenous goat breeds were used. Kacang (KC), Kejobong (KJ), and Senduro (SD) goat breeds were known for their reproductive traits. KC has a significant

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high litter size even when reared in a harsh environment and can be raised as a meat type; KJ, the does is prolific, has a short kidding interval, and can be raised as a meat type (Sodiq and Haryanto, 2007), while the litter size (LS) in SD is 1.83 ± 0.69 and perform as dual purpose (meat and dairy) type (Ciptadi *et al.*, 2019). KJ is solely located in Central Java, whereas SD goats start to spread massively in Indonesia following KC. KC is also known as Indonesian native goats (Batubara *et al.*, 2006). Half of Indonesian goat population is existed in Java, therefore a study based on goat population in Java was expected to represent the entire goat population in Indonesia, particularly in term of specific reproductive traits.

So far, the genetic structure of important economic traits has been identified, but the number of causative genes in goats has been lower than in sheep and cattle (Amills *et al.*, 2017). The phenotypic variations of goats were shaped by various artificial or natural factors such as migration of human, environmental changes and influences of socioeconomic. Further, the genomic variability of goats were constructed mostly by breeding orientation and artificial selection during domestication (Wang *et al.*, 2016). Principally, the sustainable selection and advancement of a novel traits in an environmental shifting needs the genetic diversity (Mandal *et al.*, 2020).

Reproduction is a critical function for the survival of the species, thus this function is governed by complex regulatory signals. The hypothalamic-pituitary-gonadal (HPG) axis regulates reproductive activity by modulating the secretion of inhibitory factors and pituitary gonadotropins by the hypothalamus. Gonadotropin activity in the gonad is mediated by peripheral blood circulation (Nagamalleswari *et al.*, 2004). The HPG axis is divided into three regulatory signals: 1) hypothalamus: gonadotropin-releasing hormone

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(GnRH); 2) pituitary: gonadotropin, luteinizing hormone (LH) and follicle stimulating hormone (FSH); and 3) gonads: sex steroid and peptides (Pinilla *et al.*, 2012).

Follicle Stimulating Hormone (FSH) is a pituitary gonadotropin which have essential task in reproduction. The main roles of FSH in female are maturation and development in antral follicles, encourage the antrum formation in secondary follicles and organize a response for ovulation when the LH surge (Mahdavi and Dashab, 2017).

The present study was undertaken to analyze the population structure and to explore the relative expression of KISS1 gene associated with reproductive traits through FSH level and DNA sequencing analysis from different goat breeds, litter size, haplotypes and genotypes to describe its relationship with litter size at kidding based on KISS1 gene sequences of three Indonesian indigenous goat breeds compare to other species sequences. Therefore, as KISS1 gene plays an important role on reproduction, this study was carried out.

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MATERIALS AND METHODS

Ethical Clearance

The protocol of the current research was under the standart rule of animal treatment as designated in the Republic of Indonesia's law, that is, number 41, 2014.

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2.1. Animals and samples collection

A total of 23 heads of goat does from three Indonesian indigenous goat breeds, namely KC (n=7), KJ (n=8) and SD (n=8) were utilized in the present study. The goats were healthy, unrelated and were not pregnant. They were selected randomly based on

LS, age, multiparous (2nd to 5th parities) and have phenotypic characteristic of each breed. These breeds represent different regions and altitudes, KC in Grobogan regency, KJ in Purbalingga regency, both are in Central Java while SD is from Lumajang regency East Java (Fig.1). The Grobogan, Purbalingga and Lumajang were located at 100 m, 113 m and 500 m height above mean sea level (AMSL) respectively. The goats were kept by the farmer under the homogenous environment.

2.2. Genomic DNA extraction

A total of 3 ml of blood samples were collected via the jugular vein in to sterile vacuum tubes with anticoagulant (EDTA). The blood samples were shifted to the laboratory using coolbox and freezed at -20°C until the genomic DNA extraction. Thus, GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) was applied to extract the genomic DNA from the whole blood correspond the manufacturer's guidance. The evaluation of DNA quality was set up by electrophoresis using agarose gel (1%) and Nanodrop spectrophotometer Uvidoc HD6 (UVItec Ltd., Cambridge, UK).

A clear single band on agarose (1%) electrophoresis and the optical density (OD) 260/280 ratio ranged between 1.7 and 2.0 (according to the kit protocol) verifying a good quality of DNA extraction.

2.3. PCR amplification

A 1061 bp fragment of intron 2 KISS1 gene was amplified with a pair of primer (F: 5'-GCTCAGTGGCCAGGAATCTG-3'; R: 5'-GCTCATAGCAGGGCCTCAAA-3'). The primers were designed using the sequence of KISS1 gene of *Capra hircus* breed Jining Grey (GenBank Accession Number GU142847.1) and Primer3 Plus software. Polymerase Chain Reaction (PCR) was perform in 50 µl volume containing 4 µl DNA extraction (20-30 ng/ µl), 1 µl for each primer (10 pmol/ µl), 19 µl ddH2O and 25 µl of

MyTaq Red Mix Bioline (1st BASE) on MJ Mini Personal Thermal Cycler (Bio-Rad, USA). PCR cycling program contain of pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72 °C for 30 s, and post extension at 72 °C for 7 min. The amplicon was performed by electrophoresis in 2 % agarose gel (Invitrogen, Life Technologies Co, CA) at 100V for 30 min.

2.4. DNA sequencing and analysis.

The amplicon of 23 goats reflecting three indigenous Indonesian goat breeds were sequenced both forward and reverse direction using commercial service (1st BASE). The goats were selected based on breeds, litter size, parity, age and goats which treated with estrus synchronization. The goat sequences were categorized into five group, which are LS 1(KC1a, KC1b, KJ1a, KJ1b, SD1a and SD1b), LS 2 (KJ1a, KJ1b, KJ1c, KJ1a, KJ1b, SD1a, SD1b, SD 1c and SD1d), LS 3 (KC3a, KC3b, KJ3a, KJ3b, KJ3c and SD3a), LS 4 (KJ4a) and LS 5 (SD5a). Alignment of multiple-sequence were performed by software MEGA X 10.1 version to explore the variation of single nucleotide polymorphisms (SNPs).

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2.5. Estrus synchronization, blood samples and hormonal assay (ELISA)

Five goat does for each KC and KJ and six SD goat does with different LS were treated with 0.30 mg medroxyprogesterone acetate (MAP) intravaginal sponges for 14 days. The blood samples were collected five times (0, 3, 6, 9 and 12 hours) after the sponge removal. A total 3 ml of blood samples were collected in plain and sterile vacutainer tubes. Then, the blood samples were centrifuged (3000 rpm/5 min) to obtain serum and stored at -20°C in eppendorf tubes until assayed for FSH profile. FSH hormone levels was estimated using Goat Follicle Stimulating Hormone Kit (Bioassay Technology

Laboratory Cat. No. E0006Go Shanghai, China) and counting using microplate reader (ZENIX-320, USA). The stand art curve ranges 0.05 mlU/ml – 15 mlU/ml and the sensitivity is 0.028 mlU/ml. The intra-assay coefficient of variance (CV) and the inter-assay CV less than 8% and 10% respectively. The ELISA was performed as per kit guidance.

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2.6. Statistical analysis

2.6.1 Population Structure

The data were analyzed using MEGA X software to acquire the singleton variable, parsimony sites, genetic distance within and between goat breed and to form phylogenetic tree. The neighbour-joining method was used to build the phylogenetic tree. Different sequences of KISS1 gene from other vary species were acquired from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) for phylogenetic analysis. The distance between sequence pairs were represented by the length of each pair of branches. The scale under the tree is indicating the nucleotide substitution number. The DnaSP software were used to calculate haplotype diversity, number of haplotypes, number of mutations, Fst and Tajima's D. The Arlequin software was utilized to obtain haplotype shares and haplotype frequencies.

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Basic Local Alignment Search Tool (BLAST) was used to detect the homology sequences in diverse breeds or species. Six different KISS1 gene sequences from different species/breed have been selected from the GenBank with accession number listed below (Tabel 1).

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2.6.2 Follicle Stimulating Hormone (FSH) Level

The data were analyzed using General Linier Model (GLM) of SAS Software. Fixed model used for FSH :

$$y_{ijklmn} = \mu + g_i + b_j + c_k + l_l + p_m + h_n + e_{ijklmn}$$

where y_{ijklmn} is FSH plasma level measured for each samples, μ is the overall mean, g_i is the fixed effect of *i*th genotype (i = 1,2,3), b_j is the fixed effect of *j*th breed (j = 1,2,3), c_k is the fixed effect of *k*th collection time (k = 1,2,3,4,5), l_l is the fixed of *l*th litter size (l = 1,2,3,4,5), p_m is the fixed of *m*th parities (m = 1,2,3), h_n is the fixed of *n*th haplotypes (n = 1,2,…,12) and e_{ij} is a random error of each observation. When P <0.05 it was verify significant statistically. In this study, multiple comparisons of the means were tested using Tukey-Kramer with significant level of 5%.

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RESULTS AND DISCUSSION

Nucleotide sequence identity and phylogenetic tree of KISS1 gene

The OD ratio 260/280 ranged from 1.7 to 2.0, indicating that extracted DNA was not contaminated and in good quality. Psifidi *et al.* (2015) confirming that the standart of OD ratio 260/280 is ≥ 1.8, depend on the extraction kit used. A higher ratio number showed higher purity of DNA extract. Therefore, the extracted DNA could be sequenced both forward and reverse directions immediately in this study.

BLAST from NCBI were used to find the degree of similarity between chosen sequences (http:// https://blast.ncbi.nlm.nih.gov/Blast.cgi). Three species/breed that have the highest similarity are Jining grey goats from China (GU. 142847.1), *Ovis aries* (KP835797.1) and *Capra hircus* (KR065750.1) for 99.69%, 99.47% and 97.66% respectively (Table 1). The homologous sequences from other species/breed were

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obtained from NCBI GenBank database. The closely related sequences could be indicated from the similarity at nucleotide level. The DNA sequences similarity interprets that the function and structure of regulatory elements or protein products of gene expression is similar (Mahdavi and Dashab, 2017) and high conservatism gene in species (Zheng *et al.*, 2018).

Homology of KISS1 gene with other species ranged between *Homo sapiens* (NG_032151.1) with 78.74% similarity to *Capra hircus* Jining Grey breed (99.69%). Zheng *et al.*, (2018) found the similar result in previous research on Jintang Black goat (JTG). The similarity between KC, KJ, SD and JTG is 99.02%. This output denoted that KISS1 gene is conserve in many species because of its significant role in reproduction.

The sequences analysis could be performed by aligning the gene sequences with specific role to determine the evolutionary correlation between unrecognized sequences and approved sequences (GenBank) to construct a phylogenetic tree, branching and discrepancy between species (Mahdavi and Dashab, 2017). The analysis of nucleotide sequences using MEGA X software between the indigenous goats represented 18 variable sites which include five singleton sites, 13 parsimony sites and 14 haplotypes.

Diversity in entire population is 1.18. Meanwhile, the mean distance is 1.39 that calculated from all DNA sequences which show the average of entire sequence pairs and the amount of base changes at each site. The distance within group is calculated by the average number of base changes between all sequences within the group. The disparity was enumerated to be 0.0046, 0.0047 and 0.0051 in KC, KJ and SD respectively, while the distance between group are shown in Table 2. The previous experiment found that the genetic distance based on BMP15 gene was counted to be 0.4 in cows, 1.0 in pig, 4.1 in sheep and 2.1 in goats (Mahdavi and Dashab, 2017). This condition indicated that

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nucleotide substitutions in goat species of BMP15 gene higher than KISS1 gene caused by evolution correlated with gene expression mechanisms, thus this condition showed that KISS1 gene more conserve than BMP15 gene.

The common haplotype in three Indonesian goat breeds is CCATAGCGGGGCAT (H1) and the haplotype frequencies are 0.143, 0.5 and 0.125 for KC, KJ and SD respectively. In addition, overall haplotype CCATAGCGGGGCAT (H1) frequency in the entire population is 26.1% and the haplotype diversity is 0.913.

The average pairwise fixation index (Fst) value is 0.021 (KC and KJ) and 0.082 (KC and SD). This value is lower than previous values resulted in south-east Asia. Barker *et al*. (2001) reported that Fst value between south-east Asian goat is 0.14. In contrary, the Fst value between KJ and SD is 0.195. This data showed that genetic structure differentiation between KJ and SD is higher than KC to KJ and SD, thus indicated that KC is an ancestor for KJ and SD. In accordance, Lestari *et al.* (2018) reported that KJ is a crossbred of KC goat and Etawah Grade (EG). Further research needed to investigate the phylogenetic relationship between KC and SD.

The nucleotide frequency of thymine/uracil, cytosine, adenine and guanine is 23.3%, 27.7%, 23.5% and 25.5% respectively. Nucleotides substitution rate between species/groups were estimated using Tamura-Nei model in MEGA X software (Table 3). These results denote the opportunity for replacement of each nucleotide with another one. The distance was estimated using the amount of bases and pair comparison method. The distance between *Homo sapiens* and *Ovis aries* were the maximum (6.393), while the closest distance was between Indonesian goats and Jining grey goats. This data could be confirmed with the phylogenetic tree, where *Homo sapiens* and *Ovis aries* found in

different branch. Furthermore, Indonesian native goats and Jining grey goat were located in the same node.

Adaptation is in reaction to selection of production methods and connected with local environmental situation. The alpine (*Capra ibex*) ibex and Spanish (*Capra pyrenaica*) have shown that both species were introgressed with domestic goat based on major histocompatibility complex (MHC) genes (Stella *et al.*, 2018). Phylogenetic tree of domestic and wild goat species based on Y-chromosome, nuclear marker or mitochondrial are discrepant (Amills *et al.*, 2017). The incongruous between nucleus and mtDNA phylogenies were caused by interspecific hybridization, rather than lineage sorting or paralogs (Ropiquet and Hassanin, 2006).

BLAST was used to identify similarity between DNA sequences. Other homolog species were used to align the nucleotide sequences of KISS1 gene to illustrate the phylogenetic tree. The nucleotide sequences of Indonesian goat breeds were identical with Jining grey goats, *Ovis aries* and *Bos indicus* for 99.69%, 99.20% and 88.98% respectively (Fig. 2). This data also confirmed the Fst value in this study (Table 2). The similarity between goats, sheeps and cattle which are ruminants, shows that KISS1 gene may have equivalent function in ruminants.

The phylogenetic tree shows two main clades of the phylogenetic relationship of all sequences. The last nodes of the phylogenetic tree denotes the current sequences of samples used, while the internal nodes pointed as suspect ancestor sequences. The nearest genetic relationship is between Indonesian native goats and Jining grey goat because it located in the same node. The other branch in the same clade with Indonesian goat breeds are *Capra hircus* and *Ovis aries*. Hereinafter the next clade consist of *Bos indicus*, *Homo sapiens* and *Sus scrofa*. The phylogenetic tree denoted a similarity and distance between

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species based on KISS1 gene. The phylogenetic tree from prior research (Zheng *et al.*, 2018) showed similar clustering among various species which acquired in the in this study even the accession numbers of NCBI used are different.

KISS1 gene expression and FSH plasma level

An estrus synchronization was used in the current research using progestagen intravaginal sponge. In accordance with Wildeus (2000) reported the previous research in different breeds such as Cashmere, Alpine, Boer, Saanen and Nubian revealed that the effectiveness of estrus synchronization using intravaginal sponges might represent significant differences led by distinct species, breeds, treatment management and mating system. The utilization of pregnant mare serum gonadotropin (PMSG) and progestagen sponge for estrus synchronization has resulted a satisfactory outcome. Internal appliances conceiving different kind of progestagen, implanted in female reproduction tract during 12-14 days were used widely (Bitaraf *et al.*, 2007).

The intravaginal sponges were implanted for 14 days in the present research. The long term progestagen intravaginal treatment (12-14 days) gave better result than short term (5-7 days) but not differ significantly, whether on estrus intensity, estrus response, onset of estrus, concentration of progesterone serum at 21 days after artificial insemination (AI), length of estrus, gestation period, kidding and fecundity rate different significantly (Ngangi *et al.*, 2002; Kor *et al.*, 2011). On the other hand, intravaginal progestagen sponge used in estrus synchronization on ewes could improve ovulation time and estrus expression, contrary it might shorten duration of estrus (Mahmoud and Senosy, 2019).

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The basal concentration of progesterone hormone is reached six hours after the sponge taken out from female reproduction tract (Ngangi *et al.,* 2002). The first three observations (0, 3 and 6 hours) were in luteal phase while other observations (9 and 12 hours) were in the earlier follicular phase. This might explain that the FSH plasma level increase slightly during the collection time (Table 5). In sheep, KISS1 gene expression in the sheep preoptic area (POA) is greater just previous to the late follicular phase GnRH/LH surge than luteal phase (Smith *et al.,* 2013). For future research, longer observation time is needed to evaluate the significant result of FSH plasma level.

KISS1 gene produces kisspeptin (Kp). This peptides were performed through their receptor, G-protein-coupled receptor (GPR54). Kp have been rised as important regulators of neurons that remain in the basal forebrain and yield gonadotropin releasing hormone (GnRH). Cells in the upstream of GnRH neurons synthesized KISS1 functionally (Knoll *et al.,* 2013). KISS1 gene stimulates GnRH neuron activity, gene expression and the release was regulated by circulating gonadal hormones (Smith, 2013). Kp has been known as key neuroendocrine gate keeper of reproduction and maintenance of adult reproduction recently (Millar *et al.,* 2010). Sequences of KISS1 gene have revealed a polymorphism related to reproductive traits. KISS1 gene might be a significant candidate gene on reproductive traits in goats (Cao *et al.,* 2010; An *et al.,* 2013; El-Tarabany *et al.*, 2017; Sahoo *et al.*, 2019).

Kp arranges the construction of preantal follicles negatively by leading the production of anti mullerian hormone (AMH) and degrade the sensitivity to FSH through preventing the induction of FSHR expression via sympathetic activators, thus lowering the recruitment of primary follicles (Panidis *et al.,* 2006; Cao *et al.,* 2019). The sympathetic nerve activity might adjust the intra ovarian Kp system and the peptides were

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needed for appropriate coordination between ovarian function both from neural or ovarian origin (Zheng *et al.*, 2018). Furthermore, the serum levels of Kp are in contrary correlation with FSH, but have a positive correlation with testosterone, LH and dehydroepiandrosterone (DHEA) (Gorkem *et al.*, 2018).

As mentioned before, fourteen haplotypes were obtained in current research. The gene flow, bottleneck and drift events, the distribution of nucleotide polymorphisms could formed by demographic history of breed (Nordborg and Tavare, 2002), therefore estimating haplotype variations is very informative to appraise the effects of the migrations, selection or admixture in goat populations (Criscione *et al.*, 2019).

The statistical analysis showed that haplotype affected FSH level significantly (Table 4). The TCAATGCGCAACGT haplotype (H9) goats had superior FSH plasma level compare to other haplotypes. The preliminary experiment revealed that TCAATGCGCAACGT haplotype (H9) of KISS1 gene also had high LS (3 ± 0^{b}). Moreover, the rest haplotype denoted a vary LS, not in linier with the FSH plasma level. This condition might be caused by the different goat breeds used to form the haplotype analysis. Nackley *et al.*, (2006) suggested the significance of haplotypes over SNPs for genetic variations analysis. In agreement with this result, another researches using IGF1 bond with IGF1 receptors (IGF1R) and IGF1R haplotypes are correlated with puberty age in Brahman heifers (Fortes *et al.*, 2013); the haplotypes of FSHβ3-c had a superior effect for the semen quality (Nikbin *et al.*, 2018); the casein complex haplotypes correlated with milk quality traits (Inostroza *et al.*, 2020). These phenotypes were related to reproductive traits. To date, there is no published journal concerning the haplotype effect to FSH plasma level. Therefore, our inference should be verified with further study.

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Table 5 shows the data of FSH based on goat breeds, sample collection time, litter size, parity and genotype. The discrepancies between breeds are significant, KC and KJ have a higher FSH concentration than SD. KC and KJ goats were collected from Grobogan and Purbalingga regency which represented lowland area (0 – 200 m), further SD goat was collected from Lumajang regency which reflected high land (500 m). In accordance, a breed type has a significant effect to fresh and post-thaw semen traits (Nikbin *et al.*, 2018). Both long term artificial and natural selection enforced by animal husbandry and environmental change resulted different goat breeds in China. The multigenic traits such as prominent cold and disease resistance, strong rough fodder resistance, adaptiveness to stressful environment and high prolificacy reflects distinct natural gene pool (Liu *et al.*, 2019).

Further, the present investigation did not find any correlation between parity and FSH plasma level (table 5). This finding is in accordance in Damascus and Zaribi goats. The TT genotype at T121A in the intron 1 of KISS1 gene had significant higher LS than TA genotype. Moreover, the difference on estradiol$_{17\beta}$ and progesterone level caused by parity is not significant (El-Tarabany *et al.*, 2017).

The data from our previous research found that there are three obtrusive novel single nucleotide polymorphisms (SNPs) correlated with reproductive traits in three Indonesian native goat breeds (unpublished data). The SNPs are g.2425C>G, g.2436A>G and at g.2459G>A. The previous research found a SNP in FSHB gene promoter region within one of the conserved hormone-response elements (HREs) were associated with divergent in serum FSH level in men (Grigorova *et al.*, 2008). Herewith we report for the first time polymorphisms of KISS1 gene in goats indicating a significant correlation with FSH level (Table 5).

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The recent research showed that goat breed influences the FSH level significantly, wherein SD goat have lower FSH plasma level. This finding is in accordance with previous research. Another study in human found that higher body mass index (BMI) had lower kisspeptin significantly. Metastin/kisspeptin levels were correlated with all indices of insulin resistance significantly and reversely, thus it can be concluded that a significant decrease in plasma metastin level is correlated with insulin resistance (Panidis *et al.*, 2006; Chen *et al.*, 2010). The LH levels were correlated with plasma metastin levels positively. In fact, average body weight for KC, KJ and SD goats are 24.7 kg, 27.87 kg and 48.50 kg (Batubara *et al.*, 2006; Sodiq and Haryanto, 2007; Ministry of Agriculture, 2014).

The mechanism of major decrease in KISS1 gene expression could lead a compensatory increase in the expression of its receptor (GPR54), causing a circumstance of sensitization to kisspeptin effects. Therefore, an enhancement of LH responses to Kp suggesting that substitution of endogenous KISS1 levels is adequate to activate GnRH-LH axis fully in spite of prejudicial metabolic conditions (Castellano *et al.*, 2005).

Adipose is a necessary endocrine tissue that influence reproduction through leptin primarily (Kawwass *et al.*, 2015; Symonds *et al.*, 2016). Leptin acts through the GPR54 which is found on kisspeptin neurons in hypothalamus (Tena-Sempere, 2006[a]; Tena-Sempere, 2006[b]). Kisspeptin binds to GnRH neurons and provoke GnRH release (Roseweir and Millar, 2009). The leptin-kisspeptin-GnRH neuron pathway presents the endocrine argument for the critical body weight hypothesis, which body weight relate to puberty in female (Keisler *et al.*, 1999). Thus, earlier result suggest that higher BMI caused lower LH surge, in turned FSH plasma level also decrease, but this finding needed further research.

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Nowadays, the effect of Kp on FSH secretion is less information. The response of KISS1 to FSH release emerge less sensitive than LH considerably. The pathway organized centrally through modulation GnRH system, moreover it conducted independently with other neuroendocrine regulators of gonadotropic axis such as excitatory amino acids (EEAs), nitric oxide (NO) and leptin (Navarro, 2005). The positive reaction of leptin in GnRH is mediated by proopiomelanocortins (POMC, precursor of α-MSH), galanin-like peptide (GALP) and kisspeptin neuropeptides (Gottsch *et al.*, 2004; Crown *et al.*, 2007; Quennell *et al.*, 2009). Kp is detected in the growing follicles at theca cells and begins to arise in the basal cells of granular layer in rodent and human (Castellano *et al.*, 2006). FSH is not under control entirely by GnRH (Charlton, 1983; Phillips, 2005), but the major stimulus for FSH is GnRH (Mason *et al.*, 1986).

In our prior study, the obtrusive genotype of KISS1 gene intron 2 that correlated with higher LS, particularly average LS at the first and third parity in Indonesian native goat breeds which are CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA genotype at g.2459G>A (unpublished data). The data above (Table 5) showed that the genotypes at g.2425C>G and g.2436A>G had the same FSH plasma level (P=0.22 and P=0.34 respectively). On the other hand, the AA genotype at g.2459G>A has a superior FSH level than GG genotype (Fig.3).

Preliminary study revealed that CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA genotype at g.2459G>A had significant greater litter size (2.58, 2.58 and 3.0 respectively). Furthermore, neither the CC genotype at g.2425C>G nor the AA genotype at g.2436A>G have significantly different FSH levels. On the other hand, GA genotype at g.2459G>A reveals a higher LS (3.0±0.18) than AA genotype which have a

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lower LS (2.0±0.21). Thus, it can be concluded that GA genotype at g.2459G>A is the most prominent genotype correlated with reproductive traits in Indonesian native goats.

CONCLUSION

The phylogenetic tree reveals a high closeness between Indonesian and Chinese goat breeds indicates the same function and tightness along the evolutionary timescale. *Capra hircus* and *Ovis aries* were also found in the same clade with Indonesian goat breed represents a significant role of KISS1 gene in reproductive traits in a variety of species.

The FSH level was influenced by breed, LS, and haplotype. The superior haplotype and genotype of KISS1 gene is TCAATGCGCAACGT haplotype and GA genotype at g.2459G>A that correlated with high LS and FSH level. These aspects could be considered in further breeding selection program for economically significant reproductive traits in goats.

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ACKNOWLEDGEMENT

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Table 1. KISS1 gene sequences of different species from the GenBank used to develope the phylogenetic tree

Species	Accession number	Similarity
Jining Grey	GU. 142847.1	99.69%
Ovis aries	KP835797.1	99.47
Capra hircus	KR065750.1	97.66
Bos indicus	XM_019976949.1	87.91
Sus scrofa	AB466320.1	81.14
Homo sapiens	NG_032151.1	67.38

Table 2. The mean genetic distance between Indonesian native goat breeds using the number of base pair in KISS1 gene

Goat	KC	KJ	SD
KC		0.021	0.082
KJ	0.0047		0.195
SD	0.0053	0.0061	

Note : the value above the diagonal are Fst and genetic distance value are under diagonal

KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

Table 3. The mean distance between species using the number of base pair in KISS1 gene

Species	Jining grey	KC	KJ	SD	*Capra hircus*	*Ovis aries*	*Bos indicus*	*Homo sapiens*	*Sus scrofa*
Jining grey									
KC	0.003								
KJ	0.004	0.005							
SD	0.003	0.005	0.006						
Capra hircus	2.261	2.294	2.282	2.293					
Ovis aries	4.325	4.312	4.285	4.296	2.701				
Homo sapiens	3.427	3.342	3.404	3.387	3.049	6.393			
Bos indicus	3.850	3.843	3.896	3.819	2.781	4.259	2.840		
Sus scrofa	4.411	4.437	4.467	4.455	3.950	4.606	3.106	4.529	

Note : KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

Table 4. Means ± SE of FSH (mIU/ml) on haplotype (P<0.0001)

Haplotype Variations		FSH
H9	CAATGCGCAACGCT	10.65 ± 1.27[a]
H4	TATTGCACAACGCT	8.99 ± 0.54[b]

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H2	CATAGCGCAACGCT	4.77 ± 0.49[c]
H8	TATAGCGGGGCGCT	2.72 ± 0.14[d]
H10	CATTGCGCAGTGCT	1.97 ± 0.08[de]
H1	CATAGCGGGGCACT	1.76 ± 0.14[de]
H6	CATTGCACAACGCT	1.54 ± 0.06[de]
H3	TCTTGCGGGGTACT	1.49 ± 0.08[de]
H7	TAATGCGCAACGTT	1.48 ± 0.12[de]
H13	CATTCTGCAATGCA	1.30 ± 0.19[e]
H14	CCTTCTGCAGTGCT	1.21 ± 0.09[f]
H11	CATTGCACAGTGCT	0.67 ± 0.05[g]
H12	CAATCCGCAATGCT	0.66 ± 0.05[h]

Table 5. Means ± SE of FSH (mIU/ml) based on goat breeds, sample collection time, litter size, parity and genotype

Specification	P Value	Category	Means ± SE
Breed	P = 0.002	KC	3.88 ± 0.63[a]
		KJ	3,73 ± 0.75[a]
		SD	1.49 ± 0.19[b]
Sample collection time	P = 0.9361	0 hours	2.48 ± 0.59
		3 hours	2.88 ± 0.74
		6 hours	2.89 ± 0.73
		9 hours	2.97 ± 0.74
		12 hours	3.45 ± 0.99
Litter size	P = 0.0175	1 kid	1.28 ± 0.15[b]
		2 kids	2.61 ± 0.47[ab]
		3 kids	4.21 ± 0.78[a]
		5 kids	3.77 ± 0.32[a]
Parity	P = 0.0352	1st parity	3.77 ± 0.32
		2nd parity	2.27 ± 0.34
		3rd parity	4.10 ± 0.79
SNP g.2425 C>G	P = 0.2226	CC	3.27 ± 0.44
		CG	2.10 ± 0.21
		GG	1.76 ± 0.13
SNP g.2436 A>G	P = 0.3447	AA	3.22 ± 0.48
		AG	2.66 ± 0.27
		GG	1.76 ± 0.14
SNP g.2459 G>A	P = 0.0027	AA	4.01 ± 0.96[a]
		GA	3.89 ± 0.68[ab]
		GG	1.65 ± 0.11[b]

Note : Values with different superscripts in the same column differ significantly at P<0.05

KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

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Figure 1. Distribution of sampling area in Java island, Indonesia

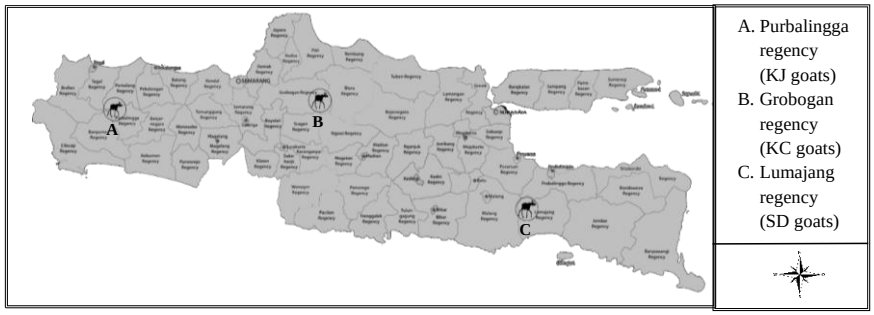


Figure 2. Phylogenetic tree of KISS1 gene of different species

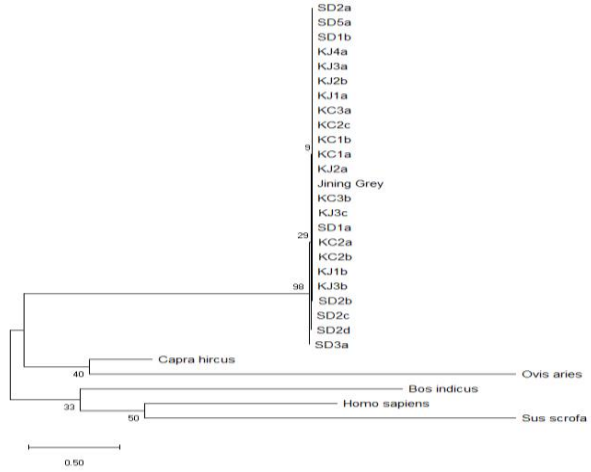
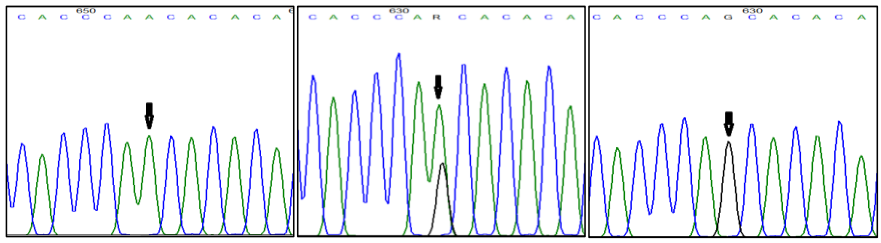


Figure 3. Genotypes of g.2459G>A



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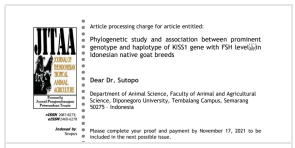
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Phylogenetic study and association between prominent genotype and haplotype of KISS1 gene with FSH level in Idonesian native goat breeds

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ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi struktur populasi dan ekspresi gen KISS1 yang berkaitan dengan sifat reproduksi menggunakan analisis level Follicle Stimulating Hormone (FSH) dan sekuensing DNA gen KISS1. Sejumlah 23 ekor induk yang terdiri dari kambing Kacang (n=7), Kejobong (n=8) and Senduro (n=8) diidentifikasi genotipenya menggunakan metode sekuensing DNA, 16 ekor diantaranya diberi perlakuan sinkronisasi estrus untuk diamati level hormon FSH menggunakan metode ELISA. *Software* MEGA X digunakan untuk menganalisa sekuens DNA, sedangkan General Linier Model (GLM) dari SAS *software* untuk menganalisa hormon FSH. Pohon filogeni memperlihatkan kesamaan yang tinggi antara kambing lokal Indonesia dengan spesies lain yang menunjukkan bahwa gen KISS1 konservatif. Analisis hormon FSH menunjukan hasil yang berbeda secara signifikan antara kambing Kacang dan Kejobong dibandingkan Senduro (P = 0.002), litter size (LS) 3 dibandingkan LS 1 (P = 0.0175), selanjutnya haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A menunjukkan hormon FSH yang lebih tinggi dibandingkan haplotipe dan genotipe yang lain (P = 0.0027; P<0.0001) dan terkait dengan LS yang tinggi (3.0±0.18). Waktu pengambilan sampel dan paritas tidak memberikan perbedaan yang signifikan terhadap hormon FSH. Penelitian ini menunjukkan bahwa haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A mempunyai asosiasi dengan sifat reproduksi.

Kata kunci : FSH, gen KISS1, haplotipe, kambing lokal Indonesia, pohon filogeni

ABSTRACT

The aim of the current research was to analyze the population structure and expression of KISS1 gene associated with reproductive traits through Follicle Stimulating Hormone (FSH) level and DNA sequencing analysis based on KISS1 gene. Genotypes of 23 goat does consist of Kacang goats (n=7), Kejobong goats (n=8) and Senduro goats (n=8) were investigated using DNA sequencing, 16 out of 23 samples were synchronized to examine their FSH level using ELISA method. The data were analyzed using MEGA X software for DNA sequences and General Linier Model (GLM) for FSH plasma level. The phylogenetic tree showed the high homology between Indonesian native goats with other species showing a gene conservatism. A significantly higher FSH plasma levels were obtained from Kacang and Kejobong than Senduro goat (P = 0.002), litter size (LS) 3 than LS 1 (P = 0.0175), further TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A have a higher FSH plasma than other haplotypes and genotypes (P = 0.0027; P<0.0001) and are associated with high LS (3.0±0.18). Neither sample collection times nor parities have different significantly. The current trial indicated that TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A were correlated with reproductive traits.

Keywords : FSH, haplotype, Indonesian native goat, KISS1 gene, phylogenetic tree

INTRODUCTION

Goats, unlike other livestock species, are adaptable animals that can survive in tropical, mountainous, and desert environments. Goats have spread widely due to their adaptability to a variety of environments and nutrition availability, small size, prolific, useful productivity for humans, and non-competitiveness with human food, and they contribute significantly, particularly in rural areas (Aziz, 2010;Guerrero *et al.*, 2019).

In Indonesia, there are more than 19 million goats, with eight goat breeds officially confessed. In Indonesia, goat population has increased over the last five years (Ministry of Agriculture, 2020). This condition could indicate that goats could be an alternative source of red meat. According to Aziz (2010), Indonesia is one of the 10 largest lamb production country in the world. This situation might represents the Indonesian preference on goat meat because most of goats were reared and consumed locally. Enhancing reproductive traits could be a way to increase the number of goat population.

Indigenous goat breeds are well adapted to agro-ecological conditions, helping to ensure goat farming's long-term viability in rural and harsh environments (Stella, 2018). Goats are traditionally bred in Indonesia for meat and dual -purpose production. In this study, three indigenous goat breeds were used. Kacang (KC), Kejobong (KJ), and Senduro (SD) goat breeds were known for their reproductive traits. KC has a significant high litter size even when reared in a harsh environment and can be raised as a meat type; KJ, the does is prolific, has a short kidding interval, and can be raised as a meat type (Sodiq and Haryanto, 2007), while the litter size (LS) in SD is 1.83 ± 0.69 and perform as dual purpose (meat and dairy) type (Ciptadi *et al.*, 2019). KJ is solely located in Central Java, whereas SD goats start to spread massively in Indonesia following KC. KC is also known as Indonesian native goats (Batubara *et al.,* 2006). Half of Indonesian goat population is existed in Java, therefore a study based on goat population in Java was expected to represent the entire goat population in Indonesia, particularly in term of specific reproductive traits.

So far, the genetic structure of important economic traits has been identified, but the number of causative genes in goats has been lower than in sheep and cattle (Amills *et al.,* 2017). The phenotypic variations of goats were shaped by various artificial or natural factors such as migration of human, environmental changes and influences of socioeconomic. Further, the genomic variability of goats were constructed mostly by breeding orientation and artificial selection during domestication (Wang *et al.,* 2016). Principally, the sustainable selection and advancement of a novel traits in an environmental shifting needs the genetic diversity (Mandal *et al.,* 2020).

Reproduction is a critical function for the survival of the species, thus this function is governed by complex regulatory signals. The hypothalamic-pituitary-gonadal (HPG) axis regulates reproductive activity by modulating the secretion of inhibitory factors and pituitary gonadotropins by the hypothalamus. Gonadotropin activity in the gonad is mediated by peripheral blood circulation (Nagamalleswari *et al.*, 2004). The HPG axis is divided into three regulatory signals: 1) hypothalamus: gonadotropin-releasing hormone (GnRH); 2) pituitary: gonadotropin, luteinizing hormone (LH) and follicle stimulating hormone (FSH); and 3) gonads: sex steroid and peptides (Pinilla *et al.,* 2012).

Follicle Stimulating Hormone (FSH) is a pituitary gonadotropin which have essential task in reproduction. The main roles of FSH in female are maturation and development in antral follicles, encourage the antrum formation in secondary follicles and organize a response for ovulation when the LH surge (Mahdavi and Dashab, 2017).

The present study was undertaken to analyze the population structure and to explore the relative expression of KISS1 gene associated with reproductive traits through FSH level and DNA sequencing analysis from different goat breeds, litter size, haplotypes and genotypes to describe its relationship with litter size at kidding based on KISS1 gene sequences of three Indonesian indigenous goat breeds compare to other species sequences. Therefore, as KISS1 gene plays an important role on reproduction, this study was carried out.

MATERIALS AND METHODS

Ethical Clearance

The protocol of the current research was under the standart rule of animal treatment as designated in the Republic of Indonesia's law, that is, number 41, 2014.

Animals and Samples Collection

A total of 23 heads of goat does from three Indonesian indigenous goat breeds, namely KC (n=7), KJ (n=8) and SD (n=8) were utilized in the present study. The goats were healthy, unrelated and were not pregnant. They were selected randomly based on LS, age, multiparous (2^{nd} to 5^{th} parities) and have phenotypic characteristic of each breed. These breeds represent different regions and altitudes, KC in Grobogan regency, KJ in Purbalingga regency, both are in Central Java while SD is from Lumajang regency East Java (Fig.1). The Grobogan, Purbalingga and Lumajang were located at 100 m, 113 m and 500 m height above mean sea level (AMSL) respectively. The goats were kept by the farmer under the homogenous environment.

Genomic DNA extraction

A total of 3 ml of blood samples were collected via the jugular vein in to sterile vacuum tubes with anticoagulant (EDTA). The blood samples were shifted to the laboratory using coolbox and freezed at -20°C until the genomic DNA extraction. Thus, GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) was applied to extract the genomic DNA from the whole blood correspond the manufacturer's guidance. The evaluation of DNA quality was set up by electrophoresis using agarose gel (1%) and Nanodrop spectrophotometer Uvidoc HD6 (UVItec Ltd*., Cambridge, UK)*.

A clear single band on agarose (1%) electrophoresis and the optical density (OD) 260/280 ratio ranged between 1.7 and 2.0 (according to the kit protocol) verifying a good quality of DNA extraction.

PCR Amplification

A 1061 bp fragment of intron 2 KISS1 gene was amplified with a pair of primer (F: 5'-GCTCAGTGGCCAGGAATCTG-3'; R: 5'-GCTCATAGCAGGGCCTCAAA-3'). The primers were designed using the sequence of KISS1 gene of *Capra hircus breed Jining Grey (GenBank Accession Number* GU142847.1) and Primer3 Plus software. Polymerase Chain Reaction (PCR) was perform in 50 µl volume containing 4 µl DNA extraction (20-30 ng/ µl), 1 µl for each primer (10 pmol/ µl), 19 µl ddH2O and 25 µl of MyTaq Red Mix Bioline (1st BASE) on MJ Mini Personal Thermal Cycler (Bio-Rad, USA). PCR cycling program contain of predenaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72 °C for 30 s, and post extension at 72 °C for 7 min. The amplicon was performed by electrophoresis in 2 % agarose gel (Invitrogen, Life Technologies Co, CA) at 100V for 30 min.

DNA Sequencing and Analysis

The amplicon of 23 goats reflecting three indigenous Indonesian goat breeds were sequenced both forward and reverse direction using commercial service (1st BASE). The goats were selected based on breeds, litter size, parity, age and goats which treated with estrus synchronization. The goat sequences were categorized into five groups, which are LS 1(KC1a, KC1b, KJ1a, KJ1b, SD1a and SD1b), LS 2 (KJ1a, KJ1b, KJ1c, KJ1a, KJ1b, SD1a, SD1b, SD 1c and SD1d), LS 3 (KC3a, KC3b, KJ3a, KJ3b, KJ3c and SD3a), LS 4 (KJ4a) and LS 5 (SD5a). Alignment of multiple-sequence were performed by software MEGA X 10.1 version to explore the variation of single nucleotide polymorphisms (SNPs).

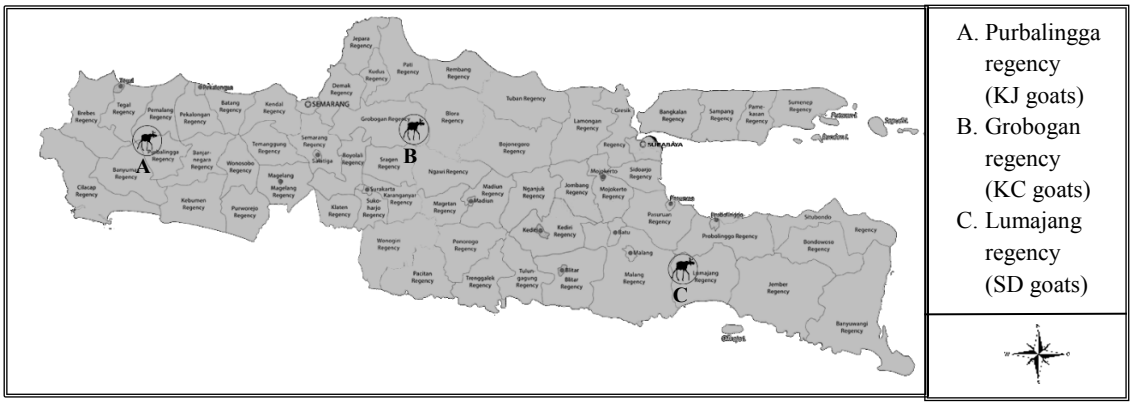

Figure 1. Distribution of sampling area in Java island, Indonesia

Estrus Synchronization, Blood Samples and Hormonal Assay (ELISA)

Five goat does for each KC and KJ and six SD goat does with different LS were treated with 0.30 mg medroxyprogesterone acetate (MAP) intravaginal sponges for 14 days. The blood samples were collected five times (0, 3, 6, 9 and 12 hours) after the sponge removal. A total 3 ml of blood samples were collected in plain and sterile vacutainer tubes. Then, the blood samples were centrifuged (3000 rpm/5 min) to obtain serum and stored at -20°C in eppendorf tubes until assayed for FSH profile. FSH hormone levels was estimated using Goat Follicle Stimulating Hormone Kit (Bioassay Technology Laboratory Cat. No. E0006Go Shanghai, China) and counting using microplate reader (ZENIX-320, USA). The stand art curve ranges 0.05 mlU/ml – 15 mlU/ml and the sensitivity is 0.028 mlU/ml. The intra-assay coefficient of variance (CV) and the inter-assay CV less than 8% and 10% respectively. The ELISA was performed as per kit guidance.

Statistical Analysis

Population Structure. The data were analyzed using MEGA X software to acquire the singleton variable, parsimony sites, genetic distance within and between goat breed and to form phylogenetic tree. The neighbour-joining method was used to build the phylogenetic tree. Different sequences of KISS1 gene from other vary species were acquired from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) for phylogenetic analysis. The distance between sequence pairs were represented by the length of each pair of branches. The scale under the tree is indicating the nucleotide substitution number. The DnaSP software were used to calculate haplotype diversity, number of haplotypes, number of mutations, Fst and Tajima's D. The Arlequin software was utilized to obtain haplotype shares and haplotype frequencies.

Basic local alignment search tool (BLAST) was used to detect the homology sequences in diverse breeds or species. Six different KISS1 gene sequences from different species/breed have been selected from the GenBank with accession number listed below (Tabel 1).

Follicle stimulating hormone (FSH) Level. The data were analyzed using General Linier Model (GLM) of SAS Software. Fixed model used for FSH :

$$y_{ijklmn} = \mu + g_i + b_j + c_k + l_l + p_m + h_n + e_{ijklmn}$$

where y_{ijklmn} is FSH plasma level measured for each samples, μ is the overall mean, g_i is the fixed effect of *i*th genotype (i = 1,2,3), b_j is the fixed effect of *j*th breed (j = 1,2,3), c_k is the fixed effect of *k*th collection time (k = 1,2,3,4,5), l_l is the fixed of *l*th litter size (l = 1,2,3,4,5), p_m is the fixed of *m*th parities (m = 1,2,3), h_n is the fixed of *n*th haplotypes (n = 1,2,…,12) and e_{ij} is a random error of each observation. When $P < 0.05$ it was verify significant statistically. In this study, multiple comparisons of the means were tested using Tukey-Kramer with significant level of 5%.

RESULTS AND DISCUSSION

Nucleotide Sequence Identity and Phylogenetic Tree of KISS1 Gene

The OD ratio 260/280 ranged from 1.7 to 2.0, indicating that extracted DNA was not contaminated and in good quality. Psifidi *et al.* (2015) confirming that the standart of OD ratio 260/280 is ≥ 1.8, depend on the extraction kit used. A higher ratio number showed higher purity of DNA extract. Therefore, the extracted DNA could be sequenced both forward and reverse directions immediately in this study.

BLAST from NCBI were used to find the degree of similarity between chosen sequences (http:// https://blast.ncbi.nlm.nih.gov/Blast.cgi). Three species/breed that have the highest similar-

Table 1. KISS1 gene sequences of different species from the GenBank used to develope the phylogenetic tree

Species	Accession number	Similarity (%)
Jining Grey	GU. 142847.1	99.69
Ovis aries	KP835797.1	99.47
Capra hircus	KR065750.1	97.66
Bos indicus	XM_019976949.1	87.91
Sus scrofa	AB466320.1	81.14
Homo sapiens	NG_032151.1	67.38

ity are Jining grey goats from China (GU. 142847.1), *Ovis aries* (KP835797.1) and *Capra hircus* (KR065750.1) for 99.69%, 99.47% and 97.66% respectively (Table 1). The homologous sequences from other species/breed were obtained from NCBI GenBank database. The closely related sequences could be indicated from the similarity at nucleotide level. The DNA sequences similarity interprets that the function and structure of regulatory elements or protein products of gene expression is similar (Mahdavi and Dashab, 2017) and high conservatism gene in species (Zheng *et al.,* 2018).

Homology of KISS1 gene with other species ranged between *Homo sapiens* (NG_032151.1) with 78.74% similarity to *Capra hircus* Jining Grey breed (99.69%). Zheng *et al.,* (2018) found the similar result in previous research on Jintang Black goat (JTG). The similarity between KC, KJ, SD and JTG is 99.02%. This output denoted that KISS1 gene is conserve in many species because of its significant role in reproduction.

The sequences analysis could be performed by aligning the gene sequences with specific role to determine the evolutionary correlation between unrecognized sequences and approved sequences (GenBank) to construct a phylogenetic tree, branching and discrepancy between species (Mahdavi and Dashab, 2017). The analysis of nucleotide sequences using MEGA X software between the indigenous goats represented 18 variable sites which include five singleton sites, 13 parsimony sites and 14 haplotypes.

Diversity in entire population is 1.18. Meanwhile, the mean distance is 1.39 that calculated from all DNA sequences which show the average of entire sequence pairs and the amount of base changes at each site. The distance within group is calculated by the average number of base changes between all sequences within the group. The disparity was enumerated to be 0.0046, 0.0047 and 0.0051 in KC, KJ and SD respectively, while the distance between group are shown in Table 2. The previous experiment found that the genetic distance based on BMP15 gene was counted to be 0.4 in cows, 1.0 in pig, 4.1 in sheep and 2.1 in goats (Mahdavi and Dashab, 2017). This condition indicated that nucleotide substitutions in goat species of BMP15 gene higher than KISS1 gene caused by evolution correlated with gene expression mechanisms, thus this condition showed that KISS1 gene more conserve than BMP15 gene.

The common haplotype in three Indonesian goat breeds is CCATAGCGGGGCAT (H1) and the haplotype frequencies are 0.143, 0.5 and 0.125 for KC, KJ and SD respectively. In addition, overall haplotype CCATAGCGGGGCAT (H1) frequency in the entire population is 26.1% and the haplotype diversity is 0.913.

The average pairwise fixation index (Fst) value is 0.021 (KC and KJ) and 0.082 (KC and SD). This value is lower than previous values resulted in south-east Asia. Barker *et al.* (2001) reported that Fst value between south-east Asian goat is 0.14. In contrary, the Fst value between KJ and SD is 0.195. This data showed that genetic structure differentiation between KJ and SD is higher than KC to KJ and SD, thus indicated that KC is an ancestor for KJ and SD. In accordance, Lestari *et al.* (2018) reported that KJ is a crossbred of KC goat and Etawah Grade (EG). Further research needed to investigate the phylogenetic relationship between KC and SD.

Table 2. The mean genetic distance between Indonesian goat breeds using the number of base pair in KISS1 gene

Goat	KC	KJ	SD
KC		0.021	0.082
KJ	0.0047		0.195
SD	0.0053	0.0061	

Values above the diagonal are Fst and genetic distance value are under diagonal; KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

The nucleotide frequency of thymine/uracil, cytosine, adenine and guanine is 23.3%, 27.7%, 23.5% and 25.5% respectively. Nucleotides substitution rate between species/groups were estimated using Tamura-Nei model in MEGA X software (Table 3). These results denote the opportunity for replacement of each nucleotide with another one. The distance was estimated using the amount of bases and pair comparison method. The distance between *Homo sapiens* and *Ovis aries* were the maximum (6.393), while the closest distance was between Indonesian goats and Jining grey goats. This data could be confirmed with the phylogenetic tree, where *Homo sapiens* and *Ovis aries* found in different branch. Furthermore, Indonesian native goats and Jining grey goat were located in the same node.

Adaptation is in reaction to selection of production methods and connected with local envi-

Table 3. The mean distance between species using the number of base pair in KISS1 gene

Species	Jining grey	KC	KJ	SD	*Capra hircus*	*Ovis aries*	*Bos indicus*	*Homo sapiens*	*Sus scrofa*
Jining grey									
KC	0.003								
KJ	0.004	0.005							
SD	0.003	0.005	0.006						
Capra hircus	2.261	2.294	2.282	2.293					
Ovis aries	4.325	4.312	4.285	4.296	2.701				
Homo sapiens	3.427	3.342	3.404	3.387	3.049	6.393			
Bos indicus	3.850	3.843	3.896	3.819	2.781	4.259	2.840		
Sus scrofa	4.411	4.437	4.467	4.455	3.950	4.606	3.106	4.529	

KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

ronmental situation. The alpine (*Capra ibex*) ibex and Spanish (*Capra pyrenaica*) have shown that both species were introgressed with domestic goat based on major histocompatibility complex (MHC) genes (Stella *et al.*, 2018). Phylogenetic tree of domestic and wild goat species based on Y-chromosome, nuclear marker or mitochondrial are discrepant (Amills *et al.,* 2017). The incongruous between nucleus and mtDNA phylogenies were caused by interspecific hybridization, rather than lineage sorting or paralogs (Ropiquet and Hassanin, 2006).

BLAST was used to identify similarity between DNA sequences. Other homolog species were used to align the nucleotide sequences of KISS1 gene to illustrate the phylogenetic tree. The nucleotide sequence of Indonesian goat breeds were identical with Jining grey goats, *Ovis aries* and *Bos indicus* for 99.69%, 99.20% and 88.98% respectively (Fig. 2). This data also confirmed the Fst value in this study (Table 2). The similarity between goats, sheeps and cattle which are ruminants, shows that KISS1 gene may have equivalent function in ruminants.

The phylogenetic tree shows two main clades of the phylogenetic relationship of all sequences. The last nodes of the phylogenetic tree denotes the current sequences of samples used,

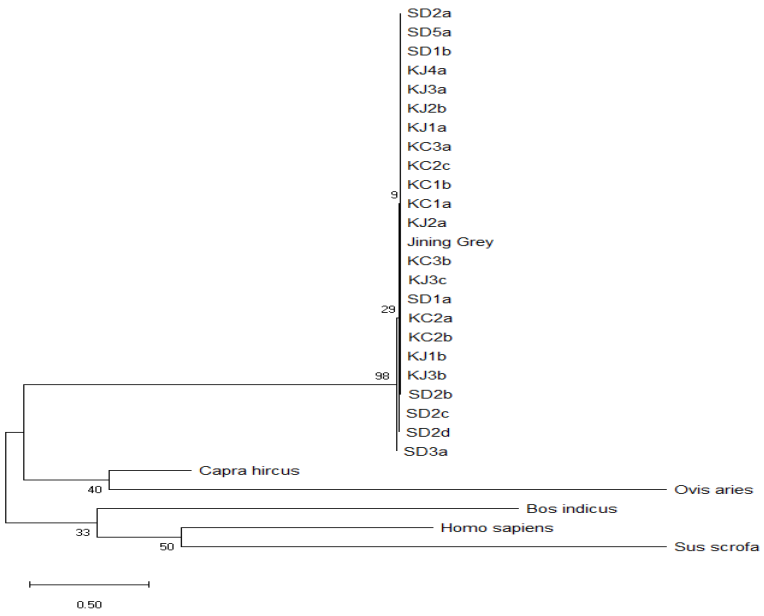

Figure 2. Phylogenetic tree of KISS1 gene of different species

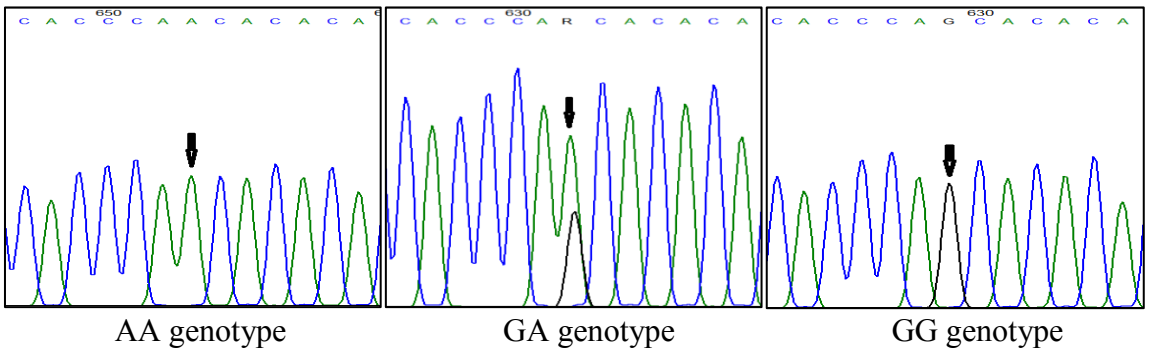

Figure 3. Genotypes of g.2459G>A

while the internal nodes pointed as suspect ancestor sequences. The nearest genetic relationship is between Indonesian native goats and Jining grey goat because it located in the same node. The other branch in the same clade with Indonesian goat breeds are *Capra hircus* and *Ovis aries*. Hereinafter the next clade consist of *Bos indicus*, *Homo sapiens* and *Sus scrofa*. The phylogenetic tree denoted a similarity and distance between species based on KISS1 gene. The phylogenetic tree from prior research (Zheng *et al.,* 2018) showed similar clustering among various species which acquired in the in this study even the accession numbers of NCBI used are different.

KISS1 Gene Expression and FSH Plasma Level

An estrus synchronization was used in the current research using progestagen intravaginal sponge Wildeus (2000) reported that the previous research in different breeds such as Cashmere, Alpine, Boer, Saanen and Nubian revealed that the effectiveness of estrus synchronization using intravaginal sponges might represent significant differences led by distinct species, breeds, treatment management and mating system. The utilization of pregnant mare serum gonadotropin (PMSG) and progestagen sponge for estrus synchronization has resulted a satisfactory outcome. Internal appliances conceiving different kind of progestagen, implanted in female reproduction tract during 12-14 days were used widely (Bitaraf *et al.,* 2007).

The intravaginal sponges were implanted for 14 days in the present research. The long term progestagen intravaginal treatment (12-14 days) gave better result than short term (5-7 days) but not differ significantly, whether on estrus intensity, estrus response, onset of estrus, concentration of progesterone serum at 21 days after artificial insemination (AI), length of estrus, gestation period, kidding and fecundity rate different significantly (Ngangi *et al.*, 2002; Kor *et al.,* 2011). On the other hand, intravaginal progestagen sponge used in estrus synchronization on ewes could

Table 4. Means ± SE of FSH (mIU/ml) on haplotype (P<0.0001)

Haplotype Variations		FSH
H9	TCAATGCGCAACGT	10.65 ± 1.27^{a}
H4	TTATTGCACAACGT	8.99 ± 0.54^{b}
H2	CCATAGCGCAACGT	4.77 ± 0.49^{c}
H8	TCATAGCGGGGCGT	2.72 ± 0.14^{d}
H10	TTATTGCGCAGTGT	1.97 ± 0.08^{de}
H1	CCATAGCGGGGCAT	1.76 ± 0.14^{de}
H6	TTATTGCACAACGT	1.54 ± 0.06^{de}
H3	TCCTTGCGGGGTAT	1.49 ± 0.08^{de}
H7	TCAATGCGCAACGT	1.48 ± 0.12^{de}
H13	TTATTCTGCAATGA	1.30 ± 0.19^{e}
H14	TTATTCTGCAATGA	1.21 ± 0.09^{f}
H11	TTATTGCACAGTGT	0.67 ± 0.05^{g}
H12	TTAATCCGCAATGT	0.66 ± 0.05^{h}

Table 5. Means ± SE of FSH (mIU/ml) based on goat breeds, sample collection time, litter size, parity and genotype

Specification	P Value	Category	Means ± SE
Breed	P = 0.002	KC	3.88 ± 0.63[a]
		KJ	3,73 ± 0.75[a]
		SD	1.49 ± 0.19[b]
Sample collection time	P = 0.9361	0 hours	2.48 ± 0.59
		3 hours	2.88 ± 0.74
		6 hours	2.89 ± 0.73
		9 hours	2.97 ± 0.74
		12 hours	3.45 ± 0.99
Litter size	P = 0.0175	1 kid	1.28 ± 0.15[b]
		2 kids	2.61 ± 0.47[ab]
		3 kids	4.21 ± 0.78[a]
		5 kids	3.77 ± 0.32[a]
Parity	P = 0.0352	1st parity	3.77 ± 0.32
		2nd parity	2.27 ± 0.34
		3rd parity	4.10 ± 0.79
SNP g.2425 C>G	P = 0.2226	CC	3.27 ± 0.44
		CG	2.10 ± 0.21
		GG	1.76 ± 0.13
SNP g.2436 A>G	P = 0.3447	AA	3.22 ± 0.48
		AG	2.66 ± 0.27
		GG	1.76 ± 0.14
SNP g.2459 G>A	P = 0.0027	AA	4.01 ± 0.96[a]
		GA	3.89 ± 0.68[ab]
		GG	1.65 ± 0.11[b]

Values with different superscripts in the same column differ significantly at P<0.05
KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

improve ovulation time and estrus expression, contrary it might shorten duration of estrus (Mahmoud and Senosy, 2019).

The basal concentration of progesterone hormone is reached six hours after the sponge taken out from female reproduction tract (Ngangi *et al.,* 2002). The first three observations (0, 3 and 6 hours) were in luteal phase while other observations (9 and 12 hours) were in the earlier follicular phase. This might explain that the FSH plasma level increase slightly during the collection time (Table 5). In sheep, KISS1 gene expression in the sheep preoptic area (POA) is greater just previous to the late follicular phase GnRH/LH surge than luteal phase (Smith *et al.,* 2013). For future research, longer observation time is needed to evaluate the significant result of FSH plasma level.

KISS1 gene produces kisspeptin (Kp). This peptides were performed through their receptor, G-protein-coupled receptor (GPR54). Kp have been rised as important regulators of neurons that remain in the basal forebrain and yield gonadotropin releasing hormone (GnRH). Cells in the upstream of GnRH neurons synthesized KISS1 functionally (Knoll *et al.*, 2013). KISS1 gene stimulates GnRH neuron activity, gene expression and the release was regulated by circulating gonadal hormones (Smith, 2013). Kp has been known as key neuroendocrine gate keeper of reproduction and maintenance of adult reproduction recently (Millar *et al.,* 2010). Sequences of KISS1 gene have revealed a polymorphism related to reproductive traits. KISS1 gene might be a significant candidate gene on reproductive traits in goats (Cao *et al.,* 2010; An *et al.,* 2013; El-Tarabany *et al.,* 2017; Sahoo *et al.,* 2019).

Kp arranges the construction of preantal follicles negatively by leading the production of anti mullerian hormone (AMH) and degrade the sensitivity to FSH through preventing the induction of FSHR expression via sympathetic activators, thus lowering the recruitment of primary follicles (Panidis *et al.,* 2006; Cao *et al.,* 2019). The sympathetic nerve activity might adjust the intra ovarian Kp system and the peptides were needed for appropriate coordination between ovarian function both from neural or ovarian origin (Zheng *et al.,* 2018). Furthermore, the serum levels of Kp are in contrary correlation with FSH, but have a positive correlation with testosterone, LH and dehydroepiandrosterone (DHEA)

(Gorkem *et al.,* 2018).

As mentioned before, fourteen haplotypes were obtained in current research. The gene flow, bottleneck and drift events, the distribution of nucleotide polymorphisms could formed by demographic history of breed (Nordborg and Tavare, 2002), therefore estimating haplotype variations is very informative to appraise the effects of the migrations, selection or admixture in goat populations (Criscione *et al.,* 2019)

The statistical analysis showed that haplotype affected FSH level significantly (Table 4). The TCAATGCGCAACGT haplotype (H9) goats had superior FSH plasma level compare to other haplotypes. The preliminary experiment revealed that TCAATGCGCAACGT haplotype (H9) of KISS1 gene also had high LS (3 ± 0^{b}). Moreover, the rest haplotype denoted a vary LS, not in linier with the FSH plasma level. This condition might be caused by the different goat breeds used to form the haplotype analysis. Nackley *et al.,* (2006) suggested the significance of haplotypes over SNPs for genetic variations analysis. In agreement with this result, another researches using IGF1 bond with IGF1 receptors (IGF1R) and IGF1R haplotypes are correlated with puberty age in Brahman heifers (Fortes *et al.,* 2013); the haplotypes of FSHβ3-c had a superior effect for the semen quality (Nikbin *et al.,* 2018); the casein complex haplotypes correlated with milk quality traits (Inostroza *et al.,* 2020). These phenotypes were related to reproductive traits. To date, there is no published journal concerning the haplotype effect to FSH plasma level. Therefore, our inference should be verified with further study.

Table 5 shows the data of FSH based on goat breeds, sample collection time, litter size, parity and genotype. The discrepancies between breeds are significant, KC and KJ have a higher FSH concentration than SD. KC and KJ goats were collected from Grobogan and Purbalingga regency which represented lowland area (0 – 200 m), further SD goat was collected from Lumajang regency which reflected high land (500 m). In accordance, a breed type has a significant effect to fresh and post-thaw semen traits (Nikbin *et al.,* 2018). Both long term artificial and natural selection enforced by animal husbandry and environmental change resulted different goat breeds in China. The multigenic traits such as prominent cold and disease resistance, strong rough fodder resistance, adaptiveness to stressful environment and high prolificacy reflects distinct natural gene pool (Liu *et al.*, 2019).

Further, the present investigation did not find any correlation between parity and FSH plasma level (table 5). This finding is in accordance in Damascus and Zaribi goats. The TT genotype at T121A in the intron 1 of KISS1 gene had significant higher LS than TA genotype. Moreover, the difference on estradiol$_{17\beta}$ and progesterone level caused by parity is not significant (El-Tarabany *et al.,* 2017).

The data from our previous research found that there are three obtrusive novel single nucleotide polymorphisms (SNPs) correlated with reproductive traits in three Indonesian native goat breeds (unpublished data). The SNPs are g.2425C>G, g.2436A>G and at g.2459G>A. The previous research found a SNP in FSHB gene promoter region within one of the conserved hormone-response elements (HREs) were associated with divergent in serum FSH level in men (Grigorova *et al.,* 2008). Herewith we report for the first time polymorphisms of KISS1 gene in goats indicating a significant correlation with FSH level (Table 5).

The recent research showed that goat breed influences the FSH level significantly, wherein SD goat have lower FSH plasma level. This finding is in accordance with previous research. Another study in human found that higher body mass index (BMI) had lower kisspeptin significantly. Metastin/kisspeptin levels were correlated with all indices of insulin resistance significantly and reversely, thus it can be concluded that a significant decrease in plasma metastin level is correlated with insulin resistance (Panidis *et al.,* 2006; Chen *et al.,* 2010). The LH levels were correlated with plasma metastin levels positively. In fact, average body weight for KC, KJ and SD goats are 24.7 kg, 27.87 kg and 48.50 kg (Batubara *et al.,* 2006; Sodiq and Haryanto, 2007; Ministry of Agriculture, 2014).

The mechanism of major decrease in KISS1 gene expression could lead a compensatory increase in the expression of its receptor (GPR54), causing a circumstance of sensitization to kisspeptin effects. Therefore, an enhancement of LH responses to Kp suggesting that substitution of endogenous KISS1 levels is adequate to activate GnRH-LH axis fully in spite of prejudicial metabolic conditions (Castellano *et al.,* 2005).

Adipose is a necessary endocrine tissue that influence reproduction through leptin primarily (Kawwass *et al.,* 2015; Symonds *et al.,* 2016). Leptin acts through the GPR54 which is found

on kisspeptin neurons in hypothalamus (Tena-Sempere, 2006[a]; Tena-Sempere, 2006[b]). Kisspeptin binds to GnRH neurons and provoke GnRH release (Roseweir and Millar, 2009). The leptin-kisspeptin-GnRH neuron pathway presents the endocrine argument for the critical body weight hypothesis, which body weight relate to puberty in female (Keisler *et al.,* 1999). Thus, earlier result suggest that higher BMI caused lower LH surge, in turned FSH plasma level also decrease, but this finding needed further research.

Nowadays, the effect of Kp on FSH secretion is less information. The response of KISS1 to FSH release emerge less sensitive than LH considerably. The pathway organized centrally through modulation GnRH system, moreover it conducted independently with other neuroendocrine regulators of gonadotropic axis such as excitatory amino acids (EEAs), nitric oxide (NO) and leptin (Navarro, 2005). The positive reaction of leptin in GnRH is mediated by proopiomelanocortins (POMC, precursor of α-MSH), galanin-like peptide (GALP) and kisspeptin neuropeptides (Gottsch *et al.,* 2004; Crown *et al.,* 2007; Quennell *et al.,* 2009). Kp is detected in the growing follicles at theca cells and begins to arise in the basal cells of granular layer in rodent and human (Castellano *et al.,* 2006). FSH is not under control entirely by GnRH (Charlton, 1983; Phillips, 2005), but the major stimulus for FSH is GnRH (Mason *et al.,* 1986).

In our prior study, the obtrusive genotype of KISS1 gene intron 2 that correlated with higher LS, particularly average LS at the first and third parity in Indonesian native goat breeds which are CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA genotype at g.2459G>A (unpublished data). The data above (Table 5) showed that the genotypes at g.2425C>G and g.2436A>G had the same FSH plasma level (P=0.22 and P=0.34 respectively). On the other hand, the AA genotype at g.2459G>A has a superior FSH level than GG genotype (Fig.3).

Preliminary study revealed that CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA genotype at g.2459G>A had significant greater litter size (2.58, 2.58 and 3.0 respectively). Furthermore, neither the CC genotype at g.2425C>G nor the AA genotype at g.2436A>G have significantly different FSH levels. On the other hand, GA genotype at g.2459G>A reveals a higher LS (3.0±0.18) than AA genotype which have a lower LS (2.0±0.21). Thus, it can be concluded that GA genotype at g.2459G>A is the most prominent genotype correlated with reproductive traits in Indonesian native goats.

CONCLUSION

The phylogenetic tree reveals a high closeness between Indonesian and Chinese goat breeds indicates the same function and tightness along the evolutionary timescale. *Capra hircus* and *Ovis aries* were also found in the same clade with Indonesian goat breed represents a significant role of KISS1 gene in reproductive traits in a variety of species.

The FSH level was influenced by breed, LS, and haplotype. The superior haplotype and genotype of KISS1 gene is TCAATGCGCAACGT haplotype and GA genotype at g.2459G>A that correlated with high LS and FSH level. These aspects could be considered in further breeding selection program for economically significant reproductive traits in goats.

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No.	Page	Column	Row	Written	Correction
1.	1	Abstrak	25	litter size	*litter size*
2.	1	Abstrak	26	TCAATGCGCAACGT and	CAATGCGCAACGCT dan
3.	1	Abstrak	30	TCAATGCGCAACGT	CAATGCGCAACGCT
4.	1	Abstract	44	TCAATGCGCAACGT	CAATGCGCAACGCT
5.	1	Abstract	46-47	TCAATGCGCAACGT	CAATGCGCAACGCT
6.	2	1	9	(Aziz, 2010;Guerrero *et al.*, 2019)	(Aziz, 2010; Guerrero *et al.*, 2019)
7.	2	1	19	This situation might represents	This situation might represent
8.	2	2	27	Follicle Stimulating Hormone	Follicle stimulating hormone
9.	3	1	7	multiparous (2nd to	multiparous (1st to
10.	3	1	10	KC in Grobogan	KC from Grobogan
11.	3	1	10-11	KJ in Purbalingga	KJ from Purbalingga
12.	3	1	18	Genomic DNA extraction	**Genomic DNA Extraction**
13.	3	1	39	fragment of intron 2 KISS1	fragment of intron 1 KISS1
14.	3	2	5	*breed Jining Grey*	breed Jining Grey
15.	3	2	6	*GenBank Accession Number*	GenBank Accession Number
16.	3	2	8	Polymerase Chain Reaction (PCR) was perform	Polymerase Chain Reaction (PCR) was performed
17.	3	2	31-33	LS 2 (KJ1a, KJ1b, KJ1c, KJ1a, KJ1b, SD1a, SD1b, SD 1c and SD1d)	LS2 (KC2a, KC2b, KC2c, KJ2a, KJ2b, SD2a, SD2b, SD2c and SD2d)
18.	3	2	34	multiple-sequence	multiple-sequences
19.	4	1	18-19	The stand art curve ranges 0.05 mlU/ml – 15 mlU/ml	The stand art curve ranges from 0.05 mlU/ml to 15 mlU/ml
20.	4	1	30	The neighbour-joining method was	The neighbor-joining method was
21.	4	2	6	stimulating hormone (FSH) Level	stimulating hormone (FSH) level
22.	4	2	21	*n*th haplotypes (n = 1,2,…,12)	*n*th haplotypes (n = 1,2,…,14)
23.	5	1	17	Zheng *et al.*, (2018)	Zheng *et al.* (2018)
24.	5	1	43	the distance between group	the distance between groups are
25.	5	2	1	CCATAGCGGGGCAT	CATAGCGGGGCACT
26.	5	2	4	CCATAGCGGGGCAT	CATAGCGGGGCACT
27.	5	2	10	values resulted in south-east	values resulted in Southeast
28.	5	2	11	south-east	Southeast

29.	5	2	35	Nucleotides substitution	Nucleotide substitution
30.	6	1	16	both species were introgressed with	both species were introgressed by
31.	6	2	27	phylogenetic tree denotes	phylogenetic tree denote
32.	7	1	4	Jining grey goat	Jining grey goats
33.	7	1	7	next clade consist	next clade consists
34.	7	1	13	which acquired in the in this study	which acquired in this study
35.	7	1	21	intravaginal sponge Wildeus (2000)	intravaginal sponge. Wildeus (2000)
36.	7	2	20	fecundity rate different	fecundity rate showed a different
37.	8	1	2	genotype	genotypes
38.	8	1	50	This peptides	These peptides
39.	8	1	52-53	Kp have been rised	Kp has been identified
40.	8	2	34	neuroendocrine gate keeper	neuroendocrine gatekeeper
41.	9	1	5	could formed by	could be formed by
42.	9	1	13	TCAATGCGCAACGT	CAATGCGCAACGCT
43.	9	1	16	TCAATGCGCAACGT	CAATGCGCAACGCT
44.	9	1	22	Nackley *et al.*,	Nackley et al.
45.	9	1	24	another researches	other researches
46.	9	1	44	m	m MSL
47.	9	1	44	SD goat	SD goats
48.	9	1	45	500 m	500 m MSL
49.	9	2	5	in	with
50.	9	2	27	goat	goats
51.	10	1	3	provoke	provokes
52.	10	1	11	needed	needs
53.	10	1	34	intron 2	intron 1
54.	10	1	41	had the same	have unsignificant
55.	10	1	48	(2.58, 2.58 and 3.0 respectively)	(2.58±0.14, 2.58±0.14 and 3.0±0.18 respectively)
56.	10	1	49-54	Furthermore, neither the CC genotype at g.2425C>G nor the AA genotype at g.2436A>G have significantly different FSH levels. On the other hand, GA genotype at g.2459G>A reveals a higher LS (3.0±0.18) than AA genotype which have a lower LS (2.0±0.21)	Furthermore, neither CC genotype at g.2425C>G nor the AA genotype at g.2436A>G have higher FSH levels eventhough not differing significantly. On the other hand, GA genotype at g.2459G>A reveals a higher LS (3.0±0.18) than AA genotype which has a lower LS (2.0±0.21). However both genotypes have the same FSH plasma level.
57.	10	2	9	indicates	indicating
58.	10	2	12	breed represents	breeds represent
59.	10	2	17	TCAATGCGCAACGT	CAATGCGCAACGCT

60.	10	2	40	Animal Genetics	Anim. Genet.
61.	10	2	54	J Anim Breed Genet.	J. Anim. Breed Genet.
62.	11	1	2	Batubara, A, M. Doloksaribu dan B. Tiesnamur-ti. 2006. Potensi keragaman sumberdaya genetik kambing lokal Indonesia. Lokakarya Nasional Pengelolaan dan Perlindungan Sumber Daya Genetik di Indonesia : Manfaat Ekonomi untuk Mewujudkan Ketahanan Nasional. Bogor, 20 Desember 2006. Badan Libang Pertanian. 206-214.	Batubara, A, M. Doloksaribu and B. Tiesnamurti. 2006. Potential diversity of Indonesian local goat genetic resources. National Workshop on Management and Protection of Genetic Resources in Indonesia: Economic Benefits for Realizing National Resilience. Bogor, 20th December 2006. Indonesian Agency for Agricultural Research and Development. 206-214.
63.	11	1	11	Bitaraf, A., M.J. Zamiri, M. Kafi and J. Izadifard, 2007.Efficacy of CIDR	Bitaraf, A., M.J. Zamiri, M. Kafi and J. Izadifard, 2007. Efficacy of CIDR
64.	11	1	16	Cao GL, Chu MX, Fang L, Di R, Feng T and Li N	Cao, G.L., M.X. Chu, L. Fan, R. Di, T. Feng and N. Li
65.	11	1	22-23	Reproductive Biology and Endocrinology.	J. Reprod. Biol. and Endocrinol.
66.	11	2	2	M. N. A. Naufal	and M. N. A. Naufal
67.	11	2	14-18	El-Tarabany MS, Zagloola AW, El-Tarabany AA, Awad A. 2017. Association analysis of polymorphism in KiSS1 gene with reproductive traits in goats. Anim Reprod Sci.180:92–99.	El-Tarabany, M.S., A. W. Zagloola, A. A. El-Tarabany and A. Awad. 2017. Association analysis of polymorphism in KiSS1 gene with reproductive traits in goats. Anim. Reprod. Sci.180:92–99.
68.	11	2	43-46	Inostroza, M.G.P., F.J.N. González, V. Landi, J.M.L. Jurado, J.V.D.B, J.F. Álvarez and M. A.M. Martínez. 2020. Bayesian analysis of the association between casein complex haplotype variants and milk yield, composi-tion, and curve shape parameters in Mur-ciano-Granadina goats. Animals. 10:1845-63.	Inostroza, M.G.P., F.J.N. González, V. Landi, J.M.L. Jurado, J.V.D. Bermejo, J.F. Álvarez and M. A. M. Martínez. 2020. Bayesian analysis of the association between casein complex haplotype variants and milk yield, composition, and curve shape parameters in Murciano-Granadina goats. Animals. 10:1845-63.
69.	11	2	50	Mol Human Reprod.	Mol. Human. Reprod.
70.	12	1	10	Kor N.M.,	Kor, N.M.,

71.	12	1	12	aieni	Raieni
72.	12	1	25	Asian Australas J Anim Sci.	Asian Australas. J. Anim. Sci.
73.	12	1	40-41	Animal Biotechnology	Anim. Biotechnol.
74.	12	1	54	Ministry of Agricuture	Ministry of Agriculture
75.	12	2	4	Ministry of Agriculture	Ministry of Agriculture of Indonesia. 2020
76.	12	2	7	2020	Please delete this
77.	12	2	24	Navarro V.M., J,M, Castellano, R. Fernandez	Navarro, V.M., J.M. Castellano, R. Fernandez
78.	12	2	36	Journal of Applied Animal Research	J. Appl. Anim. Res.
79.	12	2	50	Domestic Animal Endocrinology	Domest. Anim. Endocrinol.
80.	12	2	51-52	Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere, M.	Pinilla, L., E. Aguilar, C. Dieguez, R. P. Millar and Tena-Sempere.
81.	12	2	54	Physiol Rev.	Physiol. Rev.
82.	13	1	15	Hassanin, A.	A. Hassanin.
83.	13	2	10	dan	and
84.	13	2	13	J.Anim.Prod.	J. Anim. Prod.
85.	13	2	18	Genet Sel Evol.	Genet. Sel. Evol.
86.	13	2	21	Please add the references below as written in previous manuscript	Tena-Sempere, M. 2006a. The roles of kisspeptin and G protein-coupled receptor-54 in pubertal development. Curr. Opin. Pediat. 18:442-447. Tena-Sempere, M. 2006b. KiSS-1 and reproduction: Focus on its role in the metabolic regulation of fertility. Neuroendocrinology. 83:275-281. Wang, X., J. Liu, G. Zhou, J. Guo, H. Yan, Y. Niu, Y. Li, C. Yuan, R. Geng, X. Lan, X. An, X. Tian, H. Zhou, J. Song, Y. Jiang and Y. Chen. 2016. Whole-genome sequencing of eight goat populations for the detection of selection signatures underlying production and adaptive traits. Nature. 6:38932. Wildeus, S. 2000. Current concepts in synchronization

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87. Table 4 in page 7 (all of the nucleotide base in column 2 have to revised)

Haplotype Variations		FSH
H9	CAATGCGCAACGCT	10.65 ± 1.27^{a}
H4	TATTGCACAACGCT	8.99 ± 0.54^{b}
H2	CATAGCGCAACGCT	4.77 ± 0.49^{c}
H8	TATAGCGGGGCGCT	2.72 ± 0.14^{d}
H10	CATTGCGCAGTGCT	1.97 ± 0.08^{de}
H1	CATAGCGGGGCACT	1.76 ± 0.14^{de}
H6	CATTGCACAACGCT	1.54 ± 0.06^{de}
H3	TCTTGCGGGGTACT	1.49 ± 0.08^{de}
H7	TAATGCGCAACGTT	1.48 ± 0.12^{de}
H13	CATTCTGCAATGCA	1.30 ± 0.19^{e}
H14	CCTTCTGCAGTGCT	1.21 ± 0.09^{f}
H11	CATTGCACAGTGCT	0.67 ± 0.05^{g}
H12	CAATCCGCAATGCT	0.66 ± 0.05^{h}

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Phylogenetic study and association between prominent genotype and haplotype of KISS1 gene with FSH level in Idonesian native goat breeds

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ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi struktur populasi dan ekspresi gen KISS1 yang berkaitan dengan sifat reproduksi menggunakan analisis level Follicle Stimulating Hormone (FSH) dan sekuensing DNA gen KISS1. Sejumlah 23 ekor induk yang terdiri dari kambing Kacang (n=7), Kejobong (n=8) and Senduro (n=8) diidentifikasi genotipenya menggunakan metode sekuensing DNA, 16 ekor diantaranya diberi perlakuan sinkronisasi estrus untuk diamati level hormon FSH menggunakan metode ELISA. *Software* MEGA X digunakan untuk menganalisa sekuens DNA, sedangkan General Linier Model (GLM) dari SAS *software* untuk menganalisa hormon FSH. Pohon filogeni memperlihatkan kesamaan yang tinggi antara kambing lokal Indonesia dengan spesies lain yang menunjukkan bahwa gen KISS1 konservatif. Analisis hormon FSH menunjukan hasil yang berbeda secara signifikan antara kambing Kacang dan Kejobong dibandingkan Senduro (P = 0.002), litter size (LS) 3 dibandingkan LS 1 (P = 0.0175), selanjutnya haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A menunjukkan hormon FSH yang lebih tinggi dibandingkan haplotipe dan genotipe yang lain (P = 0.0027; P<0.0001) dan terkait dengan LS yang tinggi (3.0±0.18). Waktu pengambilan sampel dan paritas tidak memberikan perbedaan yang signifikan terhadap hormon FSH. Penelitian ini menunjukkan bahwa haplotipe TCAATGCGCAACGT and genotipe GA pada g.2459 G>A mempunyai asosiasi dengan sifat reproduksi.

Kata kunci : FSH, gen KISS1, haplotipe, kambing lokal Indonesia, pohon filogeni

ABSTRACT

The aim of the current research was to analyze the population structure and expression of KISS1 gene associated with reproductive traits through Follicle Stimulating Hormone (FSH) level and DNA sequencing analysis based on KISS1 gene. Genotypes of 23 goat does consist of Kacang goats (n=7), Kejobong goats (n=8) and Senduro goats (n=8) were investigated using DNA sequencing, 16 out of 23 samples were synchronized to examine their FSH level using ELISA method. The data were analyzed using MEGA X software for DNA sequences and General Linier Model (GLM) for FSH plasma level. The phylogenetic tree showed the high homology between Indonesian native goats with other species showing a gene conservatism. A significantly higher FSH plasma levels were obtained from Kacang and Kejobong than Senduro goat (P = 0.002), litter size (LS) 3 than LS 1 (P = 0.0175), further TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A have a higher FSH plasma than other haplotypes and genotypes (P = 0.0027; P<0.0001) and are associated with high LS (3.0±0.18). Neither sample collection times nor parities have different significantly. The current trial indicated that TCAATGCGCAACGT haplotype and GA genotype at g.2459 G>A were correlated with reproductive traits.

Keywords : FSH, haplotype, Indonesian native goat, KISS1 gene, phylogenetic tree

INTRODUCTION

Goats, unlike other livestock species, are adaptable animals that can survive in tropical, mountainous, and desert environments. Goats have spread widely due to their adaptability to a variety of environments and nutrition availability, small size, prolific, useful productivity for humans, and non-competitiveness with human food, and they contribute significantly, particularly in rural areas (Aziz, 2010;Guerrero *et al.*, 2019).

In Indonesia, there are more than 19 million goats, with eight goat breeds officially confessed. In Indonesia, goat population has increased over the last five years (Ministry of Agriculture, 2020). This condition could indicate that goats could be an alternative source of red meat. According to Aziz (2010), Indonesia is one of the 10 largest lamb production country in the world. This situation might represents the Indonesian preference on goat meat because most of goats were reared and consumed locally. Enhancing reproductive traits could be a way to increase the number of goat population.

Indigenous goat breeds are well adapted to agro-ecological conditions, helping to ensure goat farming's long-term viability in rural and harsh environments (Stella, 2018). Goats are traditionally bred in Indonesia for meat and dual -purpose production. In this study, three indigenous goat breeds were used. Kacang (KC), Kejobong (KJ), and Senduro (SD) goat breeds were known for their reproductive traits. KC has a significant high litter size even when reared in a harsh environment and can be raised as a meat type; KJ, the does is prolific, has a short kidding interval, and can be raised as a meat type (Sodiq and Haryanto, 2007), while the litter size (LS) in SD is 1.83 ± 0.69 and perform as dual purpose (meat and dairy) type (Ciptadi *et al.*, 2019). KJ is solely located in Central Java, whereas SD goats start to spread massively in Indonesia following KC. KC is also known as Indonesian native goats (Batubara *et al.,* 2006). Half of Indonesian goat population is existed in Java, therefore a study based on goat population in Java was expected to represent the entire goat population in Indonesia, particularly in term of specific reproductive traits.

So far, the genetic structure of important economic traits has been identified, but the number of causative genes in goats has been lower than in sheep and cattle (Amills *et al.,* 2017). The phenotypic variations of goats were shaped by various artificial or natural factors such as migration of human, environmental changes and influences of socioeconomic. Further, the genomic variability of goats were constructed mostly by breeding orientation and artificial selection during domestication (Wang *et al.,* 2016). Principally, the sustainable selection and advancement of a novel traits in an environmental shifting needs the genetic diversity (Mandal *et al.,* 2020).

Reproduction is a critical function for the survival of the species, thus this function is governed by complex regulatory signals. The hypothalamic-pituitary-gonadal (HPG) axis regulates reproductive activity by modulating the secretion of inhibitory factors and pituitary gonadotropins by the hypothalamus. Gonadotropin activity in the gonad is mediated by peripheral blood circulation (Nagamalleswari *et al.*, 2004). The HPG axis is divided into three regulatory signals: 1) hypothalamus: gonadotropin-releasing hormone (GnRH); 2) pituitary: gonadotropin, luteinizing hormone (LH) and follicle stimulating hormone (FSH); and 3) gonads: sex steroid and peptides (Pinilla *et al.,* 2012).

Follicle Stimulating Hormone (FSH) is a pituitary gonadotropin which have essential task in reproduction. The main roles of FSH in female are maturation and development in antral follicles, encourage the antrum formation in secondary follicles and organize a response for ovulation when the LH surge (Mahdavi and Dashab, 2017).

The present study was undertaken to analyze the population structure and to explore the relative expression of KISS1 gene associated with reproductive traits through FSH level and DNA sequencing analysis from different goat breeds, litter size, haplotypes and genotypes to describe its relationship with litter size at kidding based on KISS1 gene sequences of three Indonesian indigenous goat breeds compare to other species sequences. Therefore, as KISS1 gene plays an important role on reproduction, this study was carried out.

MATERIALS AND METHODS

Ethical Clearance

The protocol of the current research was under the standart rule of animal treatment as designated in the Republic of Indonesia's law, that is, number 41, 2014.

Animals and Samples Collection

A total of 23 heads of goat does from three Indonesian indigenous goat breeds, namely KC (n=7), KJ (n=8) and SD (n=8) were utilized in the present study. The goats were healthy, unrelated and were not pregnant. They were selected randomly based on LS, age, multiparous (2nd to 5th parities) and have phenotypic characteristic of each breed. These breeds represent different regions and altitudes, KC in Grobogan regency, KJ in Purbalingga regency, both are in Central Java while SD is from Lumajang regency East Java (Fig.1). The Grobogan, Purbalingga and Lumajang were located at 100 m, 113 m and 500 m height above mean sea level (AMSL) respectively. The goats were kept by the farmer under the homogenous environment.

Genomic DNA extraction

A total of 3 ml of blood samples were collected via the jugular vein in to sterile vacuum tubes with anticoagulant (EDTA). The blood samples were shifted to the laboratory using coolbox and freezed at -20°C until the genomic DNA extraction. Thus, GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) was applied to extract the genomic DNA from the whole blood correspond the manufacturer's guidance. The evaluation of DNA quality was set up by electrophoresis using agarose gel (1%) and Nanodrop spectrophotometer Uvidoc HD6 (UVItec Ltd., *Cambridge, UK)*.

A clear single band on agarose (1%) electrophoresis and the optical density (OD) 260/280 ratio ranged between 1.7 and 2.0 (according to the kit protocol) verifying a good quality of DNA extraction.

PCR Amplification

A 1061 bp fragment of intron 2 KISS1 gene was amplified with a pair of primer (F: 5'-GCTCAGTGGCCAGGAATCTG-3'; R: 5'-GCTCATAGCAGGGCCTCAAA-3'). The primers were designed using the sequence of KISS1 gene of *Capra hircus breed Jining Grey (GenBank Accession Number* GU142847.1) and Primer3 Plus software. Polymerase Chain Reaction (PCR) was perform in 50 µl volume containing 4 µl DNA extraction (20-30 ng/ µl), 1 µl for each primer (10 pmol/ µl), 19 µl ddH2O and 25 µl of MyTaq Red Mix Bioline (1st BASE) on MJ Mini Personal Thermal Cycler (Bio-Rad, USA). PCR cycling program contain of predenaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72 °C for 30 s, and post extension at 72 °C for 7 min. The amplicon was performed by electrophoresis in 2 % agarose gel (Invitrogen, Life Technologies Co, CA) at 100V for 30 min.

DNA Sequencing and Analysis

The amplicon of 23 goats reflecting three indigenous Indonesian goat breeds were sequenced both forward and reverse direction using commercial service (1st BASE). The goats were selected based on breeds, litter size, parity, age and goats which treated with estrus synchronization. The goat sequences were categorized into five groups, which are LS 1(KC1a, KC1b, KJ1a, KJ1b, SD1a and SD1b), LS 2 (KJ1a, KJ1b, KJ1c, KJ1a, KJ1b, SD1a, SD1b, SD 1c and SD1d), LS 3 (KC3a, KC3b, KJ3a, KJ3b, KJ3c and SD3a), LS 4 (KJ4a) and LS 5 (SD5a). Alignment of multiple-sequence were performed by software MEGA X 10.1 version to explore the variation of single nucleotide polymorphisms (SNPs).

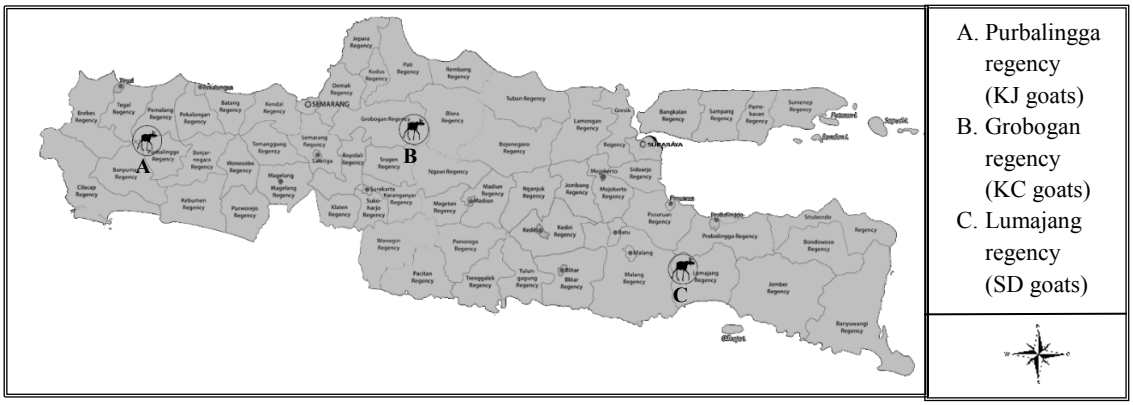

Figure 1. Distribution of sampling area in Java island, Indonesia

Estrus Synchronization, Blood Samples and Hormonal Assay (ELISA)

Five goat does for each KC and KJ and six SD goat does with different LS were treated with 0.30 mg medroxyprogesterone acetate (MAP) intravaginal sponges for 14 days. The blood samples were collected five times (0, 3, 6, 9 and 12 hours) after the sponge removal. A total 3 ml of blood samples were collected in plain and sterile vacutainer tubes. Then, the blood samples were centrifuged (3000 rpm/5 min) to obtain serum and stored at -20°C in eppendorf tubes until assayed for FSH profile. FSH hormone levels was estimated using Goat Follicle Stimulating Hormone Kit (Bioassay Technology Laboratory Cat. No. E0006Go Shanghai, China) and counting using microplate reader (ZENIX-320, USA). The stand art curve ranges 0.05 mlU/ml – 15 mlU/ml and the sensitivity is 0.028 mlU/ml. The intra-assay coefficient of variance (CV) and the inter-assay CV less than 8% and 10% respectively. The ELISA was performed as per kit guidance.

Statistical Analysis

Population Structure. The data were analyzed using MEGA X software to acquire the singleton variable, parsimony sites, genetic distance within and between goat breed and to form phylogenetic tree. The neighbour-joining method was used to build the phylogenetic tree. Different sequences of KISS1 gene from other vary species were acquired from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) for phylogenetic analysis. The distance between sequence pairs were represented by the length of each pair of branches. The scale under the tree is indicating the nucleotide substitution number. The DnaSP software were used to calculate haplotype diversity, number of haplotypes, number of mutations, Fst and Tajima's D. The Arlequin software was utilized to obtain haplotype shares and haplotype frequencies.

Basic local alignment search tool (BLAST) was used to detect the homology sequences in diverse breeds or species. Six different KISS1 gene sequences from different species/breed have been selected from the GenBank with accession number listed below (Tabel 1).

Follicle stimulating hormone (FSH) Level. The data were analyzed using General Linier Model (GLM) of SAS Software. Fixed model used for FSH :

$$y_{ijklmn} = \mu + g_i + b_j + c_k + l_l + p_m + h_n + e_{ijklmn}$$

where y_{ijklmn} is FSH plasma level measured for each samples, μ is the overall mean, g_i is the fixed effect of *i*th genotype (i = 1,2,3), b_j is the fixed effect of *j*th breed (j = 1,2,3), c_k is the fixed effect of *k*th collection time (k = 1,2,3,4,5), l_l is the fixed of *l*th litter size (l = 1,2,3,4,5), p_m is the fixed of *m*th parities (m = 1,2,3), h_n is the fixed of *n*th haplotypes (n = 1,2,…,12) and e_{ij} is a random error of each observation. When P <0.05 it was verify significant statistically. In this study, multiple comparisons of the means were tested using Tukey-Kramer with significant level of 5%.

RESULTS AND DISCUSSION

Nucleotide Sequence Identity and Phylogenetic Tree of KISS1 Gene

The OD ratio 260/280 ranged from 1.7 to 2.0, indicating that extracted DNA was not contaminated and in good quality. Psifidi *et al.* (2015) confirming that the standart of OD ratio 260/280 is ≥ 1.8, depend on the extraction kit used. A higher ratio number showed higher purity of DNA extract. Therefore, the extracted DNA could be sequenced both forward and reverse directions immediately in this study.

BLAST from NCBI were used to find the degree of similarity between chosen sequences (http:// https://blast.ncbi.nlm.nih.gov/Blast.cgi). Three species/breed that have the highest similar-

Table 1. KISS1 gene sequences of different species from the GenBank used to develope the phylogenetic tree

Species	Accession number	Similarity (%)
Jining Grey	GU. 142847.1	99.69
Ovis aries	KP835797.1	99.47
Capra hircus	KR065750.1	97.66
Bos indicus	XM_019976949.1	87.91
Sus scrofa	AB466320.1	81.14
Homo sapiens	NG_032151.1	67.38

ity are Jining grey goats from China (GU. 142847.1), *Ovis aries* (KP835797.1) and *Capra hircus* (KR065750.1) for 99.69%, 99.47% and 97.66% respectively (Table 1). The homologous sequences from other species/breed were obtained from NCBI GenBank database. The closely related sequences could be indicated from the similarity at nucleotide level. The DNA sequences similarity interprets that the function and structure of regulatory elements or protein products of gene expression is similar (Mahdavi and Dashab, 2017) and high conservatism gene in species (Zheng *et al.,* 2018).

Homology of KISS1 gene with other species ranged between *Homo sapiens* (NG_032151.1) with 78.74% similarity to *Capra hircus* Jining Grey breed (99.69%). Zheng *et al.,* (2018) found the similar result in previous research on Jintang Black goat (JTG). The similarity between KC, KJ, SD and JTG is 99.02%. This output denoted that KISS1 gene is conserve in many species because of its significant role in reproduction.

The sequences analysis could be performed by aligning the gene sequences with specific role to determine the evolutionary correlation between unrecognized sequences and approved sequences (GenBank) to construct a phylogenetic tree, branching and discrepancy between species (Mahdavi and Dashab, 2017). The analysis of nucleotide sequences using MEGA X software between the indigenous goats represented 18 variable sites which include five singleton sites, 13 parsimony sites and 14 haplotypes.

Diversity in entire population is 1.18. Meanwhile, the mean distance is 1.39 that calculated from all DNA sequences which show the average of entire sequence pairs and the amount of base changes at each site. The distance within group is calculated by the average number of base changes between all sequences within the group. The disparity was enumerated to be 0.0046, 0.0047 and 0.0051 in KC, KJ and SD respectively, while the distance between group are shown in Table 2. The previous experiment found that the genetic distance based on BMP15 gene was counted to be 0.4 in cows, 1.0 in pig, 4.1 in sheep and 2.1 in goats (Mahdavi and Dashab, 2017). This condition indicated that nucleotide substitutions in goat species of BMP15 gene higher than KISS1 gene caused by evolution correlated with gene expression mechanisms, thus this condition showed that KISS1 gene more conserve than BMP15 gene.

The common haplotype in three Indonesian goat breeds is CCATAGCGGGGCAT (H1) and the haplotype frequencies are 0.143, 0.5 and 0.125 for KC, KJ and SD respectively. In addition, overall haplotype CCATAGCGGGGCAT (H1) frequency in the entire population is 26.1% and the haplotype diversity is 0.913.

The average pairwise fixation index (Fst) value is 0.021 (KC and KJ) and 0.082 (KC and SD). This value is lower than previous values resulted in south-east Asia. Barker *et al.* (2001) reported that Fst value between south-east Asian goat is 0.14. In contrary, the Fst value between KJ and SD is 0.195. This data showed that genetic structure differentiation between KJ and SD is higher than KC to KJ and SD, thus indicated that KC is an ancestor for KJ and SD. In accordance, Lestari *et al.* (2018) reported that KJ is a crossbred of KC goat and Etawah Grade (EG). Further research needed to investigate the phylogenetic relationship between KC and SD.

Table 2. The mean genetic distance between Indonesian goat breeds using the number of base pair in KISS1 gene

Goat	KC	KJ	SD
KC		0.021	0.082
KJ	0.0047		0.195
SD	0.0053	0.0061	

Values above the diagonal are Fst and genetic distance value are under diagonal; KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

The nucleotide frequency of thymine/uracil, cytosine, adenine and guanine is 23.3%, 27.7%, 23.5% and 25.5% respectively. Nucleotides substitution rate between species/groups were estimated using Tamura-Nei model in MEGA X software (Table 3). These results denote the opportunity for replacement of each nucleotide with another one. The distance was estimated using the amount of bases and pair comparison method. The distance between *Homo sapiens* and *Ovis aries* were the maximum (6.393), while the closest distance was between Indonesian goats and Jining grey goats. This data could be confirmed with the phylogenetic tree, where *Homo sapiens* and *Ovis aries* found in different branch. Furthermore, Indonesian native goats and Jining grey goat were located in the same node.

Adaptation is in reaction to selection of production methods and connected with local envi-

Table 3. The mean distance between species using the number of base pair in KISS1 gene

Species	Jining grey	KC	KJ	SD	*Capra hircus*	*Ovis aries*	*Bos indicus*	*Homo sapiens*	*Sus scrofa*
Jining grey									
KC	0.003								
KJ	0.004	0.005							
SD	0.003	0.005	0.006						
Capra hircus	2.261	2.294	2.282	2.293					
Ovis aries	4.325	4.312	4.285	4.296	2.701				
Homo sapiens	3.427	3.342	3.404	3.387	3.049	6.393			
Bos indicus	3.850	3.843	3.896	3.819	2.781	4.259	2.840		
Sus scrofa	4.411	4.437	4.467	4.455	3.950	4.606	3.106	4.529	

KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

ronmental situation. The alpine (*Capra ibex*) ibex and Spanish (*Capra pyrenaica*) have shown that both species were introgressed with domestic goat based on major histocompatibility complex (MHC) genes (Stella *et al.*, 2018). Phylogenetic tree of domestic and wild goat species based on Y-chromosome, nuclear marker or mitochondrial are discrepant (Amills *et al.,* 2017). The incongruous between nucleus and mtDNA phylogenies were caused by interspecific hybridization, rather than lineage sorting or paralogs (Ropiquet and Hassanin, 2006).

BLAST was used to identify similarity between DNA sequences. Other homolog species were used to align the nucleotide sequences of KISS1 gene to illustrate the phylogenetic tree. The nucleotide sequence of Indonesian goat breeds were identical with Jining grey goats, *Ovis aries* and *Bos indicus* for 99.69%, 99.20% and 88.98% respectively (Fig. 2). This data also confirmed the Fst value in this study (Table 2). The similarity between goats, sheeps and cattle which are ruminants, shows that KISS1 gene may have equivalent function in ruminants.

The phylogenetic tree shows two main clades of the phylogenetic relationship of all sequences. The last nodes of the phylogenetic tree denotes the current sequences of samples used,

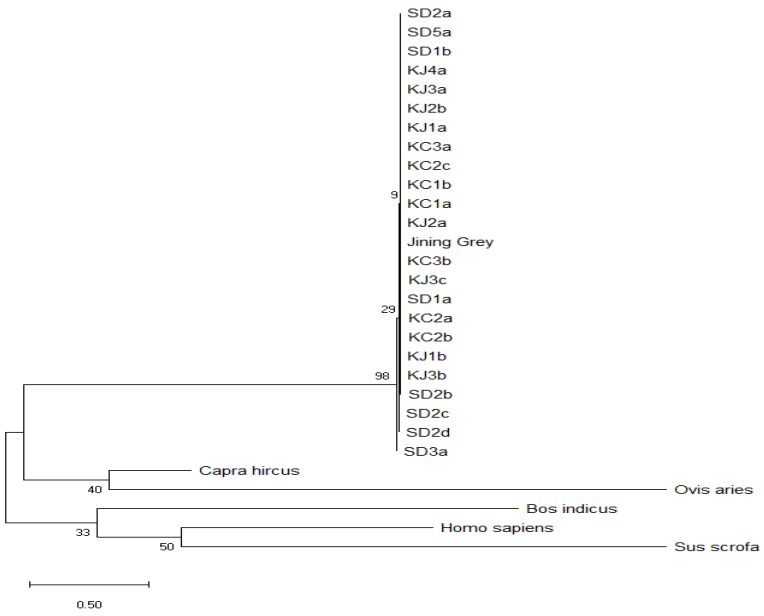

Figure 2. Phylogenetic tree of KISS1 gene of different species

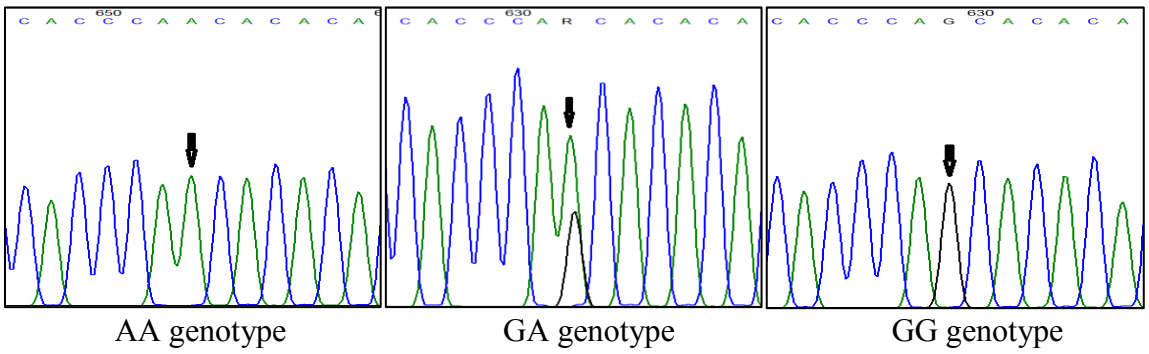

Figure 3. Genotypes of g.2459G>A

while the internal nodes pointed as suspect ancestor sequences. The nearest genetic relationship is between Indonesian native goats and Jining grey goat because it located in the same node. The other branch in the same clade with Indonesian goat breeds are *Capra hircus* and *Ovis aries*. Hereinafter the next clade consist of *Bos indicus*, *Homo sapiens* and *Sus scrofa*. The phylogenetic tree denoted a similarity and distance between species based on KISS1 gene. The phylogenetic tree from prior research (Zheng *et al.,* 2018) showed similar clustering among various species which acquired in the in this study even the accession numbers of NCBI used are different.

KISS1 Gene Expression and FSH Plasma Level

An estrus synchronization was used in the current research using progestagen intravaginal sponge Wildeus (2000) reported that the previous research in different breeds such as Cashmere, Alpine, Boer, Saanen and Nubian revealed that the effectiveness of estrus synchronization using intravaginal sponges might represent significant differences led by distinct species, breeds, treatment management and mating system. The utilization of pregnant mare serum gonadotropin (PMSG) and progestagen sponge for estrus synchronization has resulted a satisfactory outcome. Internal appliances conceiving different kind of progestagen, implanted in female reproduction tract during 12-14 days were used widely (Bitaraf *et al.,* 2007).

The intravaginal sponges were implanted for 14 days in the present research. The long term progestagen intravaginal treatment (12-14 days) gave better result than short term (5-7 days) but not differ significantly, whether on estrus intensity, estrus response, onset of estrus, concentration of progesterone serum at 21 days after artificial insemination (AI), length of estrus, gestation period, kidding and fecundity rate different significantly (Ngangi *et al.*, 2002; Kor *et al.,* 2011). On the other hand, intravaginal progestagen sponge used in estrus synchronization on ewes could

Table 4. Means ± SE of FSH (mIU/ml) on haplotype (P<0.0001)

Haplotype Variations		FSH
H9	TCAATGCGCAACGT	10.65 ± 1.27^{a}
H4	TTATTGCACAACGT	8.99 ± 0.54^{b}
H2	CCATAGCGCAACGT	4.77 ± 0.49^{c}
H8	TCATAGCGGGGCGT	2.72 ± 0.14^{d}
H10	TTATTGCGCAGTGT	1.97 ± 0.08^{de}
H1	CCATAGCGGGGCAT	1.76 ± 0.14^{de}
H6	TTATTGCACAACGT	1.54 ± 0.06^{de}
H3	TCCTTGCGGGGTAT	1.49 ± 0.08^{de}
H7	TCAATGCGCAACGT	1.48 ± 0.12^{de}
H13	TTATTCTGCAATGA	1.30 ± 0.19^{e}
H14	TTATTCTGCAATGA	1.21 ± 0.09^{f}
H11	TTATTGCACAGTGT	0.67 ± 0.05^{g}
H12	TTAATCCGCAATGT	0.66 ± 0.05^{h}

Table 5. Means ± SE of FSH (mIU/ml) based on goat breeds, sample collection time, litter size, parity and genotype

Specification	P Value	Category	Means ± SE
Breed	P = 0.002	KC	3.88 ± 0.63[a]
		KJ	3,73 ± 0.75[a]
		SD	1.49 ± 0.19[b]
Sample collection time	P = 0.9361	0 hours	2.48 ± 0.59
		3 hours	2.88 ± 0.74
		6 hours	2.89 ± 0.73
		9 hours	2.97 ± 0.74
		12 hours	3.45 ± 0.99
Litter size	P = 0.0175	1 kid	1.28 ± 0.15[b]
		2 kids	2.61 ± 0.47[ab]
		3 kids	4.21 ± 0.78[a]
		5 kids	3.77 ± 0.32[a]
Parity	P = 0.0352	1st parity	3.77 ± 0.32
		2nd parity	2.27 ± 0.34
		3rd parity	4.10 ± 0.79
SNP g.2425 C>G	P = 0.2226	CC	3.27 ± 0.44
		CG	2.10 ± 0.21
		GG	1.76 ± 0.13
SNP g.2436 A>G	P = 0.3447	AA	3.22 ± 0.48
		AG	2.66 ± 0.27
		GG	1.76 ± 0.14
SNP g.2459 G>A	P = 0.0027	AA	4.01 ± 0.96[a]
		GA	3.89 ± 0.68[ab]
		GG	1.65 ± 0.11[b]

Values with different superscripts in the same column differ significantly at P<0.05
KC : Kacang goats; KJ : Kejobong goats; SD : Senduro goats

improve ovulation time and estrus expression, contrary it might shorten duration of estrus (Mahmoud and Senosy, 2019).

The basal concentration of progesterone hormone is reached six hours after the sponge taken out from female reproduction tract (Ngangi *et al.,* 2002). The first three observations (0, 3 and 6 hours) were in luteal phase while other observations (9 and 12 hours) were in the earlier follicular phase. This might explain that the FSH plasma level increase slightly during the collection time (Table 5). In sheep, KISS1 gene expression in the sheep preoptic area (POA) is greater just previous to the late follicular phase GnRH/LH surge than luteal phase (Smith *et al.,* 2013). For future research, longer observation time is needed to evaluate the significant result of FSH plasma level.

KISS1 gene produces kisspeptin (Kp). This peptides were performed through their receptor, G-protein-coupled receptor (GPR54). Kp have been rised as important regulators of neurons that remain in the basal forebrain and yield gonadotropin releasing hormone (GnRH). Cells in the upstream of GnRH neurons synthesized KISS1 functionally (Knoll *et al.*, 2013). KISS1 gene stimulates GnRH neuron activity, gene expression and the release was regulated by circulating gonadal hormones (Smith, 2013). Kp has been known as key neuroendocrine gate keeper of reproduction and maintenance of adult reproduction recently (Millar *et al.,* 2010). Sequences of KISS1 gene have revealed a polymorphism related to reproductive traits. KISS1 gene might be a significant candidate gene on reproductive traits in goats (Cao *et al.,* 2010; An *et al.,* 2013; El-Tarabany *et al.,* 2017; Sahoo *et al.,* 2019).

Kp arranges the construction of preantal follicles negatively by leading the production of anti mullerian hormone (AMH) and degrade the sensitivity to FSH through preventing the induction of FSHR expression via sympathetic activators, thus lowering the recruitment of primary follicles (Panidis *et al.,* 2006; Cao *et al.,* 2019). The sympathetic nerve activity might adjust the intra ovarian Kp system and the peptides were needed for appropriate coordination between ovarian function both from neural or ovarian origin (Zheng *et al.,* 2018). Furthermore, the serum levels of Kp are in contrary correlation with FSH, but have a positive correlation with testosterone, LH and dehydroepiandrosterone (DHEA)

(Gorkem *et al.,* 2018).

As mentioned before, fourteen haplotypes were obtained in current research. The gene flow, bottleneck and drift events, the distribution of nucleotide polymorphisms could formed by demographic history of breed (Nordborg and Tavare, 2002), therefore estimating haplotype variations is very informative to appraise the effects of the migrations, selection or admixture in goat populations (Criscione *et al.,* 2019)

The statistical analysis showed that haplotype affected FSH level significantly (Table 4). The TCAATGCGCAACGT haplotype (H9) goats had superior FSH plasma level compare to other haplotypes. The preliminary experiment revealed that TCAATGCGCAACGT haplotype (H9) of KISS1 gene also had high LS (3 ± 0^{b}). Moreover, the rest haplotype denoted a vary LS, not in linier with the FSH plasma level. This condition might be caused by the different goat breeds used to form the haplotype analysis. Nackley *et al.,* (2006) suggested the significance of haplotypes over SNPs for genetic variations analysis. In agreement with this result, another researches using IGF1 bond with IGF1 receptors (IGF1R) and IGF1R haplotypes are correlated with puberty age in Brahman heifers (Fortes *et al.,* 2013); the haplotypes of FSHβ3-c had a superior effect for the semen quality (Nikbin *et al.,* 2018); the casein complex haplotypes correlated with milk quality traits (Inostroza *et al.,* 2020). These phenotypes were related to reproductive traits. To date, there is no published journal concerning the haplotype effect to FSH plasma level. Therefore, our inference should be verified with further study.

Table 5 shows the data of FSH based on goat breeds, sample collection time, litter size, parity and genotype. The discrepancies between breeds are significant, KC and KJ have a higher FSH concentration than SD. KC and KJ goats were collected from Grobogan and Purbalingga regency which represented lowland area (0 – 200 m), further SD goat was collected from Lumajang regency which reflected high land (500 m). In accordance, a breed type has a significant effect to fresh and post-thaw semen traits (Nikbin *et al.,* 2018). Both long term artificial and natural selection enforced by animal husbandry and environmental change resulted different goat breeds in China. The multigenic traits such as prominent cold and disease resistance, strong rough fodder resistance, adaptiveness to stressful environment and high prolificacy reflects distinct natural gene pool (Liu *et al.*, 2019).

Further, the present investigation did not find any correlation between parity and FSH plasma level (table 5). This finding is in accordance in Damascus and Zaribi goats. The TT genotype at T121A in the intron 1 of KISS1 gene had significant higher LS than TA genotype. Moreover, the difference on estradiol$_{17\beta}$ and progesterone level caused by parity is not significant (El-Tarabany *et al.,* 2017).

The data from our previous research found that there are three obtrusive novel single nucleotide polymorphisms (SNPs) correlated with reproductive traits in three Indonesian native goat breeds (unpublished data). The SNPs are g.2425C>G, g.2436A>G and at g.2459G>A. The previous research found a SNP in FSHB gene promoter region within one of the conserved hormone-response elements (HREs) were associated with divergent in serum FSH level in men (Grigorova *et al.,* 2008). Herewith we report for the first time polymorphisms of KISS1 gene in goats indicating a significant correlation with FSH level (Table 5).

The recent research showed that goat breed influences the FSH level significantly, wherein SD goat have lower FSH plasma level. This finding is in accordance with previous research. Another study in human found that higher body mass index (BMI) had lower kisspeptin significantly. Metastin/kisspeptin levels were correlated with all indices of insulin resistance significantly and reversely, thus it can be concluded that a significant decrease in plasma metastin level is correlated with insulin resistance (Panidis *et al.,* 2006; Chen *et al.,* 2010). The LH levels were correlated with plasma metastin levels positively. In fact, average body weight for KC, KJ and SD goats are 24.7 kg, 27.87 kg and 48.50 kg (Batubara *et al.,* 2006; Sodiq and Haryanto, 2007; Ministry of Agriculture, 2014).

The mechanism of major decrease in KISS1 gene expression could lead a compensatory increase in the expression of its receptor (GPR54), causing a circumstance of sensitization to kisspeptin effects. Therefore, an enhancement of LH responses to Kp suggesting that substitution of endogenous KISS1 levels is adequate to activate GnRH-LH axis fully in spite of prejudicial metabolic conditions (Castellano *et al.,* 2005).

Adipose is a necessary endocrine tissue that influence reproduction through leptin primarily (Kawwass *et al.,* 2015; Symonds *et al.,* 2016). Leptin acts through the GPR54 which is found

on kisspeptin neurons in hypothalamus (Tena-Sempere, 2006[a]; Tena-Sempere, 2006[b]). Kisspeptin binds to GnRH neurons and provoke GnRH release (Roseweir and Millar, 2009). The leptin-kisspeptin-GnRH neuron pathway presents the endocrine argument for the critical body weight hypothesis, which body weight relate to puberty in female (Keisler *et al.,* 1999). Thus, earlier result suggest that higher BMI caused lower LH surge, in turned FSH plasma level also decrease, but this finding needed further research.

Nowadays, the effect of Kp on FSH secretion is less information. The response of KISS1 to FSH release emerge less sensitive than LH considerably. The pathway organized centrally through modulation GnRH system, moreover it conducted independently with other neuroendocrine regulators of gonadotropic axis such as excitatory amino acids (EEAs), nitric oxide (NO) and leptin (Navarro, 2005). The positive reaction of leptin in GnRH is mediated by proopiomelanocortins (POMC, precursor of α-MSH), galanin-like peptide (GALP) and kisspeptin neuropeptides (Gottsch *et al.,* 2004; Crown *et al.,* 2007; Quennell *et al.,* 2009). Kp is detected in the growing follicles at theca cells and begins to arise in the basal cells of granular layer in rodent and human (Castellano *et al.,* 2006). FSH is not under control entirely by GnRH (Charlton, 1983; Phillips, 2005), but the major stimulus for FSH is GnRH (Mason *et al.,* 1986).

In our prior study, the obtrusive genotype of KISS1 gene intron 2 that correlated with higher LS, particularly average LS at the first and third parity in Indonesian native goat breeds which are CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA genotype at g.2459G>A (unpublished data). The data above (Table 5) showed that the genotypes at g.2425C>G and g.2436A>G had the same FSH plasma level (P=0.22 and P=0.34 respectively). On the other hand, the AA genotype at g.2459G>A has a superior FSH level than GG genotype (Fig.3).

Preliminary study revealed that CC genotype at g.2425C>G, AA genotype at g.2436A>G and GA genotype at g.2459G>A had significant greater litter size (2.58, 2.58 and 3.0 respectively). Furthermore, neither the CC genotype at g.2425C>G nor the AA genotype at g.2436A>G have significantly different FSH levels. On the other hand, GA genotype at g.2459G>A reveals a higher LS (3.0±0.18) than AA genotype which have a lower LS (2.0±0.21). Thus, it can be concluded that GA genotype at g.2459G>A is the most prominent genotype correlated with reproductive traits in Indonesian native goats.

CONCLUSION

The phylogenetic tree reveals a high closeness between Indonesian and Chinese goat breeds indicates the same function and tightness along the evolutionary timescale. *Capra hircus* and *Ovis aries* were also found in the same clade with Indonesian goat breed represents a significant role of KISS1 gene in reproductive traits in a variety of species.

The FSH level was influenced by breed, LS, and haplotype. The superior haplotype and genotype of KISS1 gene is TCAATGCGCAACGT haplotype and GA genotype at g.2459G>A that correlated with high LS and FSH level. These aspects could be considered in further breeding selection program for economically significant reproductive traits in goats.

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