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Identification of BMP15 Exon 2 for fecundity traits by PCR-RFLP and nucleotide sequencies in Kejobong goat

A. Febriana[1,2], Sutopo[1] and E. Kurnianto[1,*]
[1]Faculty of Animal and Agricultural Sciences, Diponegoro University, Tembalang Campus, Semarang 50275 – Indonesia
[1]Permenent Address: National Animal Husbandry Training Center (NAHTC), Ministry of Agriculture of Indonesia, Batu 65301 - Indonesia
**Corresponding E-mail:kurniantoedy17@gmail.com*

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ABSTRAK

Kambing Kejobong dikenal sebagai bangsa kambing yang mempunyai sifat prolifik dan produktivitas yang tinggi di Indonesia. PCR-RFLP dan metode sekuensing digunakan pada penelitian ini untuk mengetahui keragaman gen BMP15 ekson 2 pada kambing Kejobong. Sampel darah diperoleh dari 48 induk kambing Kejobong yang diseleksi berdasarkan *litter size*nya. Ukuran fragmen hasil amplifikasi PCR gen BMP15 ekson 2 adalah 837 pb. Amplikon PCR-RFLP direstriksi menggunakan enzim *HinfI* menghasilkan sampel yang monomorfik. Verifikasi hasil menggunakan metode sekuensing menghasilkan 4 substitusi (A391G, C464G, T828C and C830G), 1 *alignment gap* (situs ke-817) and 1 insersi nukelotida (situs ke-822). Mutasi ini menyebabkan terbentuknya 6 haplotipe. Mutasi gen BMP15 ekson 2 pada kambing Kejobong mengindikasikan bahwa bangsa ini mempunyai pola mutasi berbeda yang mengontrol sifat prolifik. Pohon filogeni yang disusun berdasarkan sekuen gen BMP15 ekson 2 pada kambing Kejobong membentuk 3 klaster. *Alignment gap* memberikan penciri khusus bagi induk kambing Kejobong yang memiliki *litter size* 2 (duplet). Insersi diduga menjadi situs pengenalan kambing Kejobong berdasarkan gen BMP15 ekson 2.

Kata Kunci: sifat fekunditas, polimorfisme, gen BMP15, prolifik, kambing Kejobong

ABSTRACT

Kejobong goat is known as prolific and high productivity goat breed in Indonesia. PCR-RFLP and sequencing technique was established in the present study to accomplish the polymorphisms of Bone Morphogenetic Protein 15 (BMP15) gene exon 2 on Kejobong goat does. The blood samples was collected from 48 Kejobong does which were selected based on their litter size. The size of PCR amplification of BMP15 gene exon 2 was 837 bp. The product of PCR-RFLP technique digested by *HinfI* enzyme showed that the samples were monomorphic. Authentication result using nucleotide sequencing found 4 substitution (A391G, C464G, T828C and C830G), 1 alignment gap (site 817) and 1 insertion nucleotide (site 822). This mutations caused 6 haplotypes formatted. The mutants of BMP15 exon 2 on Kejobong goats indicated that this breed had their own mutation controling the prolific trait. The phylogenetic tree build on the sequences of BMP15 gene exon 2 of Kejobong goats was grouped into 3 clusters. The alignment gap indicated to be the specific marker for the prolific trait (duplet) in Kejobong goat. The particular insertion site could be the recognition site of Kejobong goat based on BMP15 exon 2.

Keywords :fecundity trait, polymorphisms, BMP15 gene, prolific, Kejobong goat

INTRODUCTION

High productivity of goat is a major goal in farm business, therefore high prolific trait is one of the crucial problem to solve. Prolific is a reproduction trait or the ability to produce more than a kid in a birth. Prolific trait is regulated by different fecundity genes related to genetic factor improving ovulation rate and litter size. Some studies has indicated that this condition was caused by single gene dominates in few sheep breeds (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004; Bodin *et al.*, 2007 and Royo *et al.*, 2008).

Eight local goat breeds in Indonesia have been characterized by Ministry of Agriculture in Indonesia, namely Marica, Samosir, Muara, Kosta, Gembrong, Kacang, Ettawa Grade (EG) and Benggala (Pamungkas *et al.*, 2009) and was grouped (haplogroup) in lineage B. Haplogroup is goat's classification based on their maternal line and it spread in East Asia and South Asia (Batubara, 2011). Sodiq (2009) declared that Kejobong is crossbred of Kacang and Ettawa Grade (EG), furthermore they originated and concentrated in Purbalingga regency, Central Java Province, Indonesia (Kurnianto *et al.*, 2013). The characteristics of Kejobong are prolific, kidding interval was 268 days, roman nose and 56.49% of Kejobong population were black coat colour (Kurnianto *et al.*, 2012).

Bone Morphogenetic Protein 15 (BMP15) or Growth and Differentiation Factor 9B (GDF9B) is a gene controls prolific trait in sheep breeds such as Romney, Belcrare, Cambridge, Lacaune and Rasa Aragonesa (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004; Bodin *et al.,* 2007; Monteagudo *et al.,* 2009), but it can not be found in another sheep breeds such as Sakiz sheep (Dincel *et al.*, 2015). Chu *et al*. (2007) reported that prolific mechanism of the gene control pattern is different between sheep and goat, nevertheless controlled by BMP15 gene. Cui *et al.* (2009) and Hua *et al.* (2008) stated the important fact of BMP15 is having different response in other breed or species.

It is crucial to do this research based on molecular analysis to identify the genes with dominant effect on prolificacy in Kejobong goat in order to increase and advance the rate of genetic enhancement on litter size. BMP15 gene on *C. hircus* comprises of 2 exons of 328 bp and 856 bp in length. Heterozygous mutations of BMP15 gene engender increased ovulation rate, nevertheless infertility in homozygous mutations. Some researches of BMP15 have been conducted in Indonesian native breed such as Batang goat (Hidayat *et al.*, 2015) and Kacang, Samosir and Muara (Hasan, 2011). Both researches presented contrast result of BMP15 gene exon 2 polymorphism. The aim of this research was to identify the fecundity trait of BMP15 gene exon 2 by PCR – RFLP and DNA sequencing of Kejobong goat.

MATERIALS AND METHODS

Experimental Animals and Sample Collection

Genomic DNA samples were collected from 48 Kejobong does belonging of 3 litter size levels consisted of single (16 head), duplet (22 head) and triplet (10 head). Goats were obtained from Kejobong district in Purbalingga regency of Central Java Province, Indonesia with purposive random sampling based on the the population density, litter size in a birth, does's age and parity. Approximately 6 mL of blood were collected from the jugular vein, furthermore the collected samples punctured into vacutubes contain of EDTA as an anticoagulant, also carried to the laboratory at 5ºC and kept in freezer at minus 18ºC directly until DNA isolation.

The DNA was isolated from whole blood using extraction kit (DNA gSYNC^{TM} extraction Kit) following the procedure described in standart protocol of Geneaid Biotech Ltd. Genomic DNA's were run on agarose gel electrophoresis using 1 % agarose in 1x TBE Buffer and visualised with Etidium Bromide (EtBr). It can be monitored by UV spectrophotometry.

PCR Amplification

A 837 bp fragment of BMP15 gene exon 2 from 48 Kejobong does were amplified by PCR method using forward primer (5'-GGCACATACAGACCCTGGAC-3') and reverse primer (5'-CAGGACTGGGCAATCATACC-3'). The primer was designed by Primer3Plus software based on the *C. hircus* Ganjam breed from GenBank database (GenBank Accesion No.: JQ350892.1) and was formed by PT Genetika Science Indonesia.

The PCR reaction was performed in 50 µL reaction volume containing of 25 µL Kappa ready mix, 3 µL DNA template, 1 µL forward primer, 1 µL reverse primer and 20 µL ddH_2O. The PCR reaction was accomplished with an initial denaturation at 95ºC for 5 min, followed by 35 cycles each of denaturation at 95ºC for 45 sec,

annealing temperature at 55°C for 45 sec and extension at 72°C for 1 min, then holding the final extension (post elongation) at 72°C for 10 min. The PCR amplified products were proved by determining on 1% agarose gel in paralell with 1 kb DNA ladder and visualised with Etidium Bromide (EtBr). Gel agarose was running in a constant voltage of 90 V for 30 minutes and soaked in 1x TBE buffer, then be monitored by UV spectrophotometry.

PCR-RFLP and DNA Sequencing

To obtain 10 µL of PCR-RFLP, the PCR fragments were digested with *HinfI* restriction enzyme. Four µL of amplicon fragment of BMP15 gene exon 2 were mixed with 1 µL *HinfI* enzyme, 1 µL buffer and 4 µL ddH^2O. Then, the mixtures were incubated in the oven at 37°C for 4 hours, and the resulting products were loaded on 2% of agarose gel in paralell with 100 bp DNA ladder and visualised with Etidium Bromide (EtBr). The wild type products can not be cutted by *HinfI* (GA↓NTC). The PCR fragment of BMP15 gene exon 2 were sequenced in both directions by 1st BASE – Asia in Malaysia.

Data analysis

Allele frequencies and genotype frequencies were calculated by the formula of Nei (1987) and Nei and Kumar (2000):

$$x_i = \frac{(2n_{ii} + \sum_{j=1} n_{ij})}{2N} \quad \text{.....(1)}$$

Where x_i : allele frequencies and n_{ii} : the number of sample with genotype ii, n_{ij} : the number of sample with genotype ij, and N : the total number of sample.

Genotype frequencies is :

$$x_i = \frac{\sum_{i=1}^{n} n_i}{N} \quad \text{..... .... (2)}$$

Where x_i : genotype frequencies and n_i : the number of sample with genotype i and N : the total number of sample.

The DNA sequences were edited manually and aligned with MEGA 6.06. The edited DNA sequences then were aligned and compared with BMP15 gene sequences exon 2 of *C. hircus* from GenBank (http://ncbi.nlm.nih.gov) using ClustalW. The results were used to measure haplotype diversity and nucleotide diversity using DnaSP 5.1. Calculation of genetic distance was based on the model of Kimura 2 parameter substitution. Reconstruction of the phylogeny tree was carried by the UPGMA method using bootstrap value 1,000 times.

RESULTS AND DISCUSSION

BMP15 is one of fecundity genes significantly affect prolificacy. In the current research, the BMP15 gene exon 2 mutations were probed in Kejobong goat breed. The amplification of BMP15 exon 2 gene acquired the DNA fragment along 837 bp then it was translated into amino acid (AA) sequence for 276 AA (Figure 1).

The amplicon was digested using *HinfI* and was cutted into 3 fragments with 250 bp, 26 bp and 561 bp in lenght (Figure 2). The samples showed the identical pattern and resulted the same

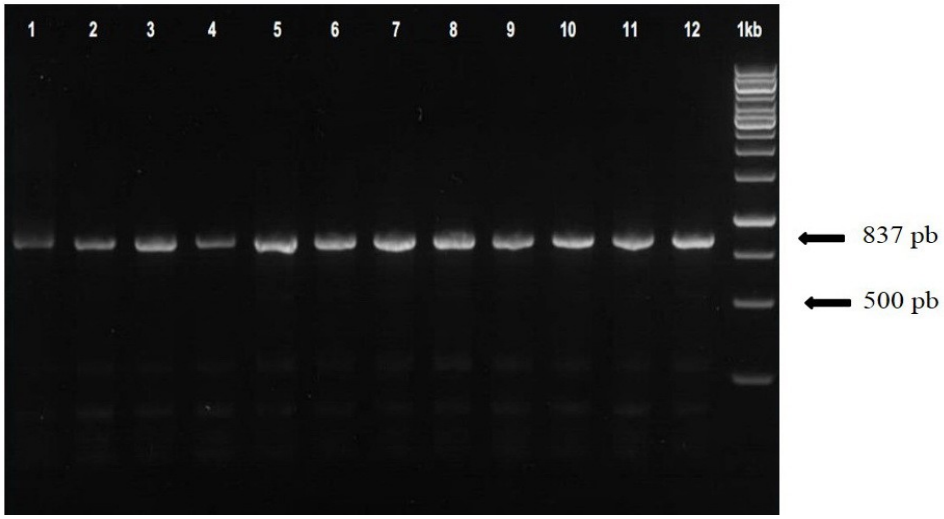

Figure 1. The fragment of BMP15 exon 2 on Kejobong goat. 1-12: samples

genotype (monomorphic). The allele and genotype frequencies estimated were 1. The annealing sites of primer and restriction sites are presented in Figure 3. This result is in line with some researches, those were Boer goat, Boer Huanghuai, Haimen, Huanghai, Nubi dan Matou (Hua *et al*., 2008); Black Bengal goat (Polley *et al.*, 2009); Iran local goat (Tajangookeh *et al.*, 2009); Marwari goat (Godara *et al.*, 2012); Indian local goat (Ahlawat *et al.*, 2013); Markhoz goat (Shokrollahi, 2015).

In contrast, the polymorphisms were found in Batang goat (Hidayat *et al.*, 2015), White goat (Ran *et al.*, 2009), White Funiu and Black Taihang goat (Wang *et al.*, 2011) and Anglo-Nubian goat (Rahman *et al.*, 2013). The PCR-RFLP result showed that Kejobong goat was mutant homozygous, in which this condition caused primary ovarian failure and resulting fault in early folliculogenesis phase, eventually lead to infertility (Galloway *et al.*, 2000; Hanrahan *et al.,* 2004). In contrast, Sodiq and Haryanto (2007) also Zulkarnain *et al.* (2015) stated that Kejobong goat has 1,6 ± 0,54 heads average on litter size. It gave the opposite information from the present study's result. This finding preliminary indicated that the prolific trait of Kejobong goat is controlled by another fecundity genes too. Additionally, Hasan (2011) declared that prolific is controlled by additive genes which are pleitropic. BMP15 gene gives differences on prolific inheritance for many goat breeds even between goat and sheep breeds (Shokrollahi , 2015).

The amplicon of BMP15 exon 2 gene was verified by sequencing method. Only 9 from 48

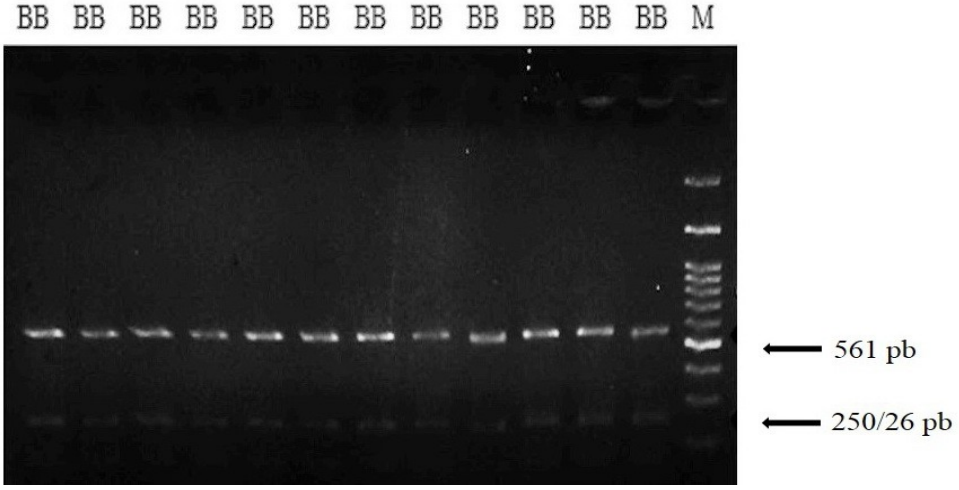

Figure 2. The DNA Fragment Digested with *HinfI* of BMP15 Exon 2 on Kejobong Goat

5641 TCCTGGCACATACAGACCCTGGACTTTCCTCTGAGACCAAACCGGGTAGCATACCAACTA
5701 GTCAGAGCCACTGTGGTTTACCGCCATCAGCTTCACCTAACTCATTCCCACCTCTCCTGC
5761 CATGTGGAGCCCTGGGGGCAGAAAAGCCCAACCAATCACTTTCCTTCTTCAGGAAGAGGC
5821 TCCCCAAAGCCTTCCCTGTTGCCCAAAACTTGGACAGAGATGGATATCATGGAACATGTT
5881 GGGCAAAAGCTCTGGAATCACAAGGGGCGCAGGGTTCTACGACTCCGCTTCGTATGTCAG
6361 GATCAGAATGTCCCTCAGCCTTCCTGTGTCCCTTATAAGTATGTTCCCATTAGCATCCTT
6421 CTGATTGAGGCAAATGGGAGTATCTTGTACAAGGAGTATGAGGGTATGATTGCCCAGTCC
6481 TGCACATGCAGGTGA

Figure 3. *C. hircus* Sequence (GenBank access code JQ350892.1.) visualized the Annealing Site of Forward and Reverse Primer, and the restriction site of *HinfI* Enzyme

Table 1. The *Alignment Gap* and Polimorfic Site on BMP15 Kejobong Goat Compared to *C. hircus* Sequence GenBank (Access Code JQ350892.1.)

No	Sample	Litter Size	Site					
			391	464	817	822	828	830
1	*C. hircus*		A	C	T	-	T	C
2	K 3A	3	G	G	.	C	.	G
3	K 3B	3	G	G	.	A	.	G
4	K 3C	3	.	.	.	C	.	A
5	K 2A	2	G	.	.	C	.	G
6	K 2B	2	.	.	-	C	.	G
7	K 2C	2	.	.	-	C	C	G
8	K 1A	1	G	G	.	C	.	G
9	K 1B	1	G	G	.	C	.	G
10	K 1C	1	.	.	.	C	.	G

(.) same with *C. Hircus;* (-) = deletion
Codon (Nucleotide base) : A (Adenine), C (Citosine), G (Guanine), T (Timine)

samples were sequenced. Three samples each represented litter size 1, 2 and 3 respectively. Thereby, the DNA sequences gave 5 polymorphisms and 1 alignment gap found from DNA sequences (Table 1).

The five single nucleotide mutations in Kejobong goat are : 1) A391G described as missense mutation from Asparagine (Asn) to be Aspartic acid (Asp), 2) T828C caused silent mutation, 3) C464G introduced a premature stop kodon in 4 samples. This finding confirmed additive genes on fecundity traits which is pleitropic on BMP15. This condition presents the unprocessed amino acid, which apparently results in total loss of BMP15 activity (Hanrahan, 2004), 4) C830G described as silent mutation, 5) Insertion site on 822. The deletion mutation found only in 2 samples (K 2B and K 2C).

The polymorphic sites based on DNA sequencing were not in recognition site of *HinfI*. This aprroved that the polymorphisms of BMP15 exon 2 can not be obtained by PCR-RFLP method using HinfI restriction enzyme. The mutations sites are not associated with single substitution known as the factor controlling fecundity in sheep and could be the particular mechanism regulated prolific trait of Kejobong goat. These mutations composed the Kejobong goat into 6 haplotypes (Table 2), moreover the haplotype diversity (Hd) index 0.889 and the nucleotide diversity (π) is 0.00214. The genetic distance coverage from 0.0000 to 0.0097.

Table 2. Haplotypes of Kejobong Goat Based on BMP15 Exon 2 Gene

Haplotype	Individual Sample
1	K 3A, K 1A, K 1B
2	K 3B
3	K 3C
4	K 2A
5	K 2B K 1C
6	K 2C

The phylogeny tree was constructed based on DNA sequence (Figure 4). All samples except K 2B and K 2C had closer genetic relationship with *C. hircus*. K 2B and K 2C had the deletion site on 817 and it was specific in litter size 2. Further, this deletion can be the DNA characteristic for twinning in Kejobong goat. The group showed that there are not differences between litter size 1 (K 1A, K 1B and K 49); litter size 2 (K 2A) , and litter size 3 (K 3A, K 3B and K 3C). This result indicated that Kejobong goat

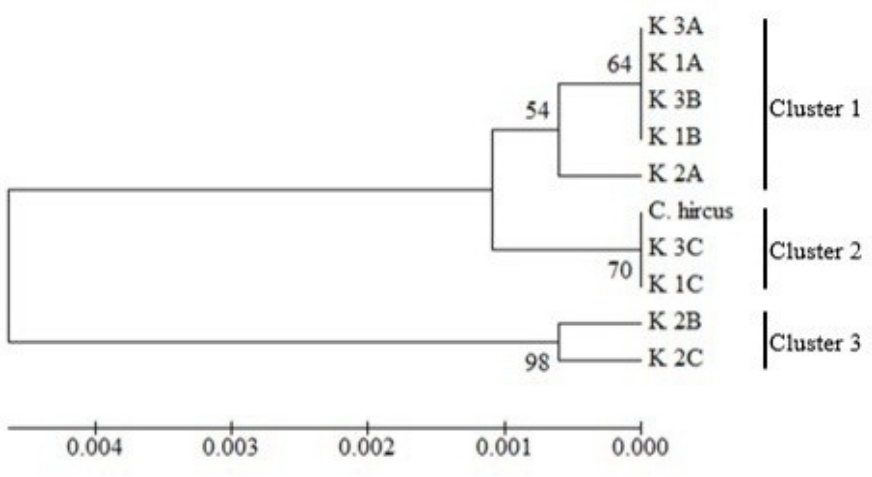

Figure 4. Phylogeny Tree of Kejobong Goat Based on BMP15 Exon 2

has a potency to be prolific, but poor breeding management caused this ability did not appear maximally. The insertion site (822) could be the recognition site of DNA sequence of Kejobong goat.

CONCLUSION

The PCR-RFLP with HinfI restriction enzyme used to detect the BMP15 gene exon 2 mutation showed that the gene was monomorfic. By sequence method found 1 missing gap alignment site and 5 polymorphic sites of BMP15 gene that formed 6 haplotypes. The deletion site can be used as a marker for twinning, therefore the insertion site is the recognition site of Kejobong goat. The genetic distance and nucleotide diversity of BMP15 gene exon 2 between Kejobong goat *C. hircus* was very low. Thereby, phylogeny tree classified Kejobong goat and *C. hircus* in the same group based on BMP15 gene exon 2.

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