

# IDENTIFICATION OF MYOSTATIN GENE c.960delG LOCUS POLYMORPHISM IN INDONESIAN LOCAL SHEEP BY USING PCR-SSCP METHOD

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## ABSTRACT

Myostatin (MSTN) is a protein inhibit muscle growth. This protein is a member of a superfamily of molecules called transforming growth factors beta (TGF- $\beta$ ). Deletion in c.960delG (1-bp deletion at position 960) disrupts the reading frame from amino acid (aa) position 320 to ending in a premature stop codon in aa position 359 have been found in Norwegian White Sheep. This deletion in the myostatin gene is responsible to increase muscle mass, also known as 'double muscling', in sheep. The purpose of this study was to identify the polymorphism of myostatin gene in c.960delG locus of local sheep in Indonesia. The 832 DNA sampels from sheep were collected from 13 populations belonging to the Priangan (86), Javanese Thin Tail (389 i.e. Jonggol, Banjar, Ciomas), Javanese Fat Tail (94), West Nusa Tenggara (136), Rote Island/East Nusa Tenggara (35), Kisar Island/Southwest Maluku (22), Donggala/South East Celebes (45) and Batur cross breed/Wonosobo, Central Java (25). A gene fragment of MSTN c.960delG length 299 bp was successfully amplified by using the technique of PCR (polymerase chain reaction) and genotyped by SSCP (single strand conformation polymorphism). The result showed no polymorphism in this gene. All sheep tested had G/G genotype for c.960delG locus.

*Keywords: local sheep, myostatin c.960delG locus, PCR-SSCP*

## INTRODUCTION

Local sheep has its own advantages to be protected and developed because it has several advantages, such as being able to adapt well in tropical environments, capable of lambing all year round, resistant to some kinds of diseases and parasites. Some weaknesses of the local sheep such as body weight and other body measurements have a very high diversity and the quality of meat has not yet met international market standards. Genetic improvement of local sheep through selection towards high productivity and quality of mutton is needed to be developed nationally due to the contribution of lamb on the national meat production is only 54,175 tonnes (2.5%) of total domestic meat production (Directorate General of Livestock Services, 2009). Progress in the field of molecular biology allows the selection efforts which can be made at the DNA level, that is by looking for genes that control the diversity of economic nature. Simple DNA test has to be applied to the detection of positive alleles at locus-locus economic value

(ETL / Economic Trait Loci). Selection using a marker gene is an alternative livestock carrier biotechnology to produce desirable traits (according to the marker gene). Mapping genes in the sheep genome has marked the commencement of new horizons in the field of livestock breeding. The genes that can be used as genetic marker for selecting the properties of production and have high economic value are known as auxiliary selection markers or Marker Assisted Selection (MAS). Identification of genetic markers is an early and critical step to obtain markers auxiliary selection (MAS). Auxiliary selection markers have been shown to increase the genetic value of breeding livestock in the program as stated Parmentier *et al.* (2001).

At Texel sheep, Quantitative Traits Loci (QTL) markers for callipygian trait (increased muscle mass) have been identified in specific regions in chromosome number two (OAR2) (Marcq *et al.*, 2002; Walling *et al.*, 2004; Johnson *et al.*, 2005). Mutation which occurs in the gene encoding myostatin (MSTN) has been found affecting the phenotype of increased muscle mass

in mice (McPherron *et al.*, 1997), cattle (Grobet *et al.*, 1997), humans (Schuelke *et al.*, 2004) and dogs (Mosher *et al.*, 2007). Myostatin gene (GDF-8) in cattle has been mapped at the distal end of chromosome number two position by position 2q14-q15. The gene consists of three exons and two introns (Grobet *et al.*, 1998). Research on myostatin gene has been reported by Boman *et al.* (2009) by using Norwegian white sheep and found a new point mutation in the 3<sup>rd</sup> exon region. Mutation that occurs is 1-bp deletion at position 960 (position based on translational starting point) namely c.960delG. The deletion (c.960delG) disrupts the reading frame from amino acid (aa) position 320, ending in a premature stop codon in aa position 359. The incidence of deletion at this locus has not been reported in sheep in Indonesia. Therefore, the purpose of this study was to identify the deletion in c.960delG myostatin gene locus in Indonesian local sheep using PCR-SSCP method.

## MATERIALS AND METHODS

### Blood Sample

Blood sample as a source of DNA were taken from vena jugularis by venoject needle with vacutainer tube. The blood samples were used as much as 832 samples originating from 13 subpopulations belonging to the Priangan (i.e. fighting type 68 and meat type 18 samples), Javanese Thin Tail (i.e. Jonggol 271, Banjar 20 and Ciomas 98 samples), Javanese Fat Tail (i.e. East Java 64 and Madura Island 30 samples), West Nusa Tenggara (Lombok Island 111 and Sumbawa Island 25 samples), Rote Island/East Nusa Tenggara (35 samples), Kisar Island/Southwest Maluku (22 samples), Donggala/South East Celebes (45 samples) and Batur cross breed/Wonosobo, Central Java (25 samples). Blood samples were preserved in ethanol 96% (blood : ethanol = 1 : 1) and stored in room temperature.

### DNA Extraction

DNA was extracted from blood in ethanol. Extraction procedure followed the phenol-chloroform method (Sambrook and Russell, 2001) was modified by Andreas *et al.* (2010), with the following procedure:

**Sample preparation.** Blood sample in the alcohol as much as 200 µl was poured to a 1.5 ml tube. Alcohol is eliminated from the sample by

adding distilled water until 1000 µl, and left in room temperature for 20 minutes. Then it was precipitated by centrifugation at a speed of 8000 rpm for 5 minutes.

**Protein degradation.** The samples cleared from alcohol were added by 200 µl 1x STE (sodium tris EDTA), 40 µl sodium dodecyl sulfate 10%, and 20 µl proteinase K (5 mg/ml). The mixture was incubated overnight at 55 °C temperature while shaken gently.

**Organic material degradation.** After incubated, samples were added by 400 µl phenol solution, 400 µl chloroform:isoamyl alcohol (24:1), and 40 µl 5M NaCl. Then, the mixture was shaken at room temperature for one hour.

**DNA precipitation.** Samples were centrifuged at a speed of 5000 rpm for 10 minutes to separate the water phase with phenol phase. Water phase was transferred in a new tube with the measured volume DNA molecules were deposited by adding a 2x volume of alcohol absolute and 0.1 x volume of 5M NaCl. The mixture was incubated at a temperature of -20°C during the night. Then, subsequent DNA precipitation was centrifuged at 12 000 rpm for 10 minutes. Obtained DNA precipitate was washed by 70% alcohol, and then precipitated again. Precipitated DNA clean from alcohol was restored by adding 100 µl TE (Tris EDTA). DNA samples were stored at -20°C and then was ready for use.

### Amplification of MSTN c.del960G Locus

Primers to amplify MSTN c. 960delG locus followed Boman *et al.* (2009), with forward primer 5'-CTC CTT GCG GTA GGA GAG TG-3' and reverse primer 5'-GGT GCA CAA GAT GGG TAT GAG-3'. The amplified product (amplicon) length was 299 bp. Amplification of MSTN c. 960delG locus was done by using PCR (polymerase chain reaction) methods. Reagents used for amplification of both of the target fragment are a 2 µl sample of DNA, each primer 25 pmol, 200 µM dNTPs mixture, 1 mM MgCl<sub>2</sub>, and 0.5 units of DreamTaq™ DNA Polymerase and 1x buffer (Fermentas) in total solution 25 µl. Amplification in vitro within GeneAmp® PCR System 9700 (Applied Biosystems™) was done with the condition of pre-denaturation at 94 °C for 5 minutes, 35 cycles consisting of denaturation at 94 °C for 45 seconds, annealing primers at 60 °C for 45 seconds and extension of new DNA at 72 °C for 1 minute, and the final extension at 72 °C for 5 minutes.

### Identification of Polymorphism MSTN gene c.del960G Locus by using PCR-SSCP Methods.

Identification polymorphism by using PCR-SSCP approach was performed to identify the c.del960G locus and another polymorphism in MSTN gene in Indonesian local sheep. PCR-SSCP method was used as an alternative method from previous study which used PCR-RFLP with *MaeIII* endonuclease (Boman *et al.*, 2009). PCR-SSCP analysis was done by resolved the single pieces of DNA in PAGE (polyacrilamide gel electrophoresis) 12%. Electrophoresis process was conducted at 100 V for 17 hours. Visualized of single strand DNA bands were done by sensitive silver staining methods (Byun *et al.*, 2009).

### Sequencing and Sequence Analysis

Prior to sequencing, each band was cut out from PAGE and purified (Hu *et al.*, 2010). This was then used as the DNA template for reamplification then sent to sequencing platform (Macrogen Inc., Korea). Ensuring that amplicon product are specific, BLAST (basic local alignment search tool) methods was used to calculate sequences homology with GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>). Alignments and comparisons of sequence were carried out by using MEGA software version 4.0.

### Genotyping and Allele Frequency

Genotype frequency represents the ratio of a genotype to total population. Allele frequency is a ratio of an allele to the overall allele at a locus in the population. Mathematics model genotype and allele frequency (Nei and Kumar, 2000) are represented as follows:

$$\chi_{ii} = \frac{n_{ii}}{N} \times 100\%$$
$$\chi_i = \frac{(2 n_{ii} + \sum_{i \neq j} n_{ij})}{2 N}$$

Note :

$\chi_{ii}$  = ii<sup>th</sup> genotype frequency

$n_{ii}$  = number sample of ii genotype

$n_{ij}$  = number sample of ij genotype

$N$  = total sample

$\chi_i$  = i<sup>th</sup> allele frequency

## RESULTS AND DISCUSSION

### Amplification of MSTN c.del960G region

Amplification of MSTN c.del960G locus

was carried out on GeneAmp® PCR System 9700 (Applied Biosystems™) with annealing in the same temperature, which was 60°C. The amplified gene fragments were visualized on 1.5% agarose gel (Figure 1). The amplified product (amplicon) length MSTN c.del960G fragment was 299 bp, including a part of 2<sup>nd</sup> intron and a part of 3<sup>rd</sup> exon (Boman *et al.*, 2009).

### Identification of MSTN c.del960G by using PCR-SSCP Methods

Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) is one further analysis method that utilizes PCR product. PCR-SSCP method is a reliable method of quickly detecting a mutation (Hayashi, 1991). This method is based on the assumption that the nucleotide acid changes would lead to change in migration patterns on polyacrylamide gel nondenaturasi (Barroso *et al.*, 1999). Mutation was detected from the differences in migration patterns from conformation of single strand DNA on polyacrylamide gels (Hayashi, 1991).

Given its relative technical simplicity and high mutation detection capacity, the PCR-coupled SSCP provides a powerful tool to specifically identify pathogens and to screen effectively for genetic variability within and among samples, specimens or populations, and importantly, to screen for unknown mutations (Sunnuck *et al.*, 2000; Doi *et al.*, 2004; Jespersgaard *et al.*, 2006; Tahira *et al.*, 2006). This approach is relatively simple technically and can be used effectively to visually display at different sequence types within and between amplicons (of ~ 100–500 bp) and to rapidly screen hundreds of samples per day (Gasser *et al.*, 2007).

It remains possible that, occasionally, some point of mutations are not detected because of the nucleotide composition (e.g. A+T or G+C richness) or excessive length of a DNA region being analyzed (Gasser *et al.*, 2007). The mutation detection rate of SSCP varies depending on the parameters, such as size of the fragment, base composition of the sequence, electrophoresis temperature and/or gel composition (i.e. pore size and cross-linking) (Hayashi, 1991; Sunnuck *et al.*, 2000; Doi *et al.*, 2004; Jespersgaard *et al.*, 2006; Tahira *et al.*, 2006; Gasser *et al.*, 2007).

Migration patterns of DNA single strand band in PAGE 12% produced four MSTN c.960delG DNA single strand banding patterns

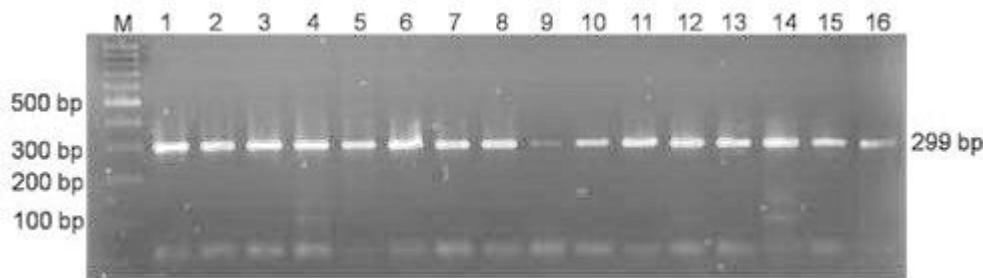


Figure 1. Visualization of MSTN c.960delG amplicon. M: DNA Ladder 100 bp, 1-16: individual samples

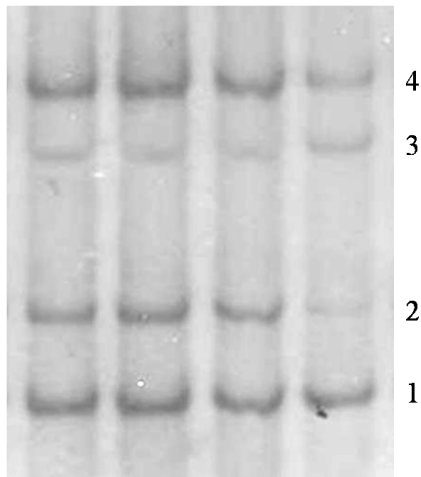


Figure 2. Visualization of Pattern of MSTN c.960delG Amplicon. 1-4: Band Pattern from Single Strand DNA Conformation.

(Figure 2). The four patterns of these bands were uniform for all samples in this study. For the purposes of sequencing analysis, each band was cut from PAGE and then purified using TE wash buffer method (Hu *et al.*, 2010). The results of purifications was used as a sample for reamplification process and sent to sequencing platform (Macrogen Inc., Korea).

### Sequence Analysis

Sequence analysis revealed that amplicons were 299 bp in length. These were the same size as previous study reported by Boman *et al.* (2009). There was not polymorphism identified from different banding pattern. All of four banding pattern in PCR-SSCP represented the same allele. Genotype found in buffalo in this research was G/G genotype. Occasionally, one strand is separated into two or more bands in the SSCP gel, even though the sequence is the same. This suggests that the strands having the same sequence can have different stable conformations. Usually, intensity of these conformer bands may be different, but the ratio of the intensity between

them is constant from sample to sample. Therefore, such sequence-different conformation situations are easily diagnosed (Hayashi, 1991).

All of our sequences in this study have very high similarity (100%) with published in ovine myostatin gene sequences (GenBank Accession no. DQ990914). There did not reveal either of any deletion in 1-bp deletion at position 960 (position based on translational starting point, Boman *et al.*, 2009) or another mutation from this region in all populations Indonesia Local Sheep (Figure 3).

### Genetic Diversity of MSTN c.del960G Locus within Indonesian Local Sheep

Genetic diversity within a population can be used as a parameter in studying the population and evolutionary genetics. In addition, it can be used to identify and preserve the peoples in the population associated with the character of a special nature. Knowledge of the genetic diversity of a nation will be very useful for food security and continuous availability (Blott *et al.*, 2003).

Level of diversity within populations can be drawn from the allele frequency. Allele frequency is a ratio of one allele relative to the overall allele found in one population. Information on genetic diversity of a population using multiple loci, can be described by the value of heterozygosity (Nei and Kumar, 2000). Genotypes and allele frequencies of MSTN c.del960G locus are presented in Table 1.

Genetic diversity based on molecular marker MSTN c.del960G locus in Indonesia local sheep are very low. This is indicated by the value of one genotype frequency and allele which has a value of 1, which marks the fixation process. This value are lower than reported by Sumantri *et al.* (2008) and Dagong *et al.* (2011) which used CAST|*MspI* locus and CAST 6<sup>th</sup> exon in Indonesian local sheep respectively.

The absent of deletion in 1-bp deletion at MSTN c.del960G can be caused by tropical

DQ990914	4480bp	ctccttgcggtaggagagtgtttggggatctattactaactcttctttcctttccataca
AM992883		.....
AM992884		.....
Band 1		.....
Band 2		.....
Band 3		.....
Band 4		.....
		N P F L E V K V T D T P K R S R R D F G
DQ990914	4540bp	<b>GAATCCTTTTTAGAAAGTCAAGGTAACAGACACACCAAAAAGATCTAGGAGAGATTTTGG</b>
AM992883		.....
AM992884		.....
Band 1		.....
Band 2		.....
Band 3		.....
Band 4		.....
		L D C D E H S T E S R C C R Y P L T V D
DQ990914	4600bp	<b>GCTTGATTGTGATGAGCACTCCACAGAATCTCGATGCTGTCGTTACCTCTAACTGTGGA</b>
AM992883		.....
AM992884		.....
Band 1		.....
Band 2		.....
Band 3		.....
Band 4		.....
		F E A F G W D W I I A P K R Y K A N Y C
DQ990914	4660bp	<b>TTTGAAGCTTTTGGATGGGATTGGATTATTGCACCTAAAAGATATAAGGCCAATTACTG</b>
AM992883		.....
AM992884		.....
Band 1		.....
Band 2		.....
Band 3		.....
Band 4		.....
		S G E C E F L F L Q K V S S Y P S C A
DQ990914	4720bp	<b>CTCTGGAGAATGTGAATTTTTATTTTTGCAAAAGTATCCTCATACCCATCTTGTGCACC</b>
AM992883		.....
AM992884		..... Y P H T H L V H
Band 1		..... <b>TATCCTCATACCCATCTTGTGCACC</b>
Band 2		.....
Band 3		.....
Band 4		.....

Figure 3. Nucleotide Sequences of the Ovine MSTN c.960delG predicted Amino Acid Sequences. DQ990914 are Ovine Myostatin Sequence Compared to the coding part of the MSTN cDNA Sequence in the NMM (GenBank accession no. AM992883) and IMM (GenBank accession no. AM992884)

adaptation process which suggested that the animal which can survive in this environmental are having small performance. In this case, the presence of the c.960delG mutation in both alleles means that no functional myostatin protein is expected to be produced (Boman *et al.*, 2009). If myostatin not to be expressed, so the negative growth regulation will failure and will increase the number of muscle fibers (hyperplasia).

### CONCLUSION

Based on these results, it can be concluded

that the diversity of MSTN c.del960G locus in Indonesian local sheep is very low and showed no polymorphisms were detected in this gene. All of sheep were tested had GG genotype for MSTN c.del960G locus. So, MSTN c.del960G locus can not be used as marker to assist selection process in Indonesian local sheep.

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Table 1. Genotype and Allele Frequency of the GH|AluI Locus

Population	Subpopulation	Genotype (%)			Allele	
		G/G	G/delG	delG/delG	G	delG
Priangan (86)	Fighting Type (68)	100.0	0.0	0.0	1.0	0.0
	Meat Type (18)	100.0	0.0	0.0	1.0	0.0
Javanese Thin Tail (389)	Jonggol (271)	100.0	0.0	0.0	1.0	0.0
	Banjar (20)	100.0	0.0	0.0	1.0	0.0
	Ciomas (98)	100.0	0.0	0.0	1.0	0.0
Javanese Fat Tail (94)	East Java (64)	100.0	0.0	0.0	1.0	0.0
	Madura Island (30)	100.0	0.0	0.0	1.0	0.0
West Nusa Tenggara (136)	Lombok Island (111)	100.0	0.0	0.0	1.0	0.0
	Sumbawa Island (25)	100.0	0.0	0.0	1.0	0.0
Rote Island (East Nusa Tenggara) (35)		100.0	0.0	0.0	1.0	0.0
Kisar Island (Southwest Maluku) (22)		100.0	0.0	0.0	1.0	0.0
Donggala (South East Celebes) (45)		100.0	0.0	0.0	1.0	0.0
Batur cross breed (Wonosobo-Central Java) (25)		100.0	0.0	0.0	1.0	0.0
Total (832)		100.0	0.0	0.0	1.0	0.0

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