

Detection of F94L mutation of the *MSTN* gene in four Indonesian local cattle breeds

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ABSTRAK

Mutasi F94L pada gen *MSTN* (*MSTN-F94L*) dianggap tidak menyebabkan terganggunya fungsi gen *myostatin* secara drastis. Menariknya, mutasi ini memiliki efek yang sangat signifikan terhadap massa otot, karkas atau produksi daging dan kualitas daging tanpa ada masalah negatif yang parah. Penelitian ini bertujuan untuk mengkonfirmasi mutasi *MSTN-F94L* pada empat bangsa sapi lokal di Indonesia. Penelitian ini menggunakan sebanyak 518 individu yang meliputi sapi Bali (140 sampel), Sumbawa (107 sampel), Pasundan (168 sampel) dan Friesian Holstein (103 sampel). Identifikasi genotipe dilakukan dengan metode PCR-RFLP. Pada penelitian ini, alel C *wild-type* ditemukan *fixed* (1,000) pada sapi Bali, Sumbawa, dan HF. Namun, alel C *wild-type* maupun alel A mutan ditemukan pada sapi Pasundan, meskipun frekuensi alel A mutan sangat rendah (0,012). Kesimpulannya, mutasi *MSTN-F94L* terdeteksi pada sapi Pasundan tetapi tidak pada ketiga bangsa sapi yang lain. Namun, keberadaan alel A mutan pada sapi Pasundan diduga berasal dari sapi Limousin. Investigasi lebih lanjut pada bangsa sapi lokal dan eksotik lainnya maupun hasil persilangannya akan menjawab status mutasi *MSTN-F94L* pada bangsa sapi lokal di Indonesia.

Kata kunci : gen MSTN, mutasi F94L, PCR-RFLP, polymorphism, sapi lokal

ABSTRACT

The F94L mutation of the *MSTN* gene (*MSTN-F94L*) is considered not to cause disrupted the function of the *myostatin* gene drastically. Interestingly, this mutation has a very significant effect on muscle mass, carcass, or meat yield and meat quality without any associated severe negative problems. This study aimed to confirm the *MSTN-F94L* mutation in four local cattle breeds in Indonesia. A total of 518 individuals (140 of Bali, 107 of Sumbawa, 168 of Pasundan, and 103 of Holstein-Friesian (H-F) cattle) were used in this study. Genotype identification was performed by PCR-RFLP method. In the present study showed that the wild-type C allele was *fixed* (1.000) in Bali, Sumbawa, and HF cattle. However, the wild-type C allele and the mutant A allele were found in Pasundan cattle, even though the frequency of the mutant A allele was very low (0.012). Therefore, in conclusion, the mutation of the *MSTN-F94L* was detected in Pasundan cattle but no in all three cattle breeds. However, the presence of the mutant A allele in Pasundan cattle allegedly derived from Limousin bulls. The further investigation in other local and exotic breeds and its crossing will answer the status of the *MSTN-F94L* mutation in local cattle breeds in Indonesia.

Keywords : F94L mutation, MSTN gene, gene polymorphism, PCR-RFLP, local cattle

INTRODUCTION

Sustained and intense selective breeding for increased meat yield and leaner meat in cattle has led to delivering the double-muscling (DM). At first glance, the animals look to have an extreme muscularity, especially in the fore- and hind-quarters extremity (Arthur, 1995). Conversely, the size of internal organs (such as heart, lungs, kidneys) significantly reduced and increased in the incidence of fractures (Cieplach *et al.*, 2017). The Piedmontese and Belgian Blue breeds are the most popular cattle breeds that exhibit the DM phenotype (Arthur, 1995). These cattle have some advantages such as the higher proportion of muscle, less bone, less fat, less saturated fatty acid (SFA), more tender meat, and a higher proportion of the most desirable cuts of beef compared with normal cattle (Casas *et al.*, 1998; O'Rourke *et al.*, 2009; Fiems, 2012). However, these cattle appear to have some disadvantages, such as reduced female fertility, susceptibility to respiratory illness, high frequency of dystocia, and faster fatigued than normal cattle (Casas *et al.*, 1998; Bellinge *et al.*, 2005; Allais *et al.*, 2010).

In the previous studies, the *myostatin* (*MSTN*) gene or also known as *growth differentiation factor 8* (*GDF-8*), has been validated as a major gene that causing the DM phenotype (Grobet *et al.*, 1997; Miranda *et al.*, 2002). The *MSTN* gene encodes myostatin, a member of the TGF- β superfamily that actively represses skeletal muscle growth and leads to DM phenotype (Bellinge *et al.*, 2005). This gene has been mapped to chromosome 2 (2q14-q15) in cattle (Grobet *et al.*, 1997) that involves three exons and two intronic regions with a coding region (CDS) length of 1.128 nucleotides and protein length of 375 amino acids (<https://www.ncbi.nlm.nih.gov/gene/281187>).

Until now, there are nine mutations have been detected in coding regions of *MSTN* gene associated with DM phenotype and six mutations are considered to inhibit the function of the *MSTN* protein, including nt419(del7-ins10), nt821(del11), E226X, Q204X, E291X and C313Y (Grobet *et al.*, 1998; Dunner *et al.*, 2003; Bellinge *et al.*, 2005). Those causal mutations were found to be breed-specific and segregated in several breeds.

The mutation of nt821(del11) is the most studied disruptive mutation of *MSTN* gene because it has been shown to have a strong

influence on DM phenotype, primarily in Belgian Blue cattle (Grobet *et al.*, 1998; Dunner *et al.*, 2003; Lines *et al.*, 2009; Druet *et al.*, 2014). Unfortunately, this mutation is associated with some severe negative consequences, notably in the incidence of calving difficulty. Therefore, it is necessary to investigate the other mutations that do not drastically disrupt the function of the *MSTN* gene. The *MSTN*-F94L mutation is an alternative one. This mutation does not cause extreme DM phenotype like the other mutations within the *MSTN* gene. Interestingly, even though this mutation is not considered to cause a loss of *MSTN* function (Grobet *et al.*, 1998), this mutation significantly associated with increased muscle mass and carcass yield as well as meat quality both in pure- and crossbred-Limousin cattle without any associated reproductive problems (Sellick *et al.*, 2007; Abe *et al.*, 2009; Alexander *et al.*, 2009; Lines *et al.*, 2009; Cullen *et al.*, 2010; Lee *et al.*, 2019).

The F94L is a non-synonymous mutation located in exon 1 (c.282C>A) that cause amino acid substitution of phenylalanine to leucine at position 94 (p.Phe94Leu) (Lines *et al.*, 2009). Limousin breed is considered to be a major breed carrying the mutant A allele. The mutant A allele was found to be very high (up to 90-100%) in Limousin cattle, while it was found to be very low (such as in Simmental 0.8%, Piedmontese 2% and Droughtmaster 4%) or even absent in other breeds (Dunner *et al.*, 2003; Vankan *et al.* 2010).

Based on those reports, when the *MSTN*-F94L mutation has known in Indonesian local cattle breeds, it may be used as a DNA marker in selection to increase muscle mass and meat yield without having severe negative impacts. However, the status of the F94L in local cattle in Indonesia has not been reported previously. Therefore, this study aimed to confirm the *MSTN*-F94L mutation in four Indonesian local cattle breeds, including Bali, Sumbawa, Pasundan, and Holstein-Friesian cattle.

MATERIALS AND METHODS

Cattle and DNA Samples

This study involved a total of 518 cattle originated from four Indonesian local cattle breeds as the sample (Bali, Sumbawa, Pasundan, and Holstein-Friesian). Table 1 displays details of the sample in regarding their breeds and origin. Isolation of genomic DNA from the blood sample was assisted by DNA mini kit (Geneaid, Taiwan)

Table 1. Total Samples and Sampling Location of Four Local Cattle Breeds in Indonesia for Detection of *MSTN*-F94L Mutation

Breed	Sampling Location (Districts)	n
Bali	Barru, Enrekang, Nusa Penida island	140
Sumbawa	Sumbawa island	107
Pasundan	Ciamis, Majalengka, Tasikmalaya, Pangandaran	168
Holstein-Friesian	Enrekang, Tasikmalaya, Bogor, Sukabumi	103
	Total	518

by following every step suggested by the manufacturer's manual guide. Then, the DNA products were safely stored at -20°C for further use.

PCR Amplification

A Primer pair, which covered the mutation site of F94L were designed using Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Detailed information on primers is given in Table 2. PCR reaction was performed in a total volume of 10 µL which contains 10 ng/µL of genomic DNA, 4 µL of Go Taq Green Mastermix (Promega, USA), 0.2 µM of each primer and nuclease-free water up to a final volume of 10 µL. As for the thermal cycle conditions, it used a 94°C pre-denaturation for 5 min which successively followed by 35 cycles of amplification at 94°C temperature for 25 s, 53°C annealing for 25 s, 72°C extension for 25 s, and a final extension under a 72°C temperature for 5 min.

PCR-RFLP Genotyping

Detection of alleles and genotypes were performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using the *TaqI* restriction enzyme (Promega, USA). The digestion mixtures and conditions were performed by following the manufacturer's manual guidance. Following the previous step, the amplified and digested DNA fragments were separated using 1% and 2% agarose gels successively and then stained. GelRed®10,000X manufactured by Biotium – USA was chosen to assist this staining stage.

Fragment-visualization band patterns in gels were performed under G-BOX Gel Documentation System (Syngene, UK).

Sequencing

The presence of F94L mutation was confirmed by forward and reverse sequencing reactions from one representing a sample of CC and CA genotype, respectively. Sequencing was performed using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems, CA). Sequenced fragments were purified by DyeEX (Qiagen, Hilden, Germany) and resolved by capillary electrophoresis using an ABI 310 Genetic Analyser (Applied Biosystems, CA). The sequence data were then aligned with GenBank AB076403.1 and analyzed using the Bioedit sequence alignment editor (Hall, 1999).

Data Analysis

Polymorphism indexes such as genotypic and allelic frequencies, expected heterozygosity (H_e) and observed heterozygosity (H_o) were direct calculated according to Nei and Kumar (2000). The genotypic frequency distribution of the Hardy-Weinberg Equilibrium (HWE) deviation was analyzed by the chi-square test.

RESULTS AND DISCUSSIONS

In the present study, the expected PCR product, which covering the *MSTN*-F94L mutation, was successfully amplified with a size of 630 bp. Genotyping of *MSTN*-F94L in all individuals resulted only in two types of restriction patterns, assigned as the CC and CA genotypes, while the AA genotype was not

Table 2. The Pairs of Primers Used to Amplify *MSTN*-F94L Gene in Four Local Cattle Breeds in Indonesia

Mutation	Location	GenBank Accession	Primer sequences (5'-3')	Amplicon size (bp)	Annealing Temperature (°C)
F94L	Exon 1	AB076403.1	F : AGATTCACTGGTGTGGCAAGT R : TTTCCCCTCCTCCTTACATACA	630	53

observed. The CC genotype was characterized by four bands (414 and 216 bp), whereas the CA genotype was characterized by three bands (630, 414, and 216 bp). The restriction pattern of genotypes and its chromatograms are shown in Figure 1.

The genotypic and allelic frequencies of *MSTN*-F94L mutations are summarized in Table 3. In the previous study, the C allele of *MSTN*-F94L was described as a wild-type allele, while the A allele was a mutant allele. This statement has emerged since the C allele was fixed in the control sample (without expression of DM phenotype), which using H-F and Jersey cattle (Grobet *et al.*, 1998). In the present study, the wild-type C allele in all individuals of Bali, Sumbawa, and H-F cattle was to be fixed (1.000). It was different in Pasundan cattle, where two alleles (C and A) were found, although the frequency of the mutant A allele was very low (0.012).

As the consequent of the fixed wild-type C allele in Bali, Sumbawa, and H-F cattle, only homozygous CC genotype (100%) was found in those three cattle breeds. Meanwhile, the frequency of homozygous CC genotype in Pasundan cattle was slightly lower than in three cattle breeds (98% vs. 100%) because of the presence of the mutant A allele. Consequently, heterozygous CA genotype was observed although at a very low frequency (2%). The homozygous AA genotype was not observed in all four local cattle in Indonesia. Genotype distribution in Pasundan cattle was in the HWE. However, the value of heterozygosity is very low ($H_o = 0.024$ and $H_e = 0.024$), which indicates very low polymorphism.

The present study showed that the wild-type C allele and the homozygous CC genotype are common in Bali, Sumbawa, H-F, and Pasundan cattle breeds. The fixed wild-type C allele found in Indonesian H-F cattle was also indicated in H-F

cattle in other countries (Grobet *et al.* 1998; Dunner *et al.*, 2003; Abe *et al.*, 2009; Vankan *et al.*, 2010). It suggested that wild-type C allele has remained in the H-F population since it was first reported to date. The different case has occurred in Charolais and Angus cattle, although some studies have reported that the wild-type C allele is fixed in these cattle breeds (Allais *et al.*, 2010; Vankan *et al.*, 2010), but a mutant A allele has been reported in Charolais cattle (Dunner *et al.*, 2003) or in Angus cattle (Sellick *et al.*, 2006).

The fixed wild-type C allele was also reported occurs in Japanese Black, Jersey, Red Angus, Salers, Shorthorn, Poll Hereford, Gelbvieh, Brahman, Maine Anjou and Nellore cattle (Abe *et al.*, 2009, Vankan *et al.*, 2010; Curi *et al.*, 2012; Lee *et al.*, 2015). Meanwhile, although it has been identified in very low frequency, the Asturiana Valles, Pirenaica, Rubia Gallega, Aubrac, Blonde Aquitaine, Inra95, Parthenaise, Devon, Galloway, Marchigiana, Simmental (0.8%), Piedmontese (2%), Droughtmaster (4%), Rubia Galega x Nellore (2.3%) and Canchim (2.3%) breeds have a history of segregating the A mutant allele (Dunner *et al.*, 2003; Vankan *et al.*, 2010; Curi *et al.*, 2012). Surprisingly, some authors reported that the mutant A allele is very high in Limousin cattle (up to 94.2%) (Dunner *et al.*, 2003; Vankan *et al.*, 2010; Lee *et al.*, 2015). This evidence indicates that the *MSTN*-F94L mutation was exclusively in Limousin breed as identified by Saatchi *et al.* (2014) using high-density SNP assays in 18,274 animals from ten cattle breeds in the United States (US).

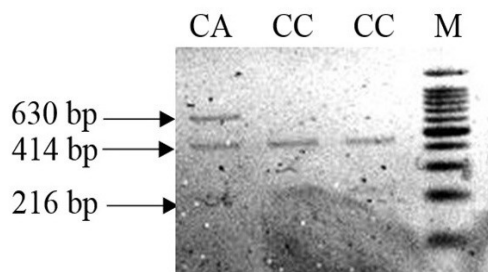
In the case of segregation of the mutant A allele in four individuals of Pasundan cattle, it may be derived from the Limousin or Simmental bulls through artificial insemination (AI). However, this mutant A allele remains allegedly derived from Limousin bulls. This assumption is proposed for two reasons. First, the Pasundan

cattle were the hybrid cattle between *B. javanicus* and *B. indicus* (Agung *et al.*, 2019). It could be seen from the result of the present study that Bali cattle as a representation of *B. javanicus* species and Sumbawa as a representation of *B. Indicus* species were not found to have the mutant A allele, as well as in Nellore cattle (*B. Indicus*) (Curi *et al.*, 2012). Second, according to Vankan *et al.* (2010) and Saatchi *et al.* (2014), the mutant A allele was exclusively in Limousin breed. However, the *MSTN*-F94L mutation in purebred Limousin and Simmental bulls in all Artificial Insemination Center (AIC) in Indonesia needs to be further confirmed.

In Indonesia, the frozen semen collected from Simmental and Limousin bulls are the first

and second of the most popular and favorable semen in the farmer communities to breed their cows. It was supported by frozen semen distribution data from Lembang AIC, where Simmental and Limousin semen straw reached 36.40% (877,415 doses) and 32.93% (793,687 doses) of total semen straw distributed in 2018 (BIB Lembang, 2019). The AI technology application in animal breeding is thought to play a role in gene flow from exotic bulls into several local cattle, including Limousin breed into the Pasundan breed population. Due to the lack of recording data obtained, this occurrence could not be ascertained. However, this presumption could be elucidated by admixture analysis. This analysis will prove the genetics admixture among breeds

a. Restriction patterns of genotypes



b. Chromatogram visualization of genotypes

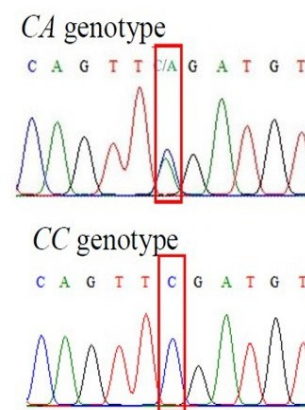


Figure 1. Genotypic visualization of *MSTN*-F94L mutation. a) Restriction patterns of genotypes; b) Chromatogram visualization of genotypes. The bands in figure (a) were separated in a 2.5% agarose gel. CA = CA genotype; CC = CC genotype; M = 100 bp DNA ladder.

Table 3. Genotypic and Allelic Frequencies of *MSTN*-F94L in Four Local Cattle Breeds in Indonesia

Breed	n	Genotypic Frequency (%)			Allelic Frequency		HWE	
		CC	CA	AA	C	A	χ^2_{test}	χ^2_{tab}
Bali	140	140 (100.0)	0 (0.0)	0 (0.0)	1.000	0.000	-	-
Sumbawa	107	107 (100.0)	0 (0.0)	0 (0.0)	1.000	0.000	-	-
Pasundan	168	164 (98.0)	4 (2.0)	0 (0.0)	0.988	0.012	0.024	3.841
Holstein-Friesian	103	103 (100.0)	0 (0.0)	0 (0.0)	1.000	0.000	-	-
Total	518							

n = number of individuals; HWE = *Hardy-Weinberg Equilibrium*; if $\chi^2_{test} < \chi^2_{tab}$ (0.05) means the genotype frequency is in HWE

of cattle even though the phenotype almost similar. Hartati *et al.* (2015) have used it and successfully explained the evidence of hybridization between *Bos javanicus* and *Bos indicus* in Peranakan Ongole cattle (PO).

Many studies have proven the benefit of *MSTN*-F94L mutation primarily in increasing meat yield and quality in pure- and crossbred Limousin cattle. Esmailizadeh *et al.* (2008) reported that the mutant A allele of *MSTN*-F94L mutation in Limousin backcross cattle (Jersey × Limousin or Limousin × Jersey) has increasing significantly to meat weight (up to 7.3%) and reducing fat depth (up to -18.7%), intramuscular fat content (up to -8.2%), and total carcass fat weight (up to -16.5%). A similar result for the superiority of the mutant A allele has been reported by Abe *et al.* (2009) in the enhancement of muscle mass and carcass yield in crossbred cattle (F2 Limousin x Japanese Black). The positive effect was also found in muscle-related traits by Sellick *et al.* (2007), where the Limousin cattle were used as a parent in the backcross population. Lines *et al.* (2009) reported that AA genotype was a 9.8% more tender than CC genotype in *semitendinosus* muscle samples collected from backcross Limousin x Jersey cattle.

Furthermore, in a genomic prediction study, the evidence has been validated by Lee *et al.* (2019) using random or fixed effects and ignoring the genotypes as treatments. Then it was reaffirmed by Purfield *et al.* (2019) using whole-genome association analyses with a high-density SNP chip (777,962 SNPs). All these findings suggested that animal were carrying the mutant A allele shown to be superior to wild-type C allele to produce high lean meat with a higher meat yield and a lower fat yield in beef cattle.

Since many studies have shown the strong evidence and validated the superiority of *MSTN*-F94L mutation, thus it has become a consideration for selecting bulls and selling frozen semen by Limousin breeders in several developed countries. Indeed it has commercially used in DNA testing service in several Limousin cattle breeder societies such British Limousin Cattle Society (limousin.co.uk), North American Limousin Foundation (nalf.org), and Australian Limousin Breeders' Society (limousin.com.au).

According to the results of the present study, it could be stated that the wild-type C allele of *MSTN*-F94L tends to be fixed in four local cattle breeds in Indonesia. It may also occur in other

local cattle breeds in which the *Bos javanicus* and *Bos indicus* as the common ancestors. Therefore, the *MSTN*-F94L mutation may not be used as a genetic marker within the Indonesian local cattle breeds. The crossbreeding program is an alternative one. The Limousin bulls that exist in several AIC in Indonesia (if it is known to carry the *MSTN*-F94L mutation) have the potential to be used in genetic improvement of meat-related traits in the commercial beef population in Indonesia. Marker-assisted introgression method may be used in the Limousin backcross breeding program to improve the heterosis effect efficiently.

CONCLUSION

In conclusion, no polymorphism of the *MSTN*-F94L was found in Bali, Sumbawa and HF cattle, but was found in very low polymorphism in Pasundan cattle. The wild-type C allele tended to be fixed in all four cattle breeds in Indonesia. Some individuals of Pasundan cattle carrying the mutant A allele may be caused by segregating from the Limousin bulls. Further investigation in other local breeds, exotic breeds, and its crossing will answer the status of the *MSTN*-F94L mutation in Indonesian local cattle breeds.

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