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Determination of Complete Sequence Mutation of Myostatin Gene in Fast- and Slow-Growing Chicken

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ABSTRACT

Myostatin plays a role in inhibiting skeletal muscle growth in vertebrates. This study aimed to investigate the full sequence of the myostatin gene in fast-growing and slow-growing chickens. Fast- and slow-growing chicken models were produced from F2 Kampung x broiler. The full sequence of the myostatin gene was identified using 24 pairs of primers covering about 8,000 bp. mRNA expression analysis of muscle tissue was performed to examine whether the expression levels of myostatin are affected by chicken lines, sex, or muscle type. The results showed 170 mutations in fast- and slow-growing chickens. One hundred and sixty-one of them are novel mutations. A total of five and twenty-two alleles were specific alleles found only in the fast-growing and slow-growing groups of chickens, respectively. There were no differences in amino acids and gene expression levels of myostatin between the fast- and slow-growing chickens. In summary, the results of this study showed that specific alleles for the fast-growing or slow-growing chicken groups were found, suggesting that these specific alleles potentially be used as genetic markers for muscle growth in chickens.

1. Introduction

Myostatin, also known as growth and development factor-8 (GDF-8), is a negative regulator of muscle growth and belongs to the transforming growth factor-beta (TGF-β) superfamily (McPherron *et al.* 1997). Myostatin suppresses myoblast proliferation and differentiation during embryogenic myogenesis (Joulia *et al.* 2003; McPherron *et al.* 1997; Thomas *et al.* 2000). It also suppresses muscle hypertrophy in postnatal muscles by regulating muscle protein synthesis and degradation (Lipina *et al.* 2010; Morissette *et al.* 2009; Welle *et al.* 2009, 2011). Myostatin is also implicated in satellite cell activation and postnatal regeneration as a negative regulator (Aiello *et al.* 2018). In mice, myostatin knockout increases muscle mass by hyperplasia and hypertrophy (Lee and McPherron 2001). Extreme muscle growth has been observed in several animal species due to natural non-functional mutations in the myostatin gene, i.e., Cattle (Grobet *et al.* 1997; Kambadur *et al.* 1997, McPherron and Lee 1997; Smith *et al.* 2000), sheep (Clop *et al.* 2006), dogs (Mosher *et al.* 2007), and humans (Schuelke *et al.* 2004). Unlike these animal species, the natural non-functional mutations in myostatin that cause extreme muscle growth have not been reported in chickens.

Like mammals, the chicken myostatin gene contains three exons (492, 374, and 1567 bp, respectively) (Aiello *et al.* 2018). The coding region (CDS) sequence of the chicken myostatin gene, starting from methionine (ATG) to stop codon (TGA), consists of 1128 bp of nucleotides (including 373, 374, and 381 bp in exons 1, 2, and 3, respectively) which are translated into 375 amino acids precursor protein (pro-myostatin). This myostatin precursor comprises a signal peptide, a propeptide region (NH_2-terminal), and a mature myostatin domain (COOH-terminal). The myostatin prodomain and mature protein regions are separated by a furin

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proteolytic site, namely RXXR (Shi *et al.* 2011). The C-terminal domain consists of 109 amino acids and nine cysteine residues (Lee 2010).

To produce mature myostatin, two proteolysis processes are required. First, the furin protease cleaves the RXXR site (it is RSRR in chicken) on the precursor protein to produce a latent myostatin complex, where mature myostatin binds to the propeptide noncovalently. Mature myostatin is still inactive at this phase. Second, the bone morphogenetic protein-1 (BMP-1)/tolloid family of metalloprotein breaks the bond between the latent myostatin complex and releases mature myostatin out of the cell (Shin *et al.* 2015). Mature myostatin is active when it is in the dimeric phase. Thus, the extracellular mature myostatin can suppress muscle growth both in the prenatal and postnatal phases (Rodriguez *et al.* 2014).

Studies have shown that myostatin genetic variations were associated with body weight and carcass traits, demonstrating that myostatin is a potential candidate gene for chicken carcass traits (Khaerunnisa *et al.* 2016; Tanjung *et al.* 2019). Significant associations were found between myostatin gene polymorphisms and carcass characteristics in the population of F2 broiler x Indonesian Kampung chicken cross (Khaerunnisa *et al.* 2016). However, at the genomic level, polymorphism information was obtained only on incomplete sequences of the myostatin gene. To get more information about mutation in the myostatin gene and its effect on chicken growth, it is important to carry out thorough, complete sequence analysis of myostatin genes. Moreover, validation at the transcriptomic level is also required for a better understanding of the role of myostatin as a candidate gene for controlling muscle growth in chickens. This study aimed to investigate the complete sequence of the myostatin gene in fast-growing and slow-growing chickens.

2. Materials and Methods

2.1. Animal Sampling and Grouping

To reduce the effects of environmental factors, all chickens were kept under the same conditions. Chickens were reared in groups with a density of 40-45 birds/m^3 and 7-10 birds/m^3 for starter (0-3 weeks) and grower (>3 weeks), respectively. Diet was prepared to contain 19.0% and 17.5% crude protein for starter and grower, respectively. The Animal Care and Use Committee at IPB University authorized all of the procedures performed in this study.

Assortative mating was carried out using male Indonesian Kampung chicken and female parent stock strain Cobb to produce a population of F1 Kampung x broiler. The males and females were mated to produce an F2 Kampung x broiler (n = 62). The F2 male and female chickens were sorted based on body weight at 12 weeks old. Grouping based on body weights was carried out with the following selection criteria: a) the fast-growing group had a bodyweight at 12 weeks above the mean plus standard deviation, and b) the slow-growing group had a bodyweight at 12 weeks below the mean value minus the standard deviation. A total of 12 chickens that met the above criteria were selected, including six for each slow- and fast-grow group, with three males and three females in each group.

At 12 weeks old, blood was collected from the wing vein. Animals were then sacrificed to collect the carcass characteristics data. Carcass component data consisted of live weight, carcass weight and percentage, weights and percentages of commercial cuts (breast, thighs, drumsticks, and wings), and weight of meat (breast, thighs, and drumsticks). In addition, physicochemical characteristics of the breast muscle (Pectoralis major) were examined by measuring pH, amount of free water, meat tenderness, and cooking loss.

2.2. Genomic DNA Isolation, Myostatin Gene Amplification, and DNA Sequencing

The genomic DNA was isolated using the phenol-chloroforms DNA isolation method. For the amplification of the myostatin gene, a PCR mix was prepared for each sample containing 50 ng/µl DNA sample, 10 µM each primer (IDT, Coralville, IA, US), 1 unit of Q5 High-Fidelity 2x Master Mix (NEB, Ipswich, MA, US), and water up to 50 µl. The C1000 Touch™ Thermal Cycler (Biorad, Hercules, CA, US) was used for amplifying the DNA target. First, the 8.7 kb of the DNA sequences of the chicken myostatin gene were amplified into 24 gene fragments, which were separated by 24 pairs of unique primers (Table 1) generated using the Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on GenBank (accession number: AF346599.2). Then, a 1.2 percent agarose gel was used to visualize the DNA. Finally, the 3730XL DNA Analyzer

Table 1. Primer sequences for chicken myostatin gene based on the GenBank (accession number: AF346599.2)

Fragment no.	Primer sequence (5'-3')		Tm (°C)	Coverage (bp)	Product size (bp)	Position in gene*
	Forward	Reverse				
01	GCCAAATCCAAAGAACCAACCAA	TCATAGCATACTGTTTTAAGCAGACTC	60	1-185	185	P
02	CGTGAATCATCCTATCCACCGT	TATTGCCTTTCCAGGGCACAG	60	73-536	464	P
03	TCAACTTTACACCATGGAAGGACA	GTATGCTTTCTGCATGAGATGCTC	60	408-960	553	P
04	GCAGCATGCCCAATGTCATC	GTGCATTAAGCAGCTCAGATTTCAG	60	861-1453	593	P
05	AGAGGGTCCAATAGTTAGCACTT	GCTCATGTCGTCAAAACCGAC	60	1336-1878	543	P
06	AATCAGTTCACCCTTGGCTGT	GCTGCTTAATAACGTCCCTGC	60	1755-2288	534	P-E1
07	GTGGCTCTGGATGGCAGTAG	GCTACAGGGCACACACGTTA	64	2111-2659	549	E1-I1
08	AGGGAGGGAGGAGAGTGTATT	GCACGACTACTGGAGCTGATT	60	2544-3052	509	I1
09	AGCACCACCCACCTCTAAGT	TGGGCACAATAGGCTGGAAT	60	2945-3463	519	I1
10	ATAATATCAGCTCCTGCAGCCA	AATAACCTCAGGGTGCTGTGT	65	3310-3896	587	I1
11	CACTGCACGTGACTTTGCTAT	ACTGTAGCTGATCCCTTGCG	64	3739-4240	502	I1
12	CTGCCTGTACACATCGCTCA	AGGATCTGCACAAACACCGT	60	4182-4665	484	I1-E2
13	CAAATGGAGGGAAAACCAAAATGTTG	AAAATGGAGGCACAGGAATGC	60	4532-5076	544	E2-I2
14	TGCTTCGAGAATGGCAGATGA	GTTTGAGCTGTTTCAGTAAGTTCAG	60	4977-5631	654	I2
15	TCCCTATTCTGCATGCATTTTCCA	GGTGGTGTGGCCAGTATTGAT	60	5318-5863	546	I2
16	GCAGTGAAGTGCAGGTGAAAA	TGTCAGCACAGTGTTCCTGTT	60	5741-6238	498	I2
17	AGTAGTGGGTAGCTGGACTGT	CCAAGCAGTGTCGGATTCCC	60	6162-6672	511	I2
18	ACCACCACTCCACCAAACTC	ACTTTCTTCCCTTCTGGGAACTT	60	6519-7036	518	I2
19	TGAGACAGTGATGATTTCAGGAAG	CTCTGGGATTTGCTTGGTGT	60	6876-7424	549	I2-E3
20	ACGGAATCCCGATGTTGTCG	TTCAAAGCCAGCCCTTAGGT	60	7253-7768	516	E3
21	GTGAAATTACGTACGCTAGGCA	AGCACCATTGCCATTGATTACT	60	7640-8187	548	E3
22	ACACCACTACACATTACCACCA	TGGCTTATCATAGTGCAAACGG	60	8007-8432	426	E3
23	AGGCAGCACGGAAGTCTGAT	ACCTGTGGTTTACTCTGTTGCAC	60	8347-8688	324	E3
24	GTAATGGTAACCTGTCCTTTAGTTTAAT	TCATGCCAATGTTAAAAAAAAAA	60	8573-8743	170	E3

*P = promoter, E1 = exon 1, I1 = intron 1, E2 = exon 2, I2 = intron 2, E3 = exon 3

(Applied Biosystems, Waltham, MA, US) was used to sequence a fragment of the myostatin gene.

2.3. Analysis of Myostatin mRNA Expression

Total mRNA was obtained from the pectoralis major and biceps femoris muscles using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Germantown, MD, US) and reverse transcriptase were performed to obtain cDNA using the First Strand cDNA Kit (Thermo Scientific, Waltham, MA, US). In addition, Myostatin and β-actin-specific primers were modified from Song *et al.* (2011) (Table 2). cDNA was used for the myostatin gene's relative quantification (qPCR) using a real-time PCR machine (StepOne Plus, Applied Biosystems). PowerUp SYBR Green Master Kit (Applied Biosystems) was used for the qPCR reaction. The mean of the ΔC_T values was used for mRNA expression profiling after all samples were evaluated in three technical replications.

Table 2. Primer sequences of myostatin and β-actin for gene expression analysis

Gene	Primer sequence (5'-3')*	PCR product (bp)
Myostatin	F: GCTTTTGATGAGACTGGACGAG	173
	R: AGCGGGTAGCGACAACATC	
β-*Actin*	F: CCTGGCACCTAGCACAATGA	194
	R: TGGGTGTTGGTAACAGTCCG	

*F = forward, R = reverse

2.4. Data Analysis

2.4.1. Myostatin Gene Sequence Alignment

All sequencing results were evaluated using MEGA X (Kumar *et al.* 2018) and BioEdit (Hall 2011). The amino acid sequences were estimated by aligning the DNA sequence with the chicken myostatin mRNA sequence in GenBank (accession number: AY448007.1). The combination of alleles in the coding sequence can be determined through haplotype analysis. A haplotype is defined as a combination of alleles at loci found on unpaired chromosomes or DNA molecules (Allendorf *et al.* 2013). Fluxus DNA Alignment software, Network 5.0.0.0 (Foerster *et al.* 2016), and DnaSP 6 were used to determine the haplotype coding sequence of the myostatin gene (Rozas *et al.* 2017).

2.4.2. Gene Expression Data Analysis

The mRNA expression of myostatin was shown in delta C_T (ΔC_T) values (Silver *et al.* 2006). The endogenous control gene (β-actin) was used to normalize myostatin gene expression, and ΔC_T was calculated by subtracting C_T values of β-actin from the myostatin gene C_T values.

2.4.3. Statistical Analysis

The difference in the average parameters of the fast and slow-growing groups of chickens was analyzed using the T-test with SAS 9.4 (SAS Institute 2013). All data are presented in mean ± standard error. To examine the effects of the line (fast- and slow-growing), sex (male and female), and muscle type (breast and thigh muscles), and their interactions with myostatin mRNA expression, the three-way ANOVA procedure was performed using SPSS ver. 28.

3. Results

3.1. Fast- and Slow- Growing Chicken Carcasses Properties

The carcass characteristics of fast and slow-growing groups were analyzed at 12 weeks old. These two groups of chickens showed a significant difference in live weight (1549 vs. 856 g for males and 1354 vs. 839 g for females), carcass weight (989 vs. 507g for males and 951 vs. 501 g for females, and carcass components, but not in the physicochemical characteristics of both male and female chickens (Table 3).

3.2. Discovery of Mutations in Myostatin Gene

A total of 170 mutations were discovered in the myostatin genes of cross-breed chicken F2 broilers, consisting of 148 point mutations (87%, Figure 1) and 22 insertions/deletions (13%, Figure 2). Of the 148 point-mutations found, 51 (34.5%) were transversion mutations and 97 (65.5%) transitional mutations. There were 40, 10, 11, 20, and 89 mutations discovered in the promoter area, 5'UTR, coding region, 3'UTR, and intron, respectively.

A total of 22 indel mutations were found in this study (Figure 2). The indel mutation varies from insertion or deletion of 1 to 11 nucleotide bases. The indel mutations were spread over the promoter, 5'UTR, intron 1, intron 2, and 3'UTR. The 11 bp insertion ($g.4923_{B}_4923_{L}$insTTAGTGTTTTT) was found at the beginning of the intron 2 sequence, which is directly adjacent to exon 2. This insertion was found in our samples but not in the reference sequences at GenBank (accession number: AF346599.2). Three indel mutations in intron 2 were specific mutations in slow-growing chickens, namely g.5356_5361delTTATGA, $g.6067_{B}_6067_{C}$insCT, and $g.6699_{B}_6699_{G}$insATTGTT (Figure 2).

Some of the mutations in this study resulted in alleles found only in the fast- or slow-growing group. A total of 44 specific alleles were found in this study (Table 4), i.e., five specific alleles for fast-growing chickens and 22 specific alleles for slow-growing chickens. The mutation findings that produce these specific alleles can be used as SNPs for genetic markers of high and low-weight chickens.

3.3. Promoter Region and 5'UTR Profile of Myostatin Gene

The length of the promoter area successfully amplified in this study was about 1.8 Kb. A total of 37 SNPs and three indels were found in this promoter area (Figures 1 and 2). The TATA and CAAT boxes were

Table 3. Carcass characteristics of fast and slow-growing chicken at 12 weeks-old

Parameter	Fast-growing (n = 6)		Slow-growing (n = 6)		Significance		
	Male	Female	Male	Female	Line	Sex	Line x sex
LW (g)	1549.00±95.04	1354.00±36.09	855.67±47.67	839.33±13.37	**	NS	NS
CW (g)	989.00±48.99	951.67±29.73	507.00±20.65	501.33±17.37	**	NS	NS
BW (g)	272.67±16.41	306.00±10.69	130.00±15.14	134.33±7.36	**	NS	NS
TW (g)	182.67±12.45	166.33±2.19	84.67±3.48	89.33±0.67	**	NS	NS
DW (g)	180.00±5.51	156.00±2.89	87.33±2.33	86.33±4.18	**	*	*
WW (g)	132.33±8.09	119.67±3.53	74.00±1.53	76.00±3.79	**	NS	NS
BMW (g)	190.67±18.89	215.00±13.89	86.33±8.37	90.33±4.48	**	NS	NS
P. Ma (g)	137.00±14.19	155.67±9.33	60.00±5.20	64.33±3.33	**	NS	NS
P. Mi (g)	54.33±4.81	61.67±2.67	25.00±2.65	27.00±2.31	**	NS	NS
TMW (g)	140.67±8.69	129.67±1.45	60.67±1.86	65.00±0.58	**	NS	NS
DMW (g)	121.00±6.66	107.33±1.45	53.33±2.60	54.00±1.53	**	NS	NS
pH	5.30±0.06	5.35±0.03	5.45±0.06	5.40±0.07	NS	NS	NS
CL (%)	37.26±1.35	33.90±0.20	32.05±1.41	35.50±0.31	NS	NS	**
WB (kg/cm^2)	2.41±0.38	2.50±0.12	2.03±0.12	2.49±0.24	NS	NS	NS
FW (mg H_20)	91.33±4.16	94.74±3.07	91.79±2.90	91.56±3.94	NS	NS	NS
FW (%mg H_20)	29.11±0.26	30.58±0.07	29.92±0.33	30.19±1.12	NS	NS	NS

Brief description of statistical analysis. LW = live weight at 12 weeks-old, CW = carcass weight, BW = breast weight, TW = thighs weight, DW = drumsticks weight, WW = wings weight, BMW = breast muscle weigh, P.Mi = Pectoralis major muscle weight, P.Mi = Pectoralis minor muscle weight, TMW = thighs muscle weight, DMW = drumsticks muscle weight, CL = cooking loss, WB = warner bratzler's shear force, FW = free water, brackets indicate the number of samples observed. Data are mean (SD or SEM), *, P<0.05, **, P<0.01, NS = not significant

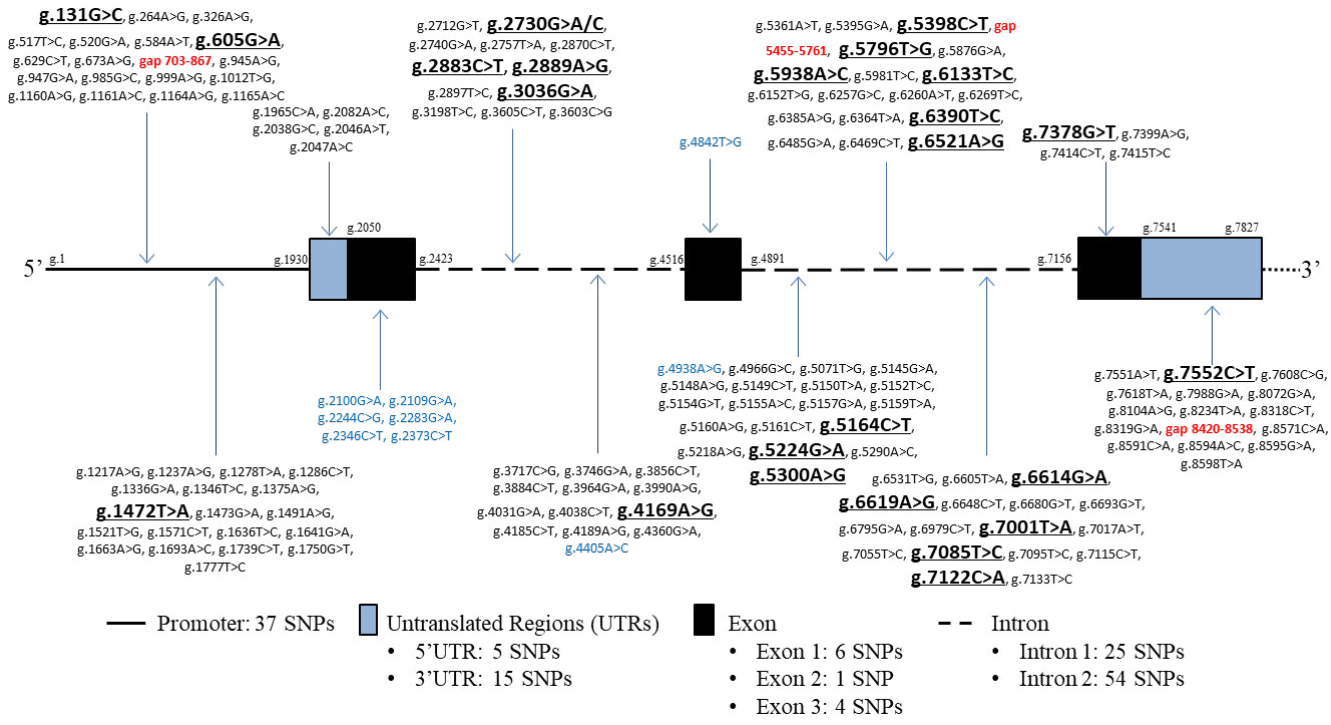

Figure 1. The distribution of novel point mutations found in the myostatin gene sequences fast- and slow-growing chicken. The mutation point number is based on GenBank (access no. AF346599.2), gap (red color) indicates unreadable sequence, blue color indicates previously reported mutation, bold with underline indicates specific mutation

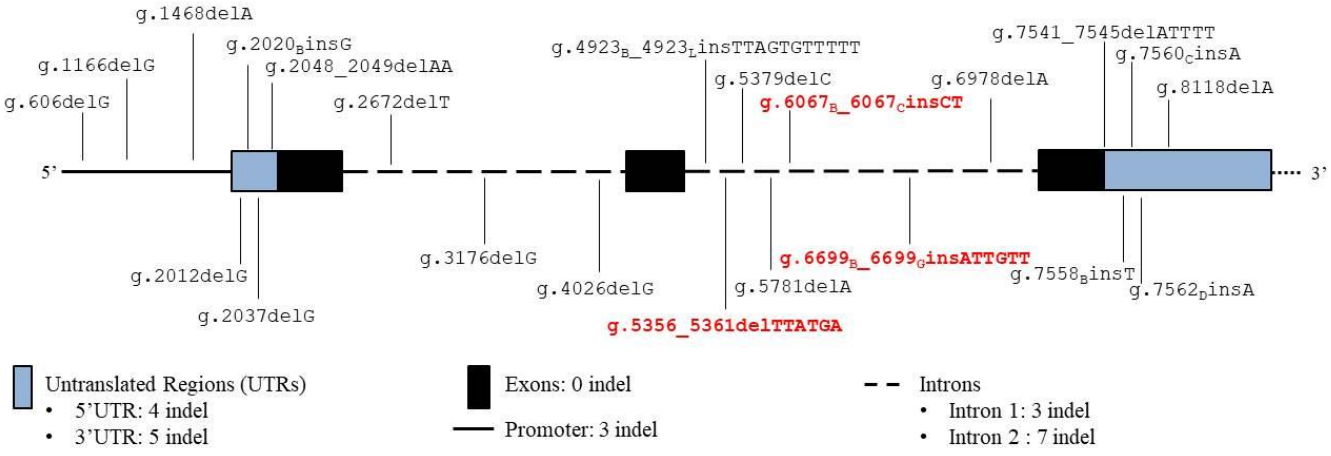

Figure 2. Distribution of indel (insertions/deletions) mutations found in the myostatin gene sequences of fast- and slow-growing chicken. The mutation point number is based on GenBank (access no. AF346599.2), red color indicates the specific mutation

also found in the core promoter area of the myostatin gene, at positions -156th and -199th from the start coding sequence, respectively, or at positions 1894 and 1851 according to GeneBank (access no: AF346599.2), respectively (Figure 3). There were no sequence differences between fast-growing, slow-growing, and Indonesian Kampung chicken groups in the TATA and CAAT box areas (Figure 3). Meanwhile, the length of the 5'UTR region successfully amplified in this study was 119 bp (Figure 3). 5 SNPs and four indels were found along the 5'UTR area (Figures 1, 2, and 3).

3.4. Mutations in the Coding Region of the Myostatin Gene

In this study, a complete coding region of myostatin has been successfully analyzed, covering 1128 bp of nucleotides, including 373, 374, and 381 bp in exons 1,

Table 4. The specific mutation of myostatin gene in fast- and slow-growing chicken groups

Mutation position	Type	Location	Distribution of alleles in chickens		
			Fast growing (n = 6)	Slow growing (n = 6)	Ref[£]
g.131G>C	Transversion	P	G	G/C*/S	G
g.605G>A	Transition	P	G	G/R*	G
g.673A>G	Transition	P	A	A	A
g.1012T>G	Transversion	P	T	T	T
g.1217A>G	Transition	P	A	A	A
g.1286C>T	Transition	P	C	C	C
g.1472T>A	Transversion	P	T	T/W*	T
g.1663A>G	Transition	P	A	A	A
g.2712G>T	Transversion	I1	G	G	G
g.2730G>A/C	Transition/transversion	I1	G/R*/S*	G	G
g.2870C>T	Transition	I1	C	C	C
g.2883C>T	Transition	I1	C/Y*	C	C
g.2889A>G	Transition	I1	A/R*	A	A
g.2897T>C	Transition	I1	T/Y	C/T/Y	T
g.3036G>A	Transition	I1	G	G/R*	G
g.3198T>C	Transition	I1	Y	Y	T
g.3717C>G	Transversion	I1	C	C	C
g.4169A>G	Transition	I1	A	A/R*	A
g.4185C>T	Transition	I1	C	C	C
g.4189A>G	Transition	I1	A	A	A
g.5164C>T	Transition	I2	C	C/Y*	C
g.5224G>A	Transition	I2	G	G/A*	G
g.5300A>G	Transition	I2	A	A/R*	A
g.5356_5361delTTATGA	Indel	I2	ins	Ins/del*	ins
g.5398C>T	Transition	I2	C	C/T*/Y	C
g.5796T>G	Transversion	I2	T	T/K*	T
g.5938A>C	Transversion	I2	A	A/C*	A
$g.6067_{B}_6067_{C}$insCT	Indel	I2	del	del/ins*	del
g.6133T>C	Transition	I2	T	T/C*	T
g.6390T>C	Transition	I2	T	T/Y*	T
g.6521A>G	Transition	I2	A	A/R*	A
g.6614G>A	Transition	I2	G/R*	G	G
g.6619A>G	Transition	I2	A	A/R*	A
$g.6699_{B}_6699_{C}$insATTGTT	Indel	I2	del	del/ins*	del
g.7001T>A	Transversion	I2	T	T/W*	T
g.7055T>C	Transition	I2	T	T	T
g.7085T>C	Transition	I2	T	T/Y*	T
g.7095T>C	Transition	I2	T	T	T
g.7122C>A	Transversion	I2	C/A*	C	C
g.7133T>C	Transition	I2	T	T	T
g.7378G>T	Transversion/synonymous	E3	G	G/K*	G
g.7552C>T	Transition	3'UTR	C	C/Y*	C
g.7618T>A	Transversion	3'UTR	T	T	T
g.7988G>A	Transition	3'UTR	G	G	G

P = promoter, E = exon, I = intron, UTR = untranslated region, A = adenine, C = cytosine, G = guanine, T = thymine, R = A or G, Y = C or T, S = G or C, W = A or T, K = G or T, M = A or C, *specific alleles of fast and slow growing chicken, [£]reference allele based on GenBank (access no.: AF346599.2)

2, and 3, respectively. In the coding area, eleven point mutations were discovered (Table 5). Since it will be translated and transcribed into mRNA and amino acids that compose the protein myostatin, the DNA sequence in the coding region can be critical when the point mutation changes the amino acid. One of the mutations, the g.4842T>G located in exon 2, is a non-synonymous mutation. This mutation causes a change of the amino acid Leucine to Arginine.

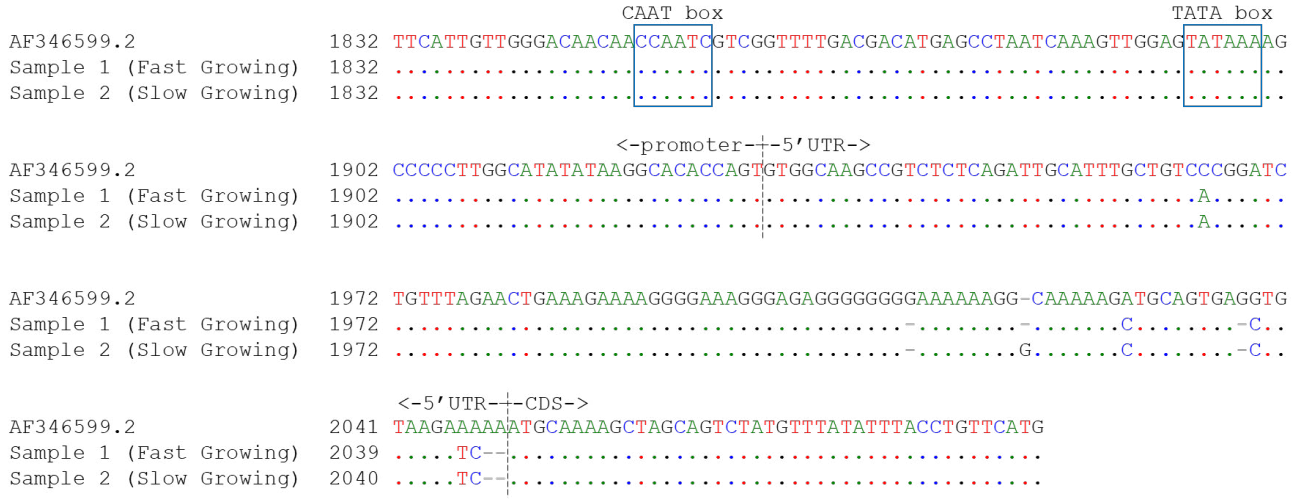

Figure 3. Nucleotide sequence of the 5'UTR and partial promoter of the myostatin gene in chicken. Boxes show the conserved CAAT and TATA boxes. Nucleotide numbering according to GeneBank (access no.: AF346599.2). CDS: coding sequence

Table 5. Distribution of alleles found in the coding region

Mutation position	Location	Distribution of alleles in chickens		
		Fast growing (n = 6)	Slow growing (n = 6)	Ref#
g.2100G>A (c.51G>A)	E1	G/R	G/A/R	G
g.2109G>A (c.60G>A)	E1	G/A/R	G/R	G
g.2244C>G (c.195C>G)	E1	C/G/S	C/G/S	C
g.2283G>A (c.234G>A)	E1	A	A	G
g.2346C>T (c.297C>T)	E1	C/Y	C/Y	C
g.2373C>T (c.324C>T)	E1	C/Y	C/T/Y	C
g.4842T>G (c.699T>G)	E2	T/K	T/G/K	T
g.7378G>T (c.966G>T)	E3	G	G/K*	G
g.7399A>G (c.987A>G)	E3	A/R	A/R	A
g.7414C>T (c.1002C>T)	E3	C	C	T
g.7415T>C (c.1003T>C)	E3	T	T	C

E = exon, A = adenine, C = cytosine, G = Guanine, T = thymine, R = A or G, Y = C or T, S = G or C, W = A or T, K = G or T, M = A or C, *specific allele of slow compared to fast growing chicken group, #reference allele based on GenBank (access no.: AF346599.2)

A total of 12 haplotypes were found in the coding sequences from a combination of eleven alleles (Table 6). Apart from SNPs, haplotypes can also be used as a marker of genetic diversity. Among the 12 haplotypes found, two were specific to the fast-growing chicken group, i.e., the H4 and H9 haplotypes. Five other haplotypes were specific to the slow-growing chicken group, i.e., H6, H7, H10, H11, and H12 haplotypes. Meanwhile, other haplotypes, i.e., H1, H2, H3, H5, and H8, were found in both fast- and slow-growing chicken groups.

3.5. Myostatin mRNA Expression

A significant sex effect on MSTN expression was observed (Table 7) with a higher ΔC_T value in male than female chickens, indicating that male chickens produced lower myostatin transcripts than female chickens. No significant effects of line and muscle type were found on myostatin expression (Table 8). The analysis also showed significant effects of line x muscle type (P<0.05) and sex x muscle type (P<0.01) on myostatin expression (Table 8). The interaction effect of sex and muscle type showed that in male chickens, ΔC_T of breast muscle was higher than that of leg muscle. Still, in female chickens, the ΔC_T of breast muscle was lower than that of leg muscle or no difference, suggesting myostatin is expressed differentially among muscles. The differential expression is affected by sex.

4. Discussion

The myostatin gene has been widely studied in humans and cattle, and the myostatin gene has been shown to have a strong role in regulating muscle tissue growth and various health conditions associated with muscle growth. However, studies on the myostatin gene in chickens are still limited.

Table 6. Nucleotide changes among haplotypes in the coding regions of the chicken myostatin gene

Haplotype	Nucleotide position in coding region										
	51	60	195	234	297	324	699	966	987	1002	1003
Ref#	G	G	C	G	C	C	T	G	A	C	T
H1	A	A	G	A	C	C	C	T	A	T	C
H2	G	G	C	A	C	T	T	T	A	T	C
H3	G	A	G	A	C	C	C	T	A	T	C
H4*	G	A	C	A	C	C	C	G	A	T	C
H5	G	G	C	A	C	C	C	T	A	T	C
H6**	G	A	G	A	C	C	C	G	A	T	C
H7**	A	G	C	A	C	T	T	T	A	T	C
H8	G	G	G	A	T	C	C	T	G	T	C
H9*	G	G	G	A	C	C	C	T	A	T	C
H10**	G	G	G	A	C	C	C	G	A	T	C
H11**	G	G	G	A	T	C	C	G	A	T	C
H12**	G	G	C	A	C	C	C	T	A	T	C

H1-H12 = Haplotypes 1-12, A = adenine, C = cytosine, G = guanine, T = thymine, *specific haplotypes of fast-growing chicken, **specific haplotypes of slow-growing chicken, #reference allele based on GenBank (access no.: AF346599.2)

Table 7. Effect of line, sex and muscle type on MSTN gene expression (delta Ct±SD)

Sex	Muscle type	Slow	
		Slow	Fast
Male	Breast	5.88±0.63	5.61±0.81
	Leg	7.21±0.38	7.03±1.00
Female	Breast	4.20±0.24	3.33±0.47
	Leg	2.38±0.29	3.78±0.79

Table 8. Result of statistical analysis

Factors	F	P-value	Sig.
L	0.008	0.931	Ns
S	136.383	<0.001	**
M	1.814	0.197	Ns
LxS	0.914	0.353	Ns
LxM	5.234	0.036	*
SxM	16.016	0.001	**
LxSxM	4.474	0.050	Ns

$F_{(0.05;1)} = 4.49$, $F_{(0.01;1)} = 8.53$, L = line (fast- and slow-growing), S = sex (male and female), M = muscle type (breast and thigh muscles). *, P<0.05, **, P<0.01

Thus this study was designed to examine the role of the myostatin gene on chicken muscle growth by full sequence analysis of the myostatin gene in fast-growing and slow-growing chickens.

In this study, we designed animal models of fast- and slow-growing chickens by crossing the Indonesian Kampung chicken with commercial broiler chicken. This crossing produced F2 populations. The average live weight of the entire F2 population was 1,089.27 g. This value is between the body weight of Kampung chickens and broilers at the same age. This average live weight is above the average live weight for Kampung chickens, but below the average live weight for broilers. The body weight of the F2-crossed chickens is 19.4% higher than the weight of the 12-week-old free-range chicken from a study reported by Suhartati *et al.* (2020), which is around 912.33 g. Other studies have reported body weights of Kampung chickens at the same age ranging from 718.34 to 742.34 g (Rahmadani *et al.* 2015). The average body weight of the F2-crossed

chickens is lower than the weight of Cobb broilers at the same age, which is 2,822.20 g (Suhartati *et al.* 2020). The physicochemical characteristics of F2-crossed chicken breast were not significantly different from Kampung chicken and Cobb strain broilers as reported by Suhartati *et al.* (2020).

Total 170 mutations were found in this study and 161 of them had not been previously reported (94%). The nine point-mutations identified in this study have been reported previously by Bhattacharya and Chatterje 2013, Dushyanth *et al.* 2016, Khaerunnisa *et al.* (2020), and Ye *et al.* (2007). Ye *et al.* (2007) reported a non-synonymous mutation, the g.4842T>G located in exon 2, which was also found in this study. This mutation alters amino acid Leucine to Arginine that affects growth traits of broiler chicken (Ye *et al.* 2007) and in Indonesian chicken (Khaerunnisa *et al.* 2016).

A total of 12 haplotypes were found in the coding sequences from a combination of eleven alleles. A total of twelve haplotypes were also reported in the PD-1 broiler, the CL layer, and the Indian native Aseel (Dushyanth *et al.* 2016). Whereas in other populations, namely PB-1 and CB broilers and IWI layer chickens, 13 haplotypes were found in the coding region of the myostatin gene (Bhattacharya and Chatterjee 2013). Both are a combination of eleven alleles. Three of the eleven alleles were also found in native F2 chickens and broilers, namely c.195, c.234, and c.324.

Most mutations found in this study was intronic mutation (more than 52%). Introns are reproduced into RNA by transcription, but intron sequences are not involved in protein-coding sequences (Jo and Choi 2015). Most mutations occur in introns since these intronic mutations can be tolerated or ignored (Chorev and Carmel 2012). Introns cover about 40% of the gene sequence, this indicates that randomly-occurring mutations happen more likely in the intron region, resulting in no change in protein sequences and their function. However, studies also indicate that many of the functional SNPs are intronic mutations, which are located less than 30 bp from the closest splicing site of genes and affect the transcription process or the splicing efficiency of these genes (Singh and Cooper 2012).

This study found specific alleles that were only found in the fast-growing chicken group or the slow-growing chicken group, i.e 5 and 22 alleles, respectively. These specific alleles may potentially be a genetic marker of muscle growth in chickens, if these alleles are proven by further studies in other chicken populations.

The results of this study indicated that there were no significant differences in gene expression and amino acid sequences of myostatin between fast- and slow-growing groups of chickens. Allegedly, the physiological function of myostatin in not only affected by the myostatin gene expression but also affected by other molecules, i.e TGF-β1(Zhu *et al.* 2007), decorin (Zhu *et al.* 2007), follistatin and follistatin associated genes (Lee and McPherron 2001), myostatin propeptide (Haq *et al.* 2013; Lee *et al.* 2016, 2017), Bone Morphogenetic Protein-1 (Lee 2010), Tolloid (Lee 2010), and Tolloid-like-1 and 2 (Lee 2010).

Myostatin regulation also appears to be influenced by the alternative splicing variations found in poultry species (Shin *et al.* 2015). Shin *et al.* (2015) found five alternative splicings in PD-21 chickens, namely MSTN-A, B, C, D, and E. Of the five, only MSTN-A and D produced mature myostatin domains. While the other three only produce prodomains. Alternative splicing of myostatin which lost the mature myostatin domain had higher myotube lengths, myotube diameters, nucleus/myotube ratios, and cell densities than individuals with intact myostatin sequences (Shin *et al.* 2015). Currently, alternative splicing is only reported in chickens but not in other mammalian species, and little is known about how alternative splicing is regulated in chickens.

Interestingly, the sex of the two groups of chickens had different mRNA expressions (Table 7), where the expression of the myostatin gene in males was lower than in females. The down-regulated of the male myostatin gene in this study likely contributed to the less mature amount of Myostatin protein. The low amount of mature Myostatin protein produced in male chickens is capable of producing higher muscle growth than in females. In humans, similar results were reported. A study by Welle *et al.* (2008) in humans explained that the high expression of the myostatin gene in female muscles was caused by the high expression of the activin A receptor IIB (ACVR2B), which is a myostatin receptor gene. The high expression of the ACVR2B gene causes high expression of the myostatin gene and decreased expression of the IGF1 gene, which contributes to sex differences in skeletal muscle growth (Welle *et al.* 2008).

In summary, the results of this study showed that specific alleles for the fast-growing or slow-growing chicken groups were found, suggesting that these specific alleles potentially be used as genetic markers for muscle growth in chickens. Given that alternative splicing is important in regulating myostatin function in chickens, future studies need to investigate whether alternative splicing is associated with intronic mutations.

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