Mutation Identification in the Complete Myostatin Sequence in Indonesian Kampung Chicken

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Abstract. Kampung chicken, an Indonesian native chicken, has a potential for meat producers to improve national food security, as well as supplying highly favored meat to Indonesian consumers. To improve the meat production of Kampung chicken, the selection at the genomic level could be a promising approach. Myostatin is well known as a negative regulator of skeletal muscle growth. The objective of this study was to identify DNA mutation in the complete sequence of the myostatin gene in Kampung chicken. Genotyping was performed in 6 Kampung chickens by PCR and direct sequencing using 24 primer pairs covering 8.3 Kb of complete myostatin sequence. A total of 95 mutations discovered in the myostatin gene, including 24 in promoter, in 5'UTR, 5 in exon 1, 19 in intron 1, 37 in intron 2, 1 in exon 3, and 8 in 3'UTR. Among all mutations, 93 mutations were classified as point mutations, and 2 mutations were classified as indels. A total of 88 of 95 mutations (92%) were novel mutations. Six point mutations were found in the coding region, including 5 SNPs in exon 1, 1 SNP in exon 3, and no mutations in exon 2. No amino acid changes within all SNPs in coding region. Further studies in a larger population are needed to confirm this potential and novel mutations and their association with growth and meat production of Kampung chicken.

1. Introduction
Native chickens contribute to the conservation of poultry genetic sources, and play an important role in the rural economy in most developing and underdeveloped countries [1, 2], including Indonesia. Kampung chicken is one of the Indonesian native chickens, which can be found easily in rural areas [3]. Sartika and Iskandar (2007) defined native chickens as chickens that do not have specific characteristics, in other words the appearance of the phenotype and genotype are still very diverse [4]. Zein and Sulandari (2012) reported that the population of native chickens is expanding with high genetic diversity [5]. Kampung chicken has strong economic and social relations with the community [3]. Kampung chicken is generally reared extensively and semi-extensively as a producer of meat and eggs.

The growth rate is a major challenge for native chicken production in Indonesia. With an intensive maintenance system, native chickens in Indonesia reach slaughter weights at 4.5 months or more [6] in contrast to broilers (Ross 308) that are capable of reaching 1.4 kg at 28 days [7]. The selection based on
molecular genetics has the potential to improve the performance of *Kampung* chickens [2, 8]. At the gene level, molecular genetics provides a fast and accurate method of identification and selection for individuals to permanently improve performance [2, 9]. In poultry breeding programs, selection based on molecular approaches can be carried out before the trait is expressed or immediately after hatching [9].

Myostatin, also known as growth and development factor-8 (GDF-8), is a member of the transforming growth factor-beta (TGF-β) superfamily and acts as a negative regulator of muscle growth [10]. Belgian Blue, a breed of double muscle cattle that have bigger muscle mass in comparison to regular cattle breeds, was shown to be resulted from a functional mutation of the myostatin gene [11]. The study of the mechanism and function of myostatin then has progressed rapidly. Several studies have reported that myostatin regulates skeletal muscle growth by inhibiting myoblast proliferation [12] and regulating skeletal muscle protein balance [13, 14]. Myostatin is mostly expressed in the skeletal muscles of young and adult animals in both the prenatal and postnatal phases [15, 16].

The myostatin gene is evolutionally well conserved and the sequence of the chicken myostatin gene is very similar to other vertebrate myostatin genes [17, 18]. The chicken myostatin gene is located on chromosome 7 [19, 20] and consists of three exons and two introns with a total length of 6693 bp, encoding 375-376 amino acids [21]. The lengths of exon fragments 1, 2, and 3 are 373, 374, and 1567 bp respectively [21]. The molecular weight of unprocessed myostatin protein is about 43 kDa while the mature protein weight is 13 kDa [22]. A study in Indonesian chicken showed that the T4842G myostatin gene polymorphism had a strong relation with carcass characteristics [23]. This information is carried out on partial sequences of the chicken myostatin gene, and it is necessary to do an analysis of the complete myostatin gene. The objective of this study was to analyze the complete sequence of the myostatin gene in *Kampung* chicken.

2. Materials and Methods

2.1. Animals
All procedures performed in this study were approved by the Animal Care and Use Committee (ACUC) of IPB University (ACUC no: 22-2016 IPB). Six *Kampung* chickens (24 months-old, unsexed) were kept under uniform maintenance conditions with a uniform feed of protein and energy balance. Feed and water were given ad libitum. The chickens were a collection of the Division of Animal Breeding and Genetics, Department of Animal Production and Technology, Faculty of Animal Husbandry, IPB University. Blood samples were collected from each chicken through a wing vein. The blood sample were then mixed with an ethylenediamine tetraacetic acid (EDTA) anticoagulant.

2.2. DNA Genome Isolation
DNA isolation was carried out using the method of Sambrook and Russel (2001) with some modifications [24]. Twenty µl fresh blood was added with 1000 µl 0.2% NaCl, then homogenized and allowed to stand for five minutes. The sample was then centrifuged at 800 rpm for five minutes to form a precipitate, while the supernatant was removed. The precipitate was added with 350 µl 1xSTE, 40 µl 10% SDS, and 10 µl Proteinase K (5 mg ml⁻¹), then incubated for two hours at 55°C. After the incubation, 400 µl phenol, 400 µl CIAA, and 40 µl 5M NaCl were added, then slowly shaken at room temperature for one hour. The next step was centrifugation for five minutes (12,000 rpm). A total of 400 µl of clear liquid in the top layer was transferred to a new tube. Then, 800 µl of absolute EtOH and 40 µl of 5 M NaCl were added to the tube and frozen stored for 12 hours. The sample was then centrifuged at 12,000 rpm for five minutes and a white precipitate was formed. The precipitate was air drained and added with 100 µl of TE 80%. The DNA sample is stored at -20°C for later use.

2.3. Amplification of Complete Myostatin Gene
The 8.3 kb DNA sequence of the *Kampung* chicken myostatin gene was amplified into 24 gene fragments (Figure 1) separated by 24 specific primer pairs (data not shown). Specific primers are
designed based on GenBank (Access No.: AF346599.2) using the Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR was performed with a total volume of 50 µL consisting of 50 ng/mL DNA template, 1 pmol primer (IDT, Coralville, IA, USA), 1 unit Q5 High-Fidelity 2X Master Mix (NEB, Ipswich, MA, USA), and water. The PCR mix was incubated using a thermocycler machine (C1000 Touch™ Thermal Cycler, BioRad) with a 23-25 cycle of PCR consisting of 98°C denaturation for 10 seconds, annealing 60-65°C for 20 seconds, and 72°C extension for 30 seconds. The FlashGel agarose 1.2% system (Lonza, Basel, Switzerland) was used for DNA visualization. Sequencing of myostatin gene fragments was carried out using Applied Biosystems 3730XL DNA Analyzer (AB Systems) at the DNA Sequencing facility, Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB), University of Hawaii, HI, USA.

![Myostatin gene fragment diagram](Figure 1. Twenty four DNA fragments separated by 24 primer pairs to cover a chicken myostatin complete sequence.)

2.4. Data Analysis

All sequencing results (ABI trace files) were analyzed using MEGA 6.0 software [25], FinchTV [26], and BioEdit [27]. Estimation of amino acid sequences were performed by aligning DNA sequences with chicken myostatin mRNA sequences in GenBank (Access No.: AY448007.1). The phylogenetic tree was prepared following UPGMA (unweighted pair grouping method of arithmetic averages) methods. The dendrograms were drawn by MEGA 6.0 Software [25].

3. Result and Discussion

3.1. Identification of Mutations in Myostatin DNA Sequences

A total of 95 mutations were found along the Kampung chicken myostatin gene, which consisted of 93 point mutations (98%) and 2 insertions/deletions (InDel, 2%). Of the 93 point mutations found, there were 62 (65%) transition mutations and 31 (33%) transversion mutations (Figure 2a.). A total of 24 mutations were found in the promoter region, 1 mutation was in 5'UTR, 6 mutations were found in the coding region, 8 mutations were in 3'UTR, and 56 mutations were in the intron region (Figure 2b, 3). Seven point mutations found in this study have also been reported by Ye et al. (2007), Bhattacharya and Chatterje (2013), and Dushyanth et al. (2016) [28, 29, 30]. While 88 other mutations are novel mutations that have never been reported before.
Figure 2. Distribution of Kampung chicken myostatin gene mutations based on mutation type (a) and position in the gene (b)

This study showed that most mutations occurred in introns (59%, Figure 3). The intron is a part of a gene sequence that does not consist of the protein-coding sequence due to its removal by RNA processing (splicing) before translation into protein [31, 32]. Chorev and Carmel (2012) illustrated that most mutations occur in introns because mutations in introns can be tolerated or ignored [33]. Similarly, Jo and Choi (2015) reported that introns are mutational buffers in the eukaryotic genome, because it protects the coding sequence from the effects of random mutations [32]. However, as studies progress, theories about the role of introns in genes continue to emerge. Cooper (2010) showed that some functional SNPs are on the intron and most of the positions are no more than 30 bp from the nearest splicing site [34]. The SNP was reported to affect the transcription activity or splicing efficiency of the gene [34].

Figure 3. Distribution of 95 mutations found in myostatin gene sequences of Kampung chicken. Numbering of mutation position based on GenBank (Access Number AF346599.2), gaps (in bold-italic) indicate unreadable sequences, *indicates mutations that have been previously reported.
3.2. Mutations in the Myostatin Coding Region Sequence

A total of six point mutations were found in the coding region (Table 1). DNA sequences in this region are very important because it will be transcribed into mRNAs and translated into the myostatin protein for eliciting physiological functions. A total of five mutation points in the coding region were reported by Ye et al. [28] in broiler chickens. Three of them were also discovered by Bhattacharya and Chatterje and Dushyanth et al. [29, 30]. All mutations that occur in the coding region were synonymous mutations, or do not cause changes in amino acids.

| No | Mutation Position | Type of Mutation | Location | Allele |
|----|-------------------|------------------|----------|--------|
| 1  | g.2100G>A         | Transition/Synonymous | E1       | G/R    |
|    | c.51G>A           |                   |          | G      |
| 2  | g.2109G>A         | Transition/Synonymous | E1       | G/R    |
|    | c.60G>A           |                   |          | G      |
| 3  | g.2244C>G         | Transversion/Synonymous | E1       | C/G/S  |
|    | c.195C>G          |                   |          | C      |
| 4  | g.2283G>A         | Transition/Synonymous | E1       | A/R    |
|    | c.195G>A          |                   |          | G      |
| 5  | g.2373C>T         | Transition/Synonymous | E1       | C/Y    |
|    | c.324C>T          |                   |          | C      |
| 6  | g.7378G>T         | Transversion/Synonymous | E3       | G/K    |
|    | c.966G>T          |                   |          | G      |

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; \(^*\) reported by Ye et al. [28]; \(^*\) reported by Bhattacharya and Chatterje [39] and Dushyanth et al. [30]; \(^*\) reference sequences based on GenBank (Access No.: AF346599.2).

3.3. Amino Acid Sequence Prediction

Estimation of amino acid sequences making up the chicken myostatin protein was carried out by aligning the DNA coding region of exon 1, exon 2, and exon 3 sequences with the mRNA sequences of myostatin genes based on GenBank as references. Throughout 1128 bp the sequence coding for the chicken myostatin gene region produced 375 amino acids and 1 stop codon (TGA). No amino acid changes were found in Kampung chickens (Figure 4). The absence of differences in amino acid sequences in this study was expected to cause no differences in the structure and function of the myostatin protein.

There are three amino acid changes found in layer and broiler chicken sequences (GenBank Access No.: GU759292.1 and GU075928.1): Alanine (A) to Valine (V) at the AA no 22, Tyrosine (Y) becomes Histidine (H) in the AA no 321, and Proline (P) becomes Leucine (L) in the AA no 365 (Figure 4). The furin proteolytic site of the amino acid RSRR (Arginine-Treonin-Arginine-Arginine) was found in the 263 to 266 amino acid sequence. Nine conserved cysteines were also found in this study. The RSRR proteolytic site and nine conserved cysteines are specific characteristics of TGF-β, including myostatin [36, 36].
### Figure 4. The amino acid sequences that compose the Myostatin protein in chicken. Amino acid D in the box indicates putative proteolytic sites BMP1/TLD proteases, RSRR = furin proteolytic sites, vertical dotted lines indicate furin cleavage points, * nine conserved cysteines. Domain naming based on Lee et al. [35] and Shi et al. [36]

#### 3.4. Phylogenetic Relationships

The UPGMA dendrograms based on myostatin complete CDS were constructed to understand the phylogenetic relationships between Kampung chicken compared to broiler (NCBI Access No: GU075928.1) and layer (NCBI Access No: GU075929.1). Present results showed a maximum genetic distance between broiler and layer; whereas, Kampung chicken showed a closer genetic distance with broiler than layer (Figure 5).
Figure 5. UPGMA dendrograms constructed using shared allele distances based on myostatin complete CDS to understand relationships between *Kampung*, broiler, and layer.

4. Conclusion
A total of 95 mutations were found along the 8.3 Kb of the *Kampung* chicken myostatin gene, 88 of 95 mutations were novel mutations. There are no changes in amino acid sequence caused by mutations in the coding region. Complete sequence information of the *Kampung* chicken myostatin gene could be the platform for further studies, such as the study of the effect of polymorphisms, function and mechanism of the myostatin gene for regulating muscle growth in chickens.

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