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Single Nucleotide Polymorphisms Identification and Genotyping Analysis of Melanocortin 1 Receptor Gene in Various Plumage Colours Magelang Ducks

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Abstract: The objective of this study was to investigate the variations in Melanocortin 1 receptor (MC1R) gene and the association with plumage colours in Magelang duck populations using single nucleotide polymorphism (SNP). Forty three genomic DNA samples of Magelang ducks consisted of 3 plumage colour groups of brown, black and white were investigated. Primer design from *Anas platyrhynchos* complete genome in GenBank Accession Number HQ699486. Primer F:5'-GCTCTTCATGCTGCTGATGG-'3, R:5'-GATGAAGACGGTGCTGGAGA-'3. A 256-bp fragment was amplified by polymorphism chain reaction then sequenced. Two SNPs identified in this study were c.376A>G and c.409G>A. Chi-square (X^2) value showed that 0.75 in Magelang ducks had GG genotype; 0.81 for GA genotype; 0.34 for AA genotype (c.376G>A) and 0 for GG genotype; 1.37 for GA genotype; 5.82 for AA genotype (c.409A>G). These results suggested that the MC1R genotype distribution in Magelang duck population with brown, black and white plumage was balanced or not deviated from Hardy Weinberg Equilibrium. Conclusively, two SNPs were identified; c.376A>G SNP that changed the amino acid from isoleucine to valine and valin/isoleucine and c.409G>A SNP that changed alanine to threonine and threonine/alanine.

Key words: Single nucleotide polymorphism, genotyping, melanocortin 1 receptor, Magelang ducks

INTRODUCTION

Magelang duck is native to Sempu, Secang district, Magelang city, Central Java Province with various plumage colour patterns. Purwantini *et al.* (2013) reported the qualitative trait of Magelang duck of bearing eleven patterns of plumage colours or more varied than other Indonesian local ducks, indicated a relatively higher polymorphism than the other native ducks in Indonesia.

Many genes influenced the plumage colour pattern and interacted with other genes to determine the phenotype; however, information on the location of gene controlling plumage in specific chromosome was still limited and mechanism underlying this pattern is absurd (Stevens, 1991). Colour formation of animal's plumage, eyes and skin is affected by melanin pigment and the synthesis is catalyzed by tyrosinase enzyme (Price and Bontrager, 2001; Liang *et al*., 2010). Single locus, melanocortin 1 receptor (MC1R), is responsible to melanic polymorphism MC1R with various roles among different species (Mundy, 2005).

Melanocortin 1 receptor (MC1R) is responsible to melanic polymorphism in at least three species namely Bananaquit, Snow Goose and Skua Arctic. Mutations in the MC1R gene have been associated with coat colour variation in chicken (Kerje *et al*., 2003; Guo *et al*., 2010; Hoque *et al*., 2013), duck (Yu *et al*., 2012), quail (Zhang *et al*., 2013), sheep (Yang *et al*., 2013), cattle (Rouzaud *et al*., 2000; Reardon *et al*., 2010), pig (Kijas *et al*., 2001), goat (Fontanesi *et al*., 2009) and rabbit (Fontanesi *et al*., 2010). No significant correlation exists between MC1R gene polymorphism and plumage colour of Chinese duck owing to the absence of amino acid change in SNP (Nenzhu *et al*., 2009). Duck's plumage colour is defined by some factors concerning different seasonal phenomena and reproduction. The amount and the way plumage colour genetics interact are still unidentified (Stevens, 1991) and the distinguished plumage colour in bird species is still beyond explanation (Purwantini *et al*., 2013).

The objective of this study was to investigate the variations in MC1R and their possible association with plumage colours in Magelang duck populations by means of single nucleotide polymorphism (SNP).

MATERIALS AND METHODS

Animals dan sampling: Forty three genomic DNA samples of Magelang ducks were assigned to 3 plumage colour groups namely 25 brown (A) with 7 patterns (*Jarakan polos*, *Bosokan*, *Kalung ombo*, *Kalung ciut*, *Gambiran*, *Jarakan kalung* and *Jowo polos* duck), 14 black (B) with 3 patterns (*Klawu borok*, *Cemani*, dan *Wiroko* duck) and 4 white (C) with 1 pattern (*Putih polos*).

Primer design, PCR amplification, dan sequencing

Primer design: Primers used in this study were designed using Primer 3 software after sequence

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alignment analysis based on GenBank Acc. No. EU877265, EU777264, EU924100, EU924101, EU924102, EU924103, EU924104, EU924105, EU924106, EU924107, HQ699486, HQ190952 and HQ699485 to seek SNPs position (Primer Biosoft, 2012). Oligonucleotide primer was constructed (free online) using GenBank Acc. No. HQ699486 as template to design the primer. The result was MC-*Anas* PF (F) as forward primer and MC-*Anas* PR (R) as reverse primer as shown in Table 1.

Amplification DNA: PCR cycle underwent pre-denaturation at 95°C for 5 min, denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, elongation (extension) at 72°C for 30 sec and post elongation at 72°C for 10 min then 30-cycle replication for optimum result. PCR reagent was composed of 20 µl KAPA2G Fast Ready Mix PCR Kit (KapaBiosystems), 14 µl aquabidest, forward and reverse primer each 2 µl (10 pmol/µl) and 2 µl DNA genom. PCR product was separated by electrophoresis in low melting agarose gel 1% using buffer 1 x TBE in Horizontal Electrophoresis (Mupid, Japan) at 50 V voltage for 15 min. The PCR products were visualized by UV light.

Sequencing DNA: Forty three PCR products of Magelang ducks were sequenced using the same primers (MC-*Anas* PF and MC-*Anas* PR) for PCR reaction by PT Genetics Science Indonesia. Sequencing results was in form of electrophoregram peaks consisted of a nucleotide sequence, each with different coloured peak namely green for nucleotide adenine (A), black for guanine (G), blue for cytosine (C) and red for thymine (T).

Data analysis: Sequencing result analysis was subject to Bioedit v 7.2.0. The SNP was confirmed based on the electrophoregram results for genotyping. Pearson's Chi-square test was used to verify the samples not deviant to Hardy-Weinberg equilibrium. The following model was:

$$X^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

where, X^2 is Chi-square value, O_i is observed frequency, E_i is expected frequency, n is the number of possible outcomes of each event. The effects of MC1R genotypes on plumage colours composition traits were under one way ANOVA procedure in SPSS version 17.0 (SPSS, USA). The model of genotype association analysis was:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where, Y is the phenotypic data (plumage colours) of sample i, μ is the overall mean, α is the genotype effect of sample i and ε is a random error. Tukey's test was performed to analyze the pair-wise differences between the genotypes.

RESULTS AND DISCUSSION

Duck grouping based on plumage colour: Melanocortin 1 Receptor gene in 43 Magelang duck was grouped into 3 plumage colour as brown (A), black (B) and white (C). Yu *et al.* (2012) grouped the duck into 3 as extended black, non-extended black and recessive white. Plumage colour in birds depends on the presence of pigments and the balance of eumelanin (black/brown pigments) and pheomelanin (yellow/red pigments) (Ha *et al.*, 2003; Rees, 2003; Simon *et al.*, 2009). Mutations blocking microphthalmia transcription factor (MITF) prevent pheomelanin and eumelanin production making the animal's coat white as controlled by the sex-linked loci B/b (albinism) and Y/y (yellow) (Zhang *et al.*, 2002).

Identification of the MC1R gene sequence variation: Sequencing PCR product 256 bp in Magelang duck MC1R gene was proceeded by alignment between GenBank Acc. No. HQ699486 and result of MC1R gene sequencing using Bioedit program as shown in Fig. 1.

SNPs found in Magelang ducks sequences were caused by mutation of a base adenine (A) to guanine (G) and guanine/adenine (H) at 376 bp (c.376A>G) and guanine (G) to adenine (A) and adenine/guanine (H) at 409 bp (c.409G>A). Polymorphisms of the MC1R gene have been reported in several mammals, such as cattle (Rouzaud *et al.*, 2000), domestic dogs (Candille *et al.*, 2007) and wolf (Anderson *et al.*, 2009), in which gain of mutation function produced black/dark coat colour, while loss of mutation function caused red/yellow or white coat. By the genotyping information, Person's Chi-square (X^2) test was used to test the Hardy-Weinberg equilibrium. Data on the identified allele and genotype frequencies in the MC1R gene investigated were all in Hardy-Weinberg equilibrium as presented in Table 2.

The results of Chi-square test (X^2) that count value was smaller than the table value (5.99), suggested that the MC1R genotype distribution in Magelang duck population of brown, black and white plume was in balance (equilibrium). Hardy-Weinberg equilibrium in this study population proposes that the allele and genotype frequencies in the duck population would remain constant from one generation to the next as long as there were no confounding factors, namely not the selection case, no mutation, no migration occurs and marriage between individuals in the population at random (Warwick *et al.*, 1983; Hardjosubroto, 1999).

Association between MC1R genotype and plumage colour: Single nucleotide polymorphism c.376A>G and c.409G>A of the sequencing results was utilized for genotyping Magelang duck whose results were subject to CRD one way ANOVA analysis to determine effect on plumage colour group of Magelang duck as shown in Table 3.

In c.376A>G SNP, high frequency of GG genotype was observed only in white (100%) plumage, while brown

Table 1: Primer from primer design

Primer set	Location	PCR product size	SNP
F:5'-GCTCTTCATGCTGCTGATGG-'3	Exon 1	256 bp	c.376A>G
R:5'-GATGAAGACGGTGCTGGAGA-'3			c.409G>A

Table 2: Chi-square test (X^2) MC1R genotype on Magelang duck

	Allele frequency (SNP)					
	c.376A>G		c.409G>A		X^2	
Fenotype	G	A	G	A	c.376A>G	c.409G>A
Brown	0.87	0.13	0	0	0.75	0
Black	0.71	0.29	0.33	0.67	0.81	1.37
White	0.67	0.33	0.67	0.33	0.34	5.82

$X^2_{0.05, 2} = 5.99$

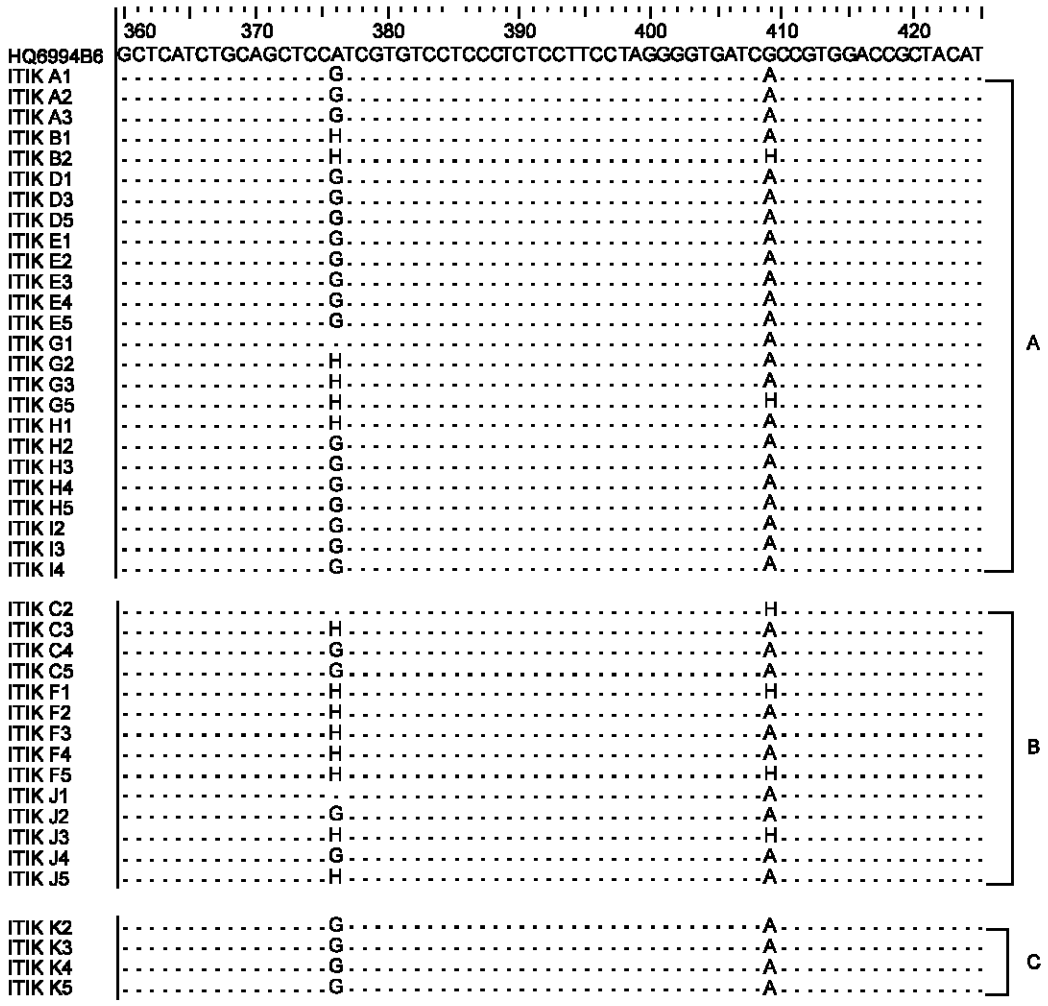

Fig. 1: Polymorphism of MC1R gene shown in SNPs (c.376A>G, c.409G>A). A = brown group, B = black group, C = white group, G = guanine, A = adenine, H = guanine/adenine

and black plumage displayed predominant GG and GA, respectively. These results were in contrast to Hoque *et al*. (2013) who reported that the GG genotypes were identified in black, black silky, yellow and red Korean chicken (c.376G>A), whereas the GG genotype was absent in the white leghorn breed. AA genotype observed only in white leghorn. Black and black silky Korean chicken were responsible for eumelanin (black/brown), while red and Yellow Korean chicken were pheomelanin (red/yellow) and white leghorn is for albino. Similarly, Yu *et al*. (2012) reported two SNPs highly significant with extended black variant of c.52G>A

Table 3: Genotyping results

		Number		Percentage (%)	
Genotype	Plumage colour	c.376A>G	c.409G>A	c.376A>G	c.409G>A
GG	Brown	18	0	70	0
	Black	4	0	15	0
	White	4	0	15	0
Sub total		26	0	60	0
GA	Brown	6	2	43	33
	Black	8	4	57	67
	White	0	0	0	0
Sub total		14	6	33	14
AA	Brown	1	23	33	62
	Black	2	10	67	27
	White	0	4	0	11
Sub total		3	37	7	86
Total		43	43	100	100

Table 4: Grouping of amino acid change

	--- Type of amino acid ---		--------- Number ---------	
Duck grouping	c.376A>G	c.409G>A	c.376A>G	c.409G>A
Brown	I to V	A to T	18	23
	I to H	A to H	6	2
	I to I	A to A	1	0
Black	I to V	A to T	6	10
	I to H	A to H	6	4
	I to I	A to A	2	0
White	I to V	A to T	4	4

dan c.376G>A (GenBank Acc. No. EU877264). Several nucleotide substitutions in chicken MC1R gene are associated with plumage colour, from the dominant extended black to the recessive yellow (Takeuchi *et al*., 1996; Kerje *et al*., 2003; Ling *et al*., 2003).

Change of amino acid: Base change in the MC1R gene sequences led to two changes in the amino acid at c.376A>G and c.409G>A SNP (Fig. 2) as follows:

1: Isoleucine (I) into Valine (V) and Valine/Isoleucine (H)
2: Alanine (A) into Threonine (T) and Threonine/ Alanine (H)

Complete amino acid change from each colour is shown in Table 4. A base change into G base indicated a transition mutation identified in the MC1R gene, was related to mutation by Ge *et al.* (2000), Di Stasio *et al.* (2005), Tatsuda *et al.* (2008), Han *et al.* (2009) and Reardon *et al.* (2010), namely the base change A into G which converted isoleucine (CAU) into valine (CGU). Mutations in MC1R gene fragment was subtitution mutation transition type, or changes in the nucleotide bases (A to G). According to Windelspecht (2007), transition mutations occurred because of substitution between the purine bases (adenine and guanine) with other purine bases or between the pyrimidine bases (thymine and cytosine) with other pyrimidine bases. Mutations or nucleotide bases changes were used as the identification base of genetic diversity.

Mutations in c.376G>A SNP were because base change from A to G and G/A d converted amino acid isoleucine

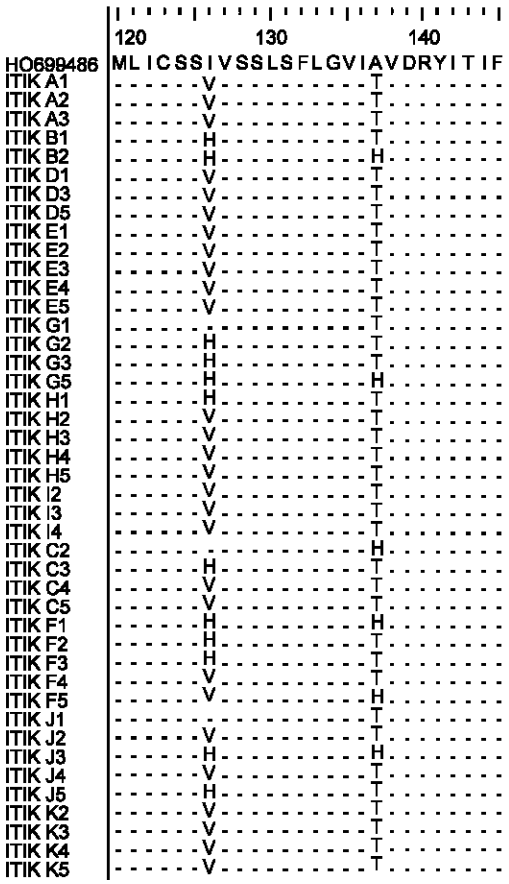

Fig. 2: Change of amino acid

to valine and valin/isoleucine while in c.409A>G SNP turned amino acid alanine into threonine and threonine/alanine. It was similar to missense mutation in c.376G>A and c.409A>G SNP according to Yang *et al.* (2012) that single base pair change causes the substitution of different amino acid in the protein produced.

Conclusively, this study identified two SNPs; c.376A>G and c.409G>A. The mutation changed amino acid

isoleucine to valine and valine/isoleucine (c.376A>G) and alanine to threonine and threonine/alanine (c.409A>G). The genotype of MC1R gene was not deviant from Hardy Weinberg Equilibrium indicating the various plumage colour of Magelang ducks population were in balance.

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