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

Ayu Rahayu1, Dattadewi Purwantini2, Dyah Maharani1 and Tety Hartatik1
1Faculty of Animal Science, University of Gadjah Mada, Yogyakarta, Indonesia
2Faculty of Animal Science, University of Jenderal Soedirman, Purwokerto, Central Java, Indonesia

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 HQ989486. Primer F:5’-GCTCTTCATGCTGCTAATGG-3’, R:5’-GATGAAGACGGTGCTGAGA-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 (X²) 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.409G>A). 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 valine/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 pigmentation 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 in at least three species namely Banaanaquit, Snow Goose and Skua Arctic. Mutations in the MC1R gene have been associated with coat colour variation in chicken (Keere 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 (Nenzhou 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 ormo, Kelung cut, Gambiran, Jarakan kalung and Jawo polos duck), 14 black (B) with 3 patterns (Klawu borck, 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
alignment analysis based on GenBank Acc. No. EU8777265, EU9777264, EU924100, EU924101, 
EU924102, EU924103, EU924104, EU924105, 
EU924106, EU924107, HQ699486, HQ190652 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 mL KAPA2G 
Fast Ready Mix PCR Kit (KapaBiosystems), 14 mL 
aquabest, forward and reverse primer each 2 mL (10 
mmol/L) and 2 mL DNA genon. 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 Magelan 
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:

\[ X^2 = \sum (O - E)^2 / E \]

where, \( X^2 \) is Chi-square value, \( O \) is observed frequency, 
\( E \) 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:

\[ Y_i = \mu + \alpha_i + \epsilon_i \]

where, \( Y \) is the phenotypic data (plumage colours) 
of sample \( i \), \( \mu \) is the overall mean, \( \alpha \) is the genotype effect 
of sample \( i \) and \( \epsilon \) 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 Magelan 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 Magelan 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 Magelan duck sequenced 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 Magelan 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 Magelan duck whose results were subject 
to CRD one way ANOVA analysis to determine effect on 
plumage colour group of Magelan 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

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Location</th>
<th>PCR product size</th>
<th>SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>F:5'-GCTCTTCATGCTGATGTGG-3</td>
<td>Exon 1</td>
<td>256 bp</td>
<td>c.376A&gt;G</td>
</tr>
<tr>
<td>R:5'-GATGAAGGCGCTGGAGA-3</td>
<td></td>
<td></td>
<td>c.409G&gt;A</td>
</tr>
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</table>

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

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>c.376A&gt;G</th>
<th>c.409G&gt;A</th>
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<tbody>
<tr>
<td>Brown</td>
<td>0.87</td>
<td>0.13</td>
</tr>
<tr>
<td>Black</td>
<td>0.71</td>
<td>0.29</td>
</tr>
<tr>
<td>White</td>
<td>0.67</td>
<td>0.33</td>
</tr>
</tbody>
</table>

$X^2_{obs} = 5.99$

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plumage colour</th>
<th>c.376G&gt;A</th>
<th>c.409G&gt;A</th>
<th>c.376G&gt;A</th>
<th>c.409G&gt;A</th>
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</thead>
<tbody>
<tr>
<td>GG</td>
<td>Brown</td>
<td>18</td>
<td>0</td>
<td>70</td>
<td>0</td>
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<td></td>
<td>Black</td>
<td>4</td>
<td>0</td>
<td>15</td>
<td>0</td>
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<tr>
<td></td>
<td>White</td>
<td>4</td>
<td>0</td>
<td>15</td>
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<tr>
<td>Sub total</td>
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<td>26</td>
<td>0</td>
<td>60</td>
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</tr>
<tr>
<td>GA</td>
<td>Brown</td>
<td>6</td>
<td>2</td>
<td>43</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>8</td>
<td>4</td>
<td>57</td>
<td>67</td>
</tr>
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<td>0</td>
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<tr>
<td>Sub total</td>
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<td>14</td>
<td>6</td>
<td>33</td>
<td>14</td>
</tr>
<tr>
<td>AA</td>
<td>Brown</td>
<td>1</td>
<td>23</td>
<td>33</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>2</td>
<td>10</td>
<td>67</td>
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<td>White</td>
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<td>4</td>
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<tr>
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<td>37</td>
<td>7</td>
<td>86</td>
</tr>
<tr>
<td>Total</td>
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<td>43</td>
<td>43</td>
<td>100</td>
<td>100</td>
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</table>

Table 4: Grouping of amino acid change

<table>
<thead>
<tr>
<th>Duck grouping</th>
<th>c.376G&gt;A</th>
<th>c.409G&gt;A</th>
<th>c.376G&gt;A</th>
<th>c.409G&gt;A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown</td>
<td>A to T</td>
<td>18</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A to H</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A to A</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>A to T</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A to H</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A to A</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>A to T</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

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.376G>A 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 substitution 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 to valine and valine/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.409G>A 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.376G>A 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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