MORPHOLOGY AND GENETIC DIVERSITY OF MITOCHONDRIAL DNA D-LOOP REGION USING PCR-RFLP ANALYSIS IN MAGELANG DUCK AND OTHER NATIVE DUCK

D. Purwantini1*, T. Yuwanta1, T. Hartatik1 and Ismoyowati2

1Faculty of Animal Science, Gadjah Mada University,
Jl. Fauna 3, Bulaksumur Yogyakarta 55281 - Indonesia.
*Permanent Address: Faculty of Animal Science, Jenderal Soedirman University,
Jl. Dr. Soeparno No. 60, Kampus Karangwangkal, Kotak Pos 110 Purwokerto 53123 - Indonesia
2Faculty of Animal Science, Jenderal Soedirman University,
Jl. Dr. Soeparno No. 60, Kampus Karangwangkal, Kotak Pos 110 Purwokerto 53123 - Indonesia
Corresponding E-mail: dattadewi2002@yahoo.com

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ABSTRAK

Penelitian ini bertujuan untuk mengetahui pengaruh warna bulu yang berbeda terhadap keragaman morfologi itik Magelang dan keragaman genetik menggunakan analisis PCR-RFLP daerah D-loop DNA mitokondria (mtDNA) pada populasi itik Magelang dan itik lokal lainnya (itik Tegal, Mojosari, Bali dan Alabio) di Indonesia. Materi penelitian terdiri atas itik Magelang sebanyak 50 ekor dan itik lokal lainnya masing-masing 20 ekor yang diambil sampel darahnya. Karakteristik morfologi ukuran tubuh, kemampuan produksi maupun kualitas telur itik Magelang dianalisis menggunakan rancangan acak lengkap dengan sebelas macam warna bulu yang berbeda sebagai perlakuan. Teknik PCR digunakan untuk mengamplifikasi fragmen pada daerah D-loop mtDNA dan produk PCR didigesti dengan enzim restriksi endonuklease AluI dan HaeIII. Penelitian ini berhasil menunjukkan bahwa secara statistik keragaman morfologi pada populasi itik Magelang dipengaruhi oleh perbedaan warna bulu. Analisis PCR-RFLP menggunakan enzim restriksi AluI dan HaeIII berhasil memperoleh enam kombinasi pola restriksi fragmen, sehingga dihasilkan enam haplotipe (A, B, C, D, E dan F). Perbedaan haplotipe tersebut menunjukkan adanya keragaman genetik pada populasi itik Magelang dan itik lokal lainnya. Disimpulkan bahwa perbedaan warna bulu mempengaruhi keragaman morfologi pada populasi itik Magelang dan keragaman genetik populasi itik lokal di Indonesia dapat diidentifikasi menggunakan analisis PCR-RFLP pada daerah D-loop mtDNA.

Kata kunci: keragaman morfologi, genetik, PCR-RFLP, D-loop mtDNA, itik Magelang

ABSTRACT

The aim of this study was to investigate the different of plumage colors on morphological diversity of Magelang duck and genetic diversity using PCR-RFLP mtDNA D-loop region analysis of Magelang duck and four others native duck population (Tegal, Mojosari, Bali and Alabio duck) in Indonesia. Blood sample was taken from 50 Magelang ducks and 20 of each native ducks. Morphological characteristics of body measurement, production ability and egg quality of Magelang duck were analyzed using Completely Randomized Design with 11 plumage colors as treatments. PCR technique was administered to amplify fragments in mtDNA D-loop region and PCR products were digested with endonuclease restriction enzyme AluI and HaeIII. The result showed that morphology diversity of Magelang duck was statistically affected by different plumage colors. PCR-RFLP analysis using AluI and HaeIII restriction enzyme resulted in six combinations of restriction fragment pattern shown in six haplotypes (A, B, C, D, E and F). Haplotype difference showed genetic diversity in the population of Magelang duck and the other native ducks. In conclusion, the different plumage colors affected morphology diversity of Magelang duck. Genetic diversity of Indonesian native duck population could be identified by using PCR-RFLP analysis on mtDNA D-loop region.

Keywords: morphology diversity, genetic, PCR-RFLP, mtDNA D-loop, Magelang duck
INTRODUCTION

Magelang duck is native duck thriving in Muntilan, Magelang, Central Java province with specific and ultimate morphological characteristics of relatively big body, high egg production and various plumage color. Ismoyowati and Purwantini (2010) reported that Magelang duck qualitatively has the highest plumage color diversity compared to other native duck.

Individual genetic variation within population is the figure of the heritable genetic expression variation. Genetic expression is the characteristic possessed by an individual as distinctive morphological feature. Through DNA marker diversity analysis on protein or enzyme, individual genetic diversity within and between population can be identified (Sakai et al., 1998). One of the analysis methods to identify genetic in several fowl species applies Polymerase Chain Reaction Restriction Fragment Lenght Polymorphism (PCR-RFLP) (Herman, 2004; Sartika, 2007). Mitochondron DNA lies outside the nucleus in one cell compartment or organelle named mitochondrion (Zhao et al., 2004), with higher mutation rate (5-10 times) than DNA nucleus (Parsons et al., 1997; Brown et al., 1979; Sigurðardóttir et al., 2000 in Sudoyo, 2004), so it has high discrimination ability (Marzuki, 2004). Likewise, there are one closed circular area with complete necluotide sequence and one non coding region called displacement loop (D-loop) measuring 1049 pb in duck (GenBank: HM010684.1, 2010). Tsai et al. (2009) proved the complete D-loop mtDNA structure in Columba livia that can be used to identify the mother and genetic linkage.

PCR-RFLP technique is a technique that can multiply certain DNA fragment to figure out whether or not restriction site difference exists in DNA fragment inter individual within one family or population (Griffiths et al., 2003), which enables to develop as alternative method to analyze genetic diversity and individual genetic uniformity and diversity in native duck population. PCR-RFLP analysis has been well applied to differ species and to detect interspecies or intraspecies variation in some animals like tuna, lobster and swine (Wolf and Hubner, 1999), that has served as verification tool of food product from various animal (Lockley and Bardsley, 2000; Rojas et al., 2011), and is used as well to identify meat characteristic on peacock (Pavocristatus) and other fowl like chicken and turkey (Saini et al., 2007). A mtDNA PCR-RFLP method also identifies genetic diversity in population of wild quail and laboratory quail (Shen et al., 1999), and other fowl species as chicken (Gallus gallus), turkey (Meleagris gallopavo), duck (Anas platyrhynchos) and goose (Anser anser) (Wisniewska and Slota, 2009; Silva et al., 2009).

Research on the effect of plumage color difference in Magelang duck morphology has never been done. In addition, information of identification and molecular genetic diversity using mtDNA D-loop region PCR-RFLP analysis of native ducks in all over Indonesia is limited as well. This research aimed to figure out the influence of different plumage colors on morphological diversity of Magelang duck and genetic diversity based on PCR-RFLP mtDNA D-loop region analysis of Magelang duck and other native duck populations in Indonesia.

MATERIALS AND METHODS

Materials used to study morphology diversity were 50 six-month-old Magelang ducks at early production with 11 different plumage colors, namely A. Jarakan polos (plain brown), B. Bosokan (dark brown), C. Klauw blorok (light brown and white), D. Kalung ombo (brown with wide white collar), E. Kalung ciut (brown with thin white collar), F. Cenani (plain black), G. Gambiran (dark brown and white), H. Jarakan kalung (brown with white collar), I. Jowo polos (brown with dark brown spot), J. Wiroko (black and white), K. Plain white (yellow bill and feet). Each color consisted of five ducks, except for A and B, each had 3 and 2 ducks, respectively.

Blood sample was taken from 50 Magelang ducks and 20 were from each native local ducks (Tegal, Mojosari, Bali and Alabio ducks). Chemical reagent used to extract DNA was DNA Isolation Kit (Geneaid). PCR-RFLP analysis used KAPA (Kit PCR), primer Forward (DL-AnasPF), primer Reverse (DL-AnasPR), dH₂O free nuclease, endonuclease restriction enzyme AluI and HaeIII (Thermo Scientific product, Lithuania) and enzyme buffer. Chemicals for agarose gel were agarose powder, buffer 0.5x TBE, good view, loading day and DNA ladder.

The experiment was conducted to figure out (1) morphology diversity and (2) genetic diversity. Morphological diversity was analyzed using Completely Randomized Design (Steel and Torrie, 1980). The treatments were 11 different
plumage color with 2-5 ducks replicates. The observed variables were (1) egg production, (2) egg weight (g/egg), (3) egg quality comprising egg length and width, egg index, eggshell thickness, albumen and egg yolk weight, albumen height, Haugh Unit (HU) and yolk color, (4) body weight (g), (5) body length (cm), (6) breast perimeter (cm) and (7) neck length (cm). Body parts measurement was done three times to avoid empirical mistakes of measurement. Egg production and weight was recorded three times and egg quality was randomly observed, in which 10 samples represented each plumage color. Data were analysed with analysis of variance.

Mathematical method used was:

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

Where:
- $$Y_{ij}$$ = result of observed variables
- $$\mu$$ = mean of population
- $$\alpha_i$$ = influence of plumage $$i^{th}$$ number
- $$\varepsilon_{ij}$$ = error

Egg production measured was hen day production (HDP), thus the number of egg according to one month of individual record was divided by the number of female ducks times 100% (Ensminger, 1992), while egg weight was the whole weight of the egg weighed on digital scale with 0.01 g accuracy. Egg length was the length from the pointed end and obtuse end of egg, while egg width was the distant of 2 sides of egg in the centre, measured with digital slide compass with 0.01 mm accuracy. Egg index was the comparison of egg width and length times 100% (Ensminger, 1992), while egg weight was divided by the number of female ducks times 100% (Reddy et al., 1979), while eggshell thickness was measured with digital slide scale from three angles: the pointed end, center and obtuse end of egg, then the score were averaged. Albumen and yolk were separated using egg separator then each weight was scaled and the height was measured with Haugh Unit (HU) (Raymond Haugh, 1937 in Monira et al., 2003). Formula used was: HU = 100 x log (h - 1.7 w^{0.37} + 7.6).

Where:
- HU = Haugh Unit
- h = albumen height (in millimeter)
- w = egg weight (in gram)

Yolk color was observed by comparing it to Roche yolk colour fan.

Genetic diversity was analyzed by extracting DNA from duck blood sample and amplifying mtDNA D-loop region with PCR technique and the analysis applied PCR-RFLP. Blood sample was taken using disposable syringe from vena axillaries, then collected in vacuntainer containing EDTA. Total genome DNA was extracted using Isolation Kit Geneaid according to the protocol. DNA extract was used as PCR template without purification process and resulted in reproducible PCR products. Amplification of mtDNA D-loop region was conducted with primer DL-AnasPF (L566) 5’ - GTTGGGATTTTTGTTA-3’ and DL-AnasPR (H773) 5’- CCATATACGCAACCGTCTC-3’, applying GenAmPPCR system thermocycler 2400 (Perkin Elmer). PCR reagent solution comprised 12.5 µl KAPA (Kit PCR), 1 µL primer Forward (DL-AnasPF) 10 pmol, 1 µL primer Reverse (DL-AnasPR) 10 pmol, 9.5 µL dH2O free nuclease and 1 µL DNA template. PCR cycle consisted of pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, elongation (extension) at 72°C for 1 minutes and post elongation at 72°C for 5 minutes. PCR reaction was repeated 35 times for optimum result. PCR products were separated by electrophoresis in 1.5% low-melting agarose gel using buffer 0.5x TBE in Submarine Electrophoresis (Hoefer, USA). RFLP analysis administered PCR products digested with endonuclease restriction enzyme Alu and HaeIII. As much as 1 µL endonuclease restriction enzyme added with buffer R 2 µL and 7 µL dH2O free nuclease was collected in PCR microtube, then added with 5 µL PCR product. The solution was then incubated at 37°C overnight for 16 hours. PCR-RFLP analysis result that has been cut with restriction enzyme and undergone electrophoresis in 2% agar gel then was observed for the DNA band resulted to know the digestion success. DNA band figure in agar gel was taken using digital camera, tabulated then analyzed for the genetic diversity. DNA band obtained from the visualization of agar gel electrophoresis on each sample was then used to determine the haplotype, genetic uniformity and diversity inter Magelang duck and other native ducks. Haplotype diversity obtained showed genetic diversity in Magelang duck and the other native ducks.

**RESULTS AND DISCUSSION**

**Morphology Diversity in Magelang Duck Population**

This research well demonstrated that plumage color affected (P>0.05) morphology diversity comprising morphological
Table 1. Mean and Standard Deviation of Morphological Characteristic in Magelang duck population based on different plumage colors

| Code | Plumage Color     | Body Weight (g) | Daily Gain (g) | Neck Length (cm) | Breast Perimeter (cm) | Body Length (cm) |
|------|-------------------|-----------------|----------------|------------------|-----------------------|------------------|
| A    | Jarakan polos     | 1563.33±221.47  | 3.29±2.13      | 19.33±0.58       | 38.33±1.15            | 38.33±1.15       |
| B    | Bosokan           | 1613.50±270.82  | 2.92±2.29      | 18.00±1.41       | 37.00±1.41            | 24.50±0.71       |
| C    | Klawu blorok      | 1625.00±101.63  | 0.40±2.49      | 17.40±1.52       | 36.40±1.14            | 24.60±1.34       |
| D    | Kalung ombo       | 1837.20±397.17  | 5.42±5.06      | 17.80±1.64       | 37.80±1.64            | 23.80±1.64       |
| E    | Kalung ciut       | 1507.60±145.67  | 2.24±2.52      | 18.20±1.30       | 37.90±0.89            | 21.00±2.34       |
| F    | Cemani            | 1936.20±291.82  | 4.36±3.04      | 20.40±1.67       | 38.80±1.30            | 22.00±5.10       |
| G    | Gambiran          | 1706.00±215.03  | 1.65±1.56      | 19.60±0.55       | 38.00±1.73            | 18.90±1.67       |
| H    | Jarakan kalung    | 1603.80±225.80  | 0.23±1.24      | 19.40±0.55       | 36.40±1.29            | 20.00±0.71       |
| I    | Jowo polos        | 1412.00±155.41  | 4.10±1.57      | 17.80±0.84       | 36.80±1.10            | 18.30±1.10       |
| J    | Wiroko            | 1565.60±250.00  | 2.04±2.47      | 17.40±0.89       | 36.20±1.48            | 18.40±0.96       |
| K    | Plain white       | 1583.20±87.63   | 0.42±1.15      | 19.80±0.84       | 36.40±1.14            | 18.80±0.84       |

Table 2. Mean and Standard Deviation of Morphological Characteristic of Egg Production and Quality in Magelang Duck Population with Different Plumage Colors

| Code | Plumage Color     | EP (%)     | EW (g)  | EL (mm)  | EW (mm) | EI (mm) | ET (mm) |
|------|-------------------|------------|---------|----------|---------|---------|---------|
| A    | Jarakan polos     | 30.37±23.42| 63.45±1.93| 55.14±0.79| 43.70±0.76| 79.26±2.04| 0.40±0.03|
| B    | Bosokan           | 23.89±16.50| 64.46±2.59| 56.75±1.07| 44.72±0.77| 78.81±1.43| 0.40±0.04|
| C    | Klawu blorok      | 49.33±18.78| 66.17±6.41| 59.22±1.67| 47.34±1.06| 79.99±2.48| 0.44±0.03|
| D    | Kalung ombo       | 25.56±19.29| 65.60±5.82| 59.24±2.00| 46.12±2.21| 77.85±2.88| 0.42±0.04|
| E    | Kalung ciut       | 48.67±9.41 | 63.74±2.83| 56.03±2.33| 44.74±0.66| 79.97±3.40| 0.42±0.01|
| F    | Cemani            | 43.33±51.51| 67.39±1.12| 56.35±0.93| 45.99±0.61| 81.62±1.12| 0.45±0.06|
| G    | Gambiran          | 33.11±27.56| 64.28±3.90| 54.97±1.38| 45.45±1.40| 82.72±2.82| 0.41±0.07|
| H    | Jarakan kalung    | 30.56±29.20| 66.72±1.93| 58.02±0.94| 46.33±0.46| 79.88±1.86| 0.43±0.03|
| I    | Jowo polos        | 35.11±19.95| 58.06±3.10| 53.45±1.75| 44.04±1.12| 82.44±2.04| 0.44±0.02|
| J    | Wiroko            | 40.22±32.08| 65.32±2.17| 56.34±2.54| 46.07±0.95| 81.87±2.96| 0.51±0.06|
| K    | Plain white       | 45.56±12.37| 62.34±1.65| 54.26±2.04| 45.23±0.59| 83.46±3.17| 0.42±0.03|

EP = Egg Production; EW = Egg eight; EL = Egg Length; EW = Egg Weight; EI = Egg Index ET = Eggshell Thickness
characteristics of body measure (Table 1), egg production (Table 2) and egg quality (Table 3) in Magelang duck population.

Many genes determined plumage color pattern and interacted with other genes to determine the phenotype; however, information on the location of gene that controls plumage in specific chromosome was still limited and mechanism underlying this pattern was not absolute either (Stevens, 1991). Color formation of animal’s plumage, eyes and skin was affected by melanin pigment and the synthesis is catalyzed by tyrosinase enzyme (Price and Bontrager, 2001 and Liang et al., 2010). Single locus, melanocortin-1 receptor (MC1R), is responsible to melanic polymorphism. MC1R has various roles among different species (Mundy, 2005). Hormone that limits plumage color expression in most birds is estrogen-dependent found in the females. Mutation point in MC1R and TYRP1 was figured to be responsible to produce pigmentation variants, and TYRP1 expression is lower in the female than in the male (Irwin, 1994). The more frequent plumage color change of the female is mostly due to dichromatism, assuming that sexual selection is more likely to happen to the female’s plumage color (Burns, 1998).

This research showed that egg production and its weight was lower but the eggshell thickness was higher (Table 2) than the result of Ismoyowati and Purwantini (2010), reporting that egg production and egg weight of Magelang duck were 70.24±14.10% and 69.19±4.05 g, respectively, and the eggshell thickness was 0.38±0.02 mm. According to Sofwah (2007), eggshell quality was affected by egg weight and mother’s age. This production diversity was influenced by the different of laying period, cage environment, and feed given.

**Genetic Diversity in mtDNA D-loop region with PCR-RFLP analysis of Magelang Duck and other Native Ducks**

This research was well conducted with PCR amplification of 718-bp fragment. PCR-RFLP analysis result showed that PCR products in mtDNA D-loop region of Magelang duck and the other native ducks were well digested by endonuclease restriction enzyme AluI (Figure 1), proven by different DNA bands size from column 1 to 6. Column 7 showed PCR products and column 8 was DNA markers. AluI-RFLP analysis resulted in four fragment restriction patterns of mtDNA D-loop region, namely pattern I measuring 428, 317, 212, 179 and 119 bp that were obtained from Alabio and Bali duck. Pattern II measured 400, 317, 179 and 119 bp that were...
obtained from Tegal duck. Pattern III measured 400, 317 and 179 bp that were obtained from Mojosari duck. Pattern IV measured 317, 179 and 119 bp that were obtained from Magelang duck. The four patterns showed genetic diversity in at least four native Indonesia ducks based on fragment restriction pattern of mtDNA D-loop region by AluI enzyme.

Result also showed that PCR products of mtDNA D-loop region in Magelang duck and the other native ducks were well digested with HaeIII enzyme (Figure 2) that was proven by different DNA bands size from column 2 to column 7. Column 8 showed PCR products and column 1 was DNA marker. HaeIII-RFLP analysis resulted in two fragment restriction patterns namely Pattern I measuring 460 bp, 274 bp, 178 bp and 166 bp that were obtained from black Cemani of Magelang duck, Tegal duck and Bali duck. Pattern II measured 274 bp, 178 bp and 166 bp that were obtained from plain white Magelang duck, Mojosari duck and Alabio duck. Both patterns showed genetic diversity in at least two other native Indonesian ducks based on fragment restriction patterns in mtDNA D-loop region by HaeIII enzyme.

PCR-RFLP analysis with AluI and HaeIII restriction enzyme resulted in six combinations or restriction patterns, leading to six haplotypes (A, B, C, D, E and F). Fragment haplotype in mtDNA D-loop region of Magelang duck and the other native ducks by AluI and HaeIII restriction enzyme is shown in Table 4. Bali duck belongs to haplotype A, Alabio ducks in haplotype B, Tegal duck in haplotype C, Mojosari duck in haplotype D, and Magelang duck in haplotype E and F. Haplotype diversity showed genetic diversity or marked polymorphism in Indonesian native duck population. Genetic diversity in mtDNA D-loop region existed in different ducks (Magelang, Tegal, Mojosari, Bali and Alabio ducks), although one type of duck (Magelang) showed polymorphism based on maternal inheritance. Hao (2009) studied haplotype diversity (Hd) based on mtDNA D-loop region fragment in Cherry Valley duck in Taiwan. Cherry Valley is classified into two haplotype subgroups, the first homologizes with Mallard duck (Anas zonorhyncha) and Jianchang duck, the second does not homologize or has high variation with Mallard duck.

The difference of restriction pattern shown in research result with PCR-RFLP analysis using restriction enzyme AluI and HaeIII could be clarified by observing polymorphism sequence or nucleotide sequence from mtDNA D-loop region fragment of Magelang duck and other native ducks. Some researchs reported that polymorphism analysis of mtDNA marker

| Haplotype (AluI pattern x HaeIII pattern) | Fragment Measurements | Breed |
|------------------------------------------|-----------------------|-------|
| Haplotype A (Pattern I x Pattern I) | 428, 317, 212, 179 and 119 | 460, 274, 178 and 166 | Bali |
| Haplotype B (Pattern I x Pattern II) | 428, 317, 212, 179 and 119 | 274, 178 and 166 | Alabio |
| Haplotype C (Pattern II x Pattern I) | 400, 317, 179 and 119 | 460, 274, 178 and 166 | Tegal |
| Haplotype D (Pattern III x Pattern II) | 400, 317 and 179 | 274, 178 and 166 | Mojosari |
| Haplotype E (Pattern IV x Pattern I) | 317, 179 and 119 | 460, 274, 178 and 166 | Magelang |
| Haplotype D (Pattern IV x Pattern II) | 317, 179 and 119 | 274, 178 and 166 | Magelang |
included ND1-6, ND4L, COX 1-3, ATP6 and ATP8 in Beijing duck in China (Zhang et al., 2010), prolactin gene polymorphism in purebred China duck (Shanma, Shaoxing, Youma, Jinyun, Jingjiang and interbred (F2) of white Liancheng and white Kaiya) (Wang et al., 2011) and polymorphism of growth hormone gene in Beijing duck, Xihu Mallards, Cherry Valley, Jingding duck, Shan Partridge duck, Jingjiang duck, Shaoxing duck and Partridge Jinyun (Hai et al., 2007) can be done by using PCR-SSCP (Single Stranded Conformation Polymorphism) and the DNA sequencing products.

CONCLUSION

Plumage color difference affected morphology diversity of Magelang duck
population, and genetic diversity can be identified using PCR-RFLP in mtDNA D-loop region of Indonesian native duck population.

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