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RESEARCH ARTICLE
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Phylogenetic Study of Black and Brown Japanese Quails In Indonesia Based On Mitochondrial D-Loop Sequence

Muhammad Rifki, Ratih Dewanti, Nuzul Widyas, Muhammad Cahyadi*

Department of Animal Science, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta 57126, Indonesia

ABSTRACT

Quails which are commonly found in the small holder farmers in Indonesia is black and brown lines of Japanese quail (*Coturnix coturnix japonica*). The objective of this study was to construct phylogenetic tree of black and brown Japanese quail based on Mitochondrial (mt) D-loop sequence. A total of nine quail blood samples has been collected to be furthermore used for DNA extraction. Polymerase chain reaction has been carried out to isolate 661 bp of D-loop region. Moreover, PCR product was analyzed to generate D-loop sequences. Nine D-loop sequences from samples and nine additional published sequences have been used to construct phylogenetic tree by MEGA 7.0. The result of phylogenetic reconstruction of 18 samples indicated that black and brown Japanese quail was located in the same clade that means no genetic variation found in the population. According to all sequences, a total of five haplotypes were observed with haplotype and nucleotide diversities of 0.68 and 0.059, respectively. In addition, the genetic distance among quails was ranged from 0.000 to 0.674 with average value of genetic distance was 0.300. In conclusion, black and brown Japanese quails in Indonesia were closely related each other and also relatively closed to published sequence of Japanese quail, and they were completely separated with other species of quails such as *Coturnix chinensis* based on D-loop sequence.

Keywords: D-loop, Genetic diversity, Japanese quail, Phylogenetic analysis

INTRODUCTION

Quail is a species from *Coturnix* genus that abundant across the lands and it is a bird species who loss the ability to fly with small body size and short shanks (Listiyowati and Roospitasari, 2009). Quail commonly raised in Indonesia is Japanese quail (*Coturnix cotunix japonica*) with brown and black plumage colors. Plumage color is totally controlled by genetic factor either dominant or recessive (Hutt and Rasmusen, 1982; North 1984). Moreover, plumage color indicates qualitative trait that controlled by one or more genes and alleles (Warwick *et al.*, 1995).

Data obtained from interview of smallholder farmers reported that they are producing final stock of commercial layer quail by mating between brown plumage male and black plumage female. It is conducted to produce brown plumage layer, which is furthermore called as commercial stock by utilizing of crisscross inheritance phenomenon. However, this activity is hereditary conducted to do auto sexing for day old quail (DOQ). Previous study reported that phenomenon of crisscross inheritance between brown plumage male and black plumage female mating system is controlled by the gene encoding black plumage color which is sex linked dominant, therefore, it produces brown female DOQ and black male DOQ (Prihtiyantoro *et al.*, 2001). This mating system is very popular in the smallholder

*Corresponding author: Dr. Muhammad Cahyadi, Tel: +62-271-637457, Fax: +62-271-637457, E-mail: mcahaydi@uns.ac.id
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farmers due to very effective and efficient method to separate male and female DOQ in the very early stage of quail life. The study of quail plumage color differences in the basis of molecular genetics in Indonesia is very rare. The characterization of genetic resources is very important to understand the genetic diversity and origin of commercial quail layer. Genetic variation between black and brown plumage quail may be found by evaluating their partial genetic materials such as Mitochondrial (mt) DNA D-loop.

The analysis of mtDNA sequence variation is widely used to evaluate phylogenetic relationships, maternal origins of farm animals, and it is a powerful tool to discriminate animal species (Mindell *et al*., 1997; Machugh and Bradley, 2001; Wayne *et al*., 2002). Analysis of mtDNA also shows 99.99% of mtDNA is maternally inherited (Lodish *et al*., 1998). Mitochondrial DNA D-loop is an effective barcode or small segments of DNA to study genetic variations of species among different populations due to hyper variations within the region (Kawahara *et al.,* 2013). In addition, D-loop is a region of mtDNA that very fast to be evolved and the most polymorphic sites within the region are focused on Hypervariable region 1 (HVR1) and Hypervariable region 2 (HVR2) (Wilkinson-Herbots *et al*., 1996). These regions can be utilized to characterize genetic diversity and phylogenetic study of black and brown Japanese quail. The objective of this study was to evaluate genetic diversity and phylogenetic study of black and brown Japanese quail based on analysis of mtDNA D-loop sequence and their genetic relationships with Japanese quail in the world.

MATERIALS AND METHODS

Quail population and management

A total of 500 Japanese quails have been raised in this study. The number of quail was equal for every plumage color. Moreover, the female quails were divided into four colony cages with 25 heads per cage. The birds was fed using commercial feed for laying quail containing 18% of crude protein, 3% of calcium, 0.9% of phosphor, and 2700 kcal ME/kg. Each quail accepted around 25 g of feed whereas water was available ad libitum. A lighting was applied for 12 hours/day. All quails in this study were reared under the same environment and management based on standard procedure for raising Japanese quail (Randall and Bolla, 2008).

Blood Collection and DNA Extraction

A total of nine blood samples consisting five black plumage quails and four brown plumage quails have been used in this study. Total of 3 ml of quail blood has been collected with tube containing ethylene diamine tetra acetic acid (EDTA) after they were slaughtered. Furthermore, the blood samples were kept in the freezer at -21°C until used for further analysis. Extraction of DNA has been conducted by putting 20 µl of bloods into 1.5 ml microtube. The DNA extraction process was according to standard procedure of Wizard Genomic DNA Purification Kit (Promega, USA). The extracted DNA was indicated by 0.8% agarose gel electrophoresis stained by ethidium bromide under the UV light.

Design of Primer and Polymerase Chain Reaction (PCR)

Primer design was performed using Primer3 (http://primer3.ut.ee/). The reference sequence used in this study was KX712089.1 obtained from National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/). It represents complete mitochondrion genome of *Coturnix japonica*. The primer pair was set to cover HVR1, HVR2 and HVR3 regions. The primers are listed in the Table 1.

Polymerase chain reaction was carried out in total volume of 25 µl containing 12.5 µl of GoTaq® Green Master Mix

(Promega, USA), 9.5 µl of nuclease free water, 1 µl of each primer, and 1 µl of DNA template. The PCR condition was started from initial denaturation at 95°C for 3 minutes and followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR was completed by final extension at 72°C for 3 minutes. The result of PCR was then checked by 2% agarose gels electrophoresis that stained with ethidium bromide in the Gel documentation (Vilber Lourmat Infinity 1100126M, France). Then, the PCR product was sequenced (1st BASE, Malaysia).

Data Analysis

Sequence of quail mtDNA D-loop was aligned using Clustal Omega program (Thompson *et al*., 1994), construction of phylogenetic tree was performed using MEGA version 7.0.2.6 (Tamura *et al*., 2011), the number of haplotype, haplotype and nucleotide diversity was determined using DNAsp version 5.10 (Rozas *et al*., 2003), and NETWORK version 5.0.0.3 was used for median-joining network profile (Bandelt *et al*., 1999). The published complete mtDNA D-loop sequences data which are exactly similar region to this study from NCBI were also used in the analysis as parenthesis with following GenBank accession number: AP003195.2, NC_003408.1, NC_004575.1, AB073301.1, NC_023939.1, KF027439.1, KR349185.1, and NC_027279.1. Genetic distance was determined using Kimura 2-parameters model and neighbor-joining (NJ) phylogenetic tree was calculated with 1000 bootstrap resampling (Kimura *et al*., 2004).

RESULTS AND DISCUSSION

Genetic distance of the populations based on mtDNA D-loop

Genetic distance among individual birds within population was ranged from 0.000 to 0.674 (Table 2). It is used as a basis in construction of phylogenetic tree. Genetic distance is the degree of nucleotide differences between populations or species which is calculated by some numerical quantity (Nei, 1987).

Genetic distance of black and brown Japanese quail with published sequences of Japanese quail was ranged 0.000 to 0.091 and the overall average of genetic distance was 0.300. This result suggested that close relationship between Japanese quail around the world has been found in this study. Small value of genetic distance indicated close genetic relationship (Kim *et al*., 2002; Odahara *et al*., 2006). On the other hand, genetic distances between black and brown Japanese quail and other bird species such as *Coturnix chinensis, Tetraogallus tibetanus* and *Tetraogallus himalayensis* was ranged from 0.491 to 0.548 (Table 2). It suggested that these birds were domesticated from different ancestors (Amano *et al*., 1981). Nei and Kumar (2000) stated that two or more individuals are not closely related if their values of genetic distance are more than 0.1.

Genetic diversity and haplotype analysis

Genetic diversity and haplotype analysis have been conducted by comparing mtDNA D-loop sequences of Black and Brown Japanese quail with reference sequences downloaded from GenBank database. Overall, information of genetic diversity covers the number of sequence used, polymorphic site, the number of haplotype, haplotype and nucleotide

Table 1. Nucleotide primers of mtDNA D-loop used in this study

Primer	Sequence of nucleotide (5' to 3')	Product size (bp)
Forward	CCAAGGACTACGGCTTGAAA	661
Reverse	TGAAGAAGCCCCAAAGAGAA	

Table 2. Genetic distance of the population used in this study

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
KX712089.1																	
P.M2	0.000																
P.M3	0.000	0.000															
P.M4	0.000	0.000	0.000														
P.M6	0.000	0.000	0.000	0.000													
P.M7	0.000	0.000	0.000	0.000	0.000												
P.M9	0.000	0.000	0.000	0.000	0.000	0.000											
P.M10	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
P.M11	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000									
P.M12	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000								
AP95	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091							
NC79	0.548	0.548	0.548	0.548	0.548	0.548	0.548	0.548	0.548	0.548	0.418						
AB01	0.491	0.491	0.491	0.491	0.491	0.491	0.491	0.491	0.491	0.491	0.494	0.402					
NC39	0.542	0.542	0.542	0.542	0.542	0.542	0.542	0.542	0.542	0.542	0.555	0.674	0.563				
NC75	0.491	0.491	0.491	0.491	0.491	0.491	0.491	0.491	0.491	0.491	0.494	0.402	0.000	0.563			
KF39	0.542	0.542	0.542	0.542	0.542	0.542	0.542	0.542	0.542	0.542	0.555	0.674	0.563	0.000	0.563		
KR85	0.548	0.548	0.548	0.548	0.548	0.548	0.548	0.548	0.548	0.548	0.418	0.000	0.402	0.674	0.402	0.674	
NC08	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.000	0.418	0.494	0.555	0.494	0.555	0.418

KX712089.1, AP95 and NC08 are reference sequences of Japanese quail; P.M4, P.M6, P.M10, P.M11, and P.M12 are Black Japanese quail; P.M2, P.M3, P.M7 and P.M9 are Brown Japanese quail; NC79 and KR85 are *Tetraogallus himalayensis*; AB01 and NC75 are *Coturnix chinensis*; NC39 and KF39 are *Tetraogallus tibetanus*

diversities (Table 3).

Data analysis showed nine sequences of Black and Brown Japanese quail were identical. They were also 100% similar with mtDNA D-loop sequence with accession KX712089.1. The number of polymorphic sites, nucleotide and haplotype diversities within Japanese quail population were also very low (Table 3). These results revealed that Japanese quail population was not heterogeneous based on mtDNA D-loop sequence. Low nucleotide and haplotype diversities in this study indicated low genetic diversity within population (Ruo-Yu *et al.*, 2006). In addition, the value of haplotype diversity increased by comparing the sequence data to other bird species obtained from GenBank database. Smith and Chesser (1981) classified $h \geq 0 < 0{,}5$ as low haplotype diversity and $h > 0{,}5 \leq 1$ as high haplotype diversity. In Indonesia, raising Japanese quail to produce egg has been conducted since long time ago, nobody exactly knows when it is introduced to farmers for the first time. The rearing system of Japanese quail is still traditional until now and there is no policy regarding breeding system, therefore the possibility of inbreeding among populations may be occurred. It may cause to low genetic diversity of Black and Brown Japanese quail. Moreover, number and origin of samples have to be improved to get more comprehensive discussion.

Phylogenetic tree and Network profile of Black and Brown Japanese quail

Table 3. Index of genetic diversity of Black and Brown Japanese quail and reference sequence of mtDNA D-loop

Population	N	S	NHap	π	h
All birds	18	109	5	0,05884	0,680
Japanese quail	12	4	2	0,00184	0,303

N is number of sequence; S is polymorphic site; NHap is number of haplotype; π is nucleotide diversity; and h is haplotype diversity

Phylogenetic tree has been constructed using nine sequences of mtDNA D-loop and nine published sequences obtained from NCBI website which are precisely started from similar forward primer and ended by reverse primer designed in this study (Figure 1). The construction of phylogenetic tree showed that Black and Brown Japanese quail are located in the same clade with reference sequence with accession number KX712089.1 whereas two Japanese quails with accession number AP003195.2 and NC_003408.1 were in the sub clade of this clade. This result may be caused different maternal lineage between them since mitochondrial genome is well known maternally inherited and no recombination occurred on it (Hayashi *et al.*, 1985; Herbert *et al.*, 2003). Additionally, Japanese quail was clearly different clade compared to other species of *Coturnix*. Moreover, the values of bootstrap in the phylogenetic tree constructed in this study was very high about 99% that means the level of confidence set for topology phylogenetic tree was also very high (Batubara *et al.*, 2011; Hall, 2011). In term of network profiling, it has been traced using five haplotypes in accordance to mtDNA D-loop nucleotides used in the analysis (Figure 2). In this current study, Black and Brown Japanese quails were included in haplotype 1 (H_1), two reference sequences of Japanese quail were grouped in haplotype 2 (H_2), and three other species were completely separated from Japanese quail. Each species grouped in different haplotypes. This study found two haplotypes of Japanese quail which is able to be used to discriminate it from other birds, however

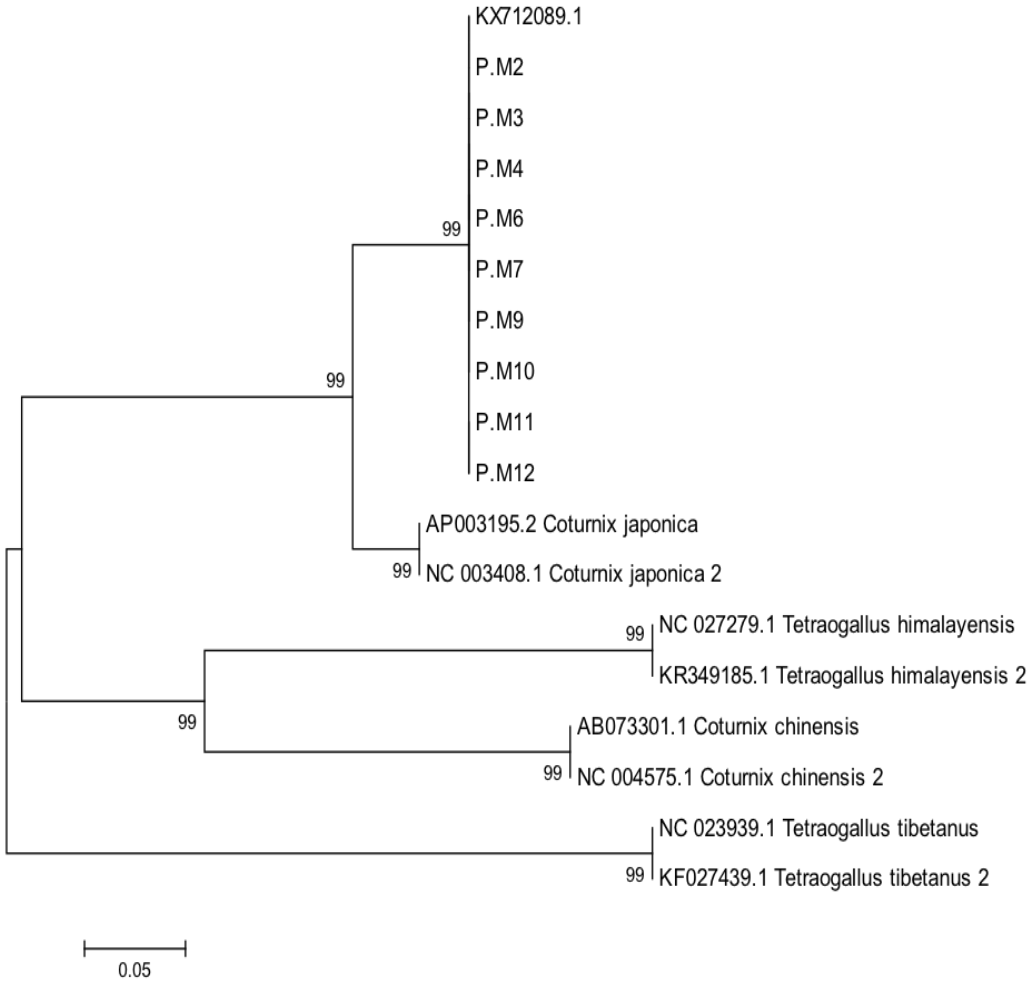

Figure 1. Phylogenetic tree of nine samples of Black and Brown Japanese quail and reference sequences obtained from NCBI database

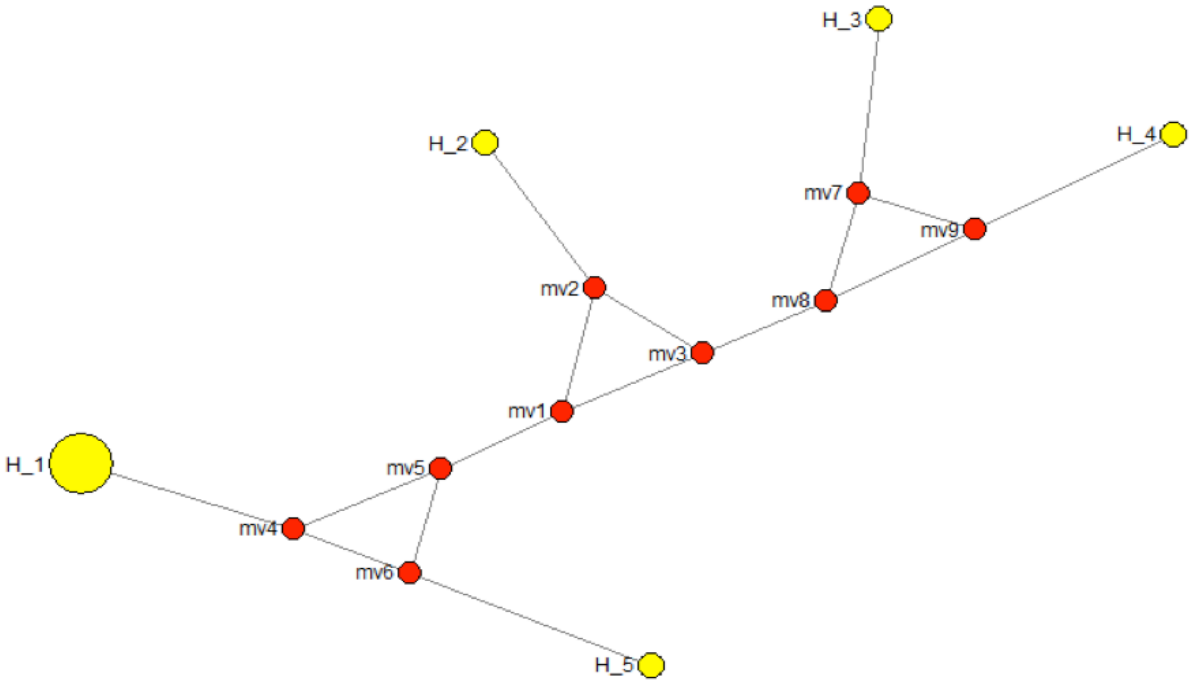

Figure 2. Network profiles of 5 haplotypes from population used in this study. The median vectors (mv) are nucleotide junctions that hypothesized as ancestral sequence

between Black and Brown Japanese quail was genetically not different based on partial region of mtDNA D-loop. The advantages of using D-loop region of mtDNA in phylogenetic study are due to maternally inherited and no recombination occurred in this region (Herbert *et al.*, 2003). In conclusion, Black and Brown Japanese quails in Indonesia were genetically not different each other based on D-loop region of mtDNA. Two haplotypes have been identified for Japanese quail that makes Japanese quail clearly discriminated form other species of birds used in this study.

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