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Comparing growth, immune and pigmentation related gene expression in three lines of Japanese and wild European quail

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This study was conducted to identify the differences and similarities among three Japanese quail (JQ) lines (JQ lines: white, brown and wild-black) and the European quail (EQ). The qRT-PCR was used to determine the expression of growth related genes: growth hormone (GH) and Insulin-like growth factor I (IGF-I), immune genes (Interleukins 1β, IL-1β, Interferon-α, IFN-α), pigmentation genes; dopachrome tautomerase, (Dct) and endothelin receptor type B2, (EdnrB2) in several quail tissues, while PCR-RFLP analysis of the mitochondrial 12S rRNA gene was conducted in quail meat.
Expression levels of the pigmentation related genes (Dct and EdnrB2) were significantly higher ($P<0.05$) in the JQBr and EQ lines than in JQwh and they were comparable between JQbr and wild EQ. Expression levels of the growth related genes (GH and IGF-1) were significantly higher in 3 JQ lines than in EQ. No differences between all 4 quail lines were found in the expression of the immune related genes. In conclusion, the PCR-RFLP method may be used to distinguish between the Japanese and the European quail, which is important for breeding programs, labeling meat products and biodiversity studies.

KEY WORDS: gene expression /growth rate / feather colour / humoral immunity / mutations / restocking

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The common European quail (*Coturnix coturnix coturnix*) is an extremely mobile quail that has spread over Europe, Eurasia and North Africa. In Europe, the European quail populations have experienced a serious decline in the last four decades because of overhunting and farm restocking practices involving farm reared hybrids [Guyomarc'h, 2003; Puigcerver *et al*. 2012]. Therefore, most European countries passed regulations to stop these practices for maintaining *Coturnix coturnix coturnix* stocks [Puigcerver *et al*. 2012]. The Japanese quail is scattered in the North-East Asia, so its range slightly overlaps that of the European quail. The two species are naturally sympatric only in the Baikal region in Russia and the Khentei region in Mongolia [Guyomarc'h, 2003]. Japanese quail strains have been established and served as animal models in avian toxicology, sexual behavior and microbiological laboratory studies [Ball and Balthazart, 2010; Nain *et al.* 2011]. Thus, plenty of data are available concerning the characteristics of the Japanese quail.

Quail are sensitive to various avian viral diseases such as Newcastle disease (ND), fowl pox and avian influenza [Lavoie *et al*., 2007; Sharawi *et al*. 2015]. Innate immune responses of chicken and ducks have been examined against viral diseases [Kwon *et al*. 2008, Adams *et al*. 2009; Reemers *et al*. 2010], whereas little data are available on the immune response of quail. Cytokines, including interferons (IFNs) and interleukins (ILs), participate in the host immune system by controlling lymphocyte activation, proliferation, differentiation and function. In addition, ILs play an important role in enhancing humoral immunity against viral diseases, *e.g.* ND [El Sabry *et al*. 2012]. Knowledge of the cytokine gene expression profile can elucidate the pathway of the interaction between host immunity and an infectious disease [Reemers *et al*. 2010]. Cytokines may be measured accurately at the mRNA level by the Real Time polymerase chain reaction (qRT-PCR) [Giotis *et al*. 2015].

From the commercial perspective, the identification of poultry species, especially quail, has been gaining in practical importance as a tool to avoid unfair competition and to assure accurate and truthful labeling. So, the need of food manufacturers to find efficient methods to confirm the validity of their products in accordance with government legislations has become extremely important [Sun 2008].

Even though there are many protein-based methods for meat identification, these methods are characterised by low sensitivity and inability to detect heat-treated meat due to the denaturation of protein during processing. On the other hand, DNA-based methods are best in species identification techniques, even in the case of processed meat. The advantage of DNA-based techniques depends on the stability of DNA molecules compared to protein ones [Lanzilao *et al*. 2005, Montowska and Pospiech 2007]. Some of these techniques have been used to identify meat of different species, *e.g.* PCR amplification [Tanabe *et al*. 2007], multiplex PCR [Asensio *et al.* 2008] and RT-PCR [Fajardo *et al*. 2008]. In addition to the above-mentioned techniques, the restriction fragment length polymorphism (PCR-RFLP) technique has been widely developed for the typing and differentiation of beef (*Bos taurus*), pork (*Sus scrofa*) and chicken (*Gallus gallus*) species [Murugaiah *et al*. 2009].

Despite the importance of the European quail, most published data appear to be performed on the Japanese quail (*Coturnix coturnix japonica*) or with hybrids between the two quail species. Therefore, the objective of this study was to evaluate the differences between lines of the Japanese quail and the European quail using two different techniques: 1) qRT-PCR to determine the expression of the growth related genes growth hormone (GH) and insulin-like growth factor I (IGF-I), the immune genes (Interleukins 1β, IL-1β, Interferon- α, IFN-α), the pigmentation genes (dopachrome tautomerase (Dct), endothelin receptor type B, EdnrB2) in several quail tissues; and 2) PCR- RFLP analysis of the mitochondrial 12S rRNA gene in quail meat (pectoralis major muscle).

Material and methods

This study was approved by the Institutional Animal Care and Use Committee, Cairo University (Protocol number: CU-II-F-6-16).

Three Japanese quail lines were obtained from the quail populations of the Quail Farm, the Faculty of Agriculture, Cairo University. These lines mainly differ in the colours of their feathers (white, brown and the wild color "black"). As for the migratory wild European Quail, these birds were caught at the North African city of El-Dabaa, which is a Mediterranean Sea city about 80 miles west of Alexandria, Egypt. These birds had been acclimated to the College of Agriculture Quail Farm's environmental conditions [Stino, 1975] for 6 months before sampling. By that time they had reached sexual maturity [Stino, 1975], the females started to lay and the males were crowing (singing). All the quail used in this study were housed in the same facilities and received the same diet and light treatment (16 L and 8 D). Sampling was performed on the same day for all the quail used. The 3 Japanese quail lines were of the same age (8 weeks old). However, we did not know the real age of the migratory European quail.

RNA extraction and RT-PCR amplification

Extraction of total RNA. Brain, liver, bursa and feather pulp cells from the different quail lines and species (European and Japanese) (5 males per group) were used to extract total RNA using the TRIzol® Reagent (cat#15596-026, Invitrogen, Germany). Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and photospectrometrically quantified at A_{260}. The purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, its integrity was assured by ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT) and then stored at -80°C.

Synthesis of cDNA using the reverse transcription (RT) reaction. The RNA samples isolated from each quail brain, bursa and liver tissues were reverse transcribed into cDNA in a total volume of 20 µl using the RevertAid™ First Strand cDNA Synthesis Kit

(MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as the master mix (MM). The MM consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec. at 1000 g and transferred to the thermocycler (Biometra GmbH, Göttingen, Germany). The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through RT-PCR.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Step one RT-PCR Cycler (Applied Biosystems, Life Technologies, USA) was used to determine the quail cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water and 5 µL of cDNA template. The primers were summarized in Table 1. The quantitative values of the RT-PCR (qRT-PCR) of GH, IGF-I, IFN-α, IL-1β, Dct and EdnrB2 genes were normalised on the basis of β-actin expression.

Table 1. Primers used for quantitative real time-polymerase chain reaction

Genes	Primer sequence (5`- 3`)	References/ Accession Nos.
GH	F: GCTGCCGAGACATACAAAGAG R: GAGCTGGGATGGTTTCTGAG	FJ458436
IGF-I	F: CACCTAAATCTGCACGCT R: CTTGTGGATGGCATGATCT	FJ977570.1
IFN-α	F: CCTTGCTCCTTCAACGACA R: CGCTGAGGATACTGAAGAGGT	AB154298
IL-1β	F: CTTCCTCCAGCCAGAAAGT R: CAGCTTGTAGCCCTTGAT	AB559570[4]
Dct	F: CCCCGTGTCTGCATGA R: CGCAGTGGGCAGCTGTTC	Niwaa *et al*. [2002]
EdnrB2	F: GCCAGACCCCAGACACCT R: GTCTTGGCCACCTCCC	Niwaa *et al*. [2002]
β-actin	F: ACCCCAAAGCC AACAGA- R: CCAGAGTCCATCACAATACC	L08165

GH – growth hormone; IGF-I – insulin-like growth factor 1; IFN-α – interferon; IL-1β – interleukin; Dct – dopachrome tautomerase; EdnrB2 – endothelin receptor type B.

Calculation of Gene Expression. First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae [Bio-Rad 2006]:

$Ef = 10^{-1/slope}$

Efficiency (%) = (Ef – 1) x 100.

The relative quantification of the target to the reference was determined using the ΔC_T method if E for the target (GH, IGF-I, IFN-α, IL-1β, Dct and EdnrB2) and the reference primers (β-actin) are the same [Bio-Rad, 2006]:

Ratio (reference/target gene) = Effe CT(reference) – CT(target).

Meat samples

Pectoralis major muscle samples were collected from the same sacrificed birds, immediately after scarifying of birds from different groups and stored at -80şC until use. Each sample was prepared and analysed in five replicates.

DNA Extraction. The DNA was extracted from the pectoralis major muscle of different quail groups using the DNeasy® tissue kit (QIAGEN, Germany) according to the manufacturer's instructions. Subsequently, the quality of DNA was assessed by agarose gel electrophoresis using 1% agarose gel stained with ethidium bromide. The purity and concentration of DNA was verified using Nanodrop at 260 and 280 nm. The DNA sample showing the OD 260:280 nm value of 1.70 to 1.90 was considered to be of good quality.

PCR amplification. Universal primers for the mitochondrial 12S r RNA gene were used for PCR amplification as described by Kocher *et al.* [1989].

Forward 5'- CAAACTGGGATTAGATACCCCACTAT- 3'

Reverse 5'- GAGGGTGACGGGCGGTGTGT-3'

Polymerase chain reaction (PCR) was performed in 25 µl of the reaction mixture containing 2 µl of genomic DNA, 12.5 µl of the master mix, 1 µl of each primer (100 ng) and 8.5 µl RNase-free water. Amplification was performed on a thermal cycler using 0.2 ml reaction tubes. The PCR program consisted of 4 min denaturation at 96°C, followed by 35 cycles of denaturation (94°C, 30 s), annealing (63°C, 30s and primer extension (72°C, 30 s). The final cycle was followed by extension at 72°C for 7 min.

Restriction fragment length polymorphism (RFLP-PCR) analysis. Five DNA samples from each group or line were individually extracted and subjected to PCR amplification for use in the PCR-RFLP trials. PCR amplicons of the mitochondrial 12S rRNA gene were subjected to restriction enzyme digestion with *Acil* restriction enzyme according to the supplier's instructions. The digested product was visualised by electrophoresis in agarose gel along with the 100 bp ladder.

Gel electrophoresis. Agarose gel (1.5%) electrophoresis was used to analyse PCR products. Electrophoresis was run for 15 min at 100 V, followed by 40 min. at 50 V. The PCR product was analysed using a UV transilluminator with the PCR fragment length determined with the bp 100 ladder.

Statistical analysis

All data were subjected to a one-way analysis of variance and the significance of the differences between means was tested using Duncan's multiple range test at ($P<0.05$). The software used was SAS, Version 9.1 [Statsoft Inc., Tulsa, USA].

Results and discussion

Expression of growth (GH, IGF-I), immune (IFN-α, IL-1β) and pigmentation (Dct, EdnrB2) related genes

To the best of our knowledge, this is the first time that the mRNA expression of GH and IGF-1 has been examined in the European quail. The GH and IGF-I gene expression of all the Japanese quail lines were higher compared to those of the European quail (EQ) ($P< 0.05$) (Fig. 1ab).

The results for the immune-related genes (IFN-α and IL-1β) showed no differences between the studied quail groups ($P> 0.05$) (Fig. 2ab).

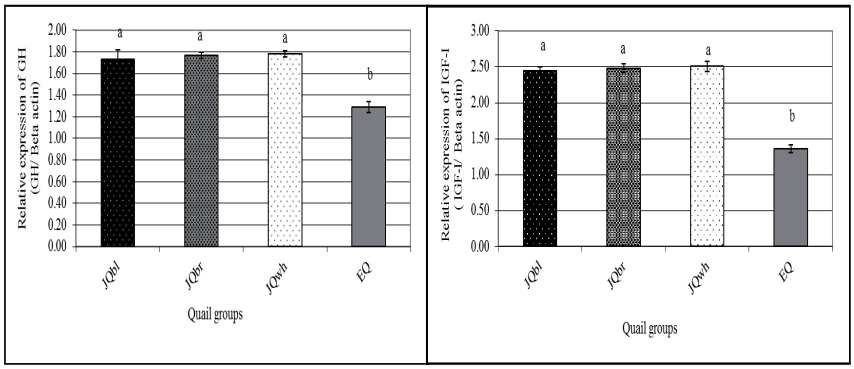

Fig. 1. Quantitative RT-PCR analysis of a) GH-mRNA in brain and b) IGF1-mRNA in liver collected from different quail groups. Columns with different letters differ significantly ($P\leq0.05$). JQbl – Japanese quail, black; JQbr – Japanese quail, brown; JQwh – Japanese quail, white; EQ – European quail.

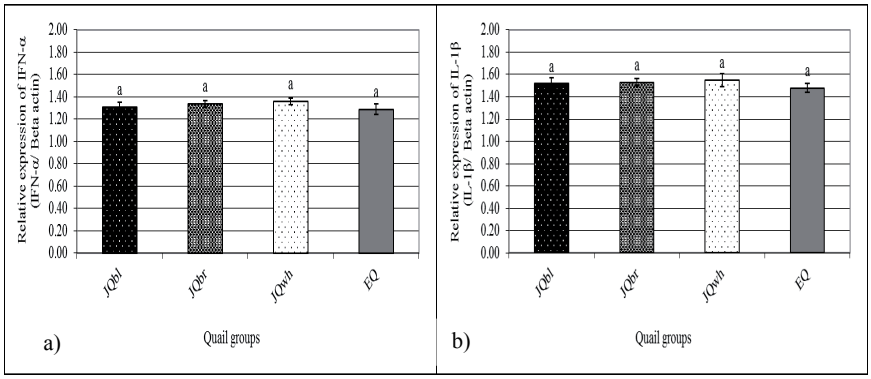

Fig. 2. Quantitative Real Time-PCR analysis of a) IFN-α-mRNAs and b) of IL-1β-mRNAs in the bursa tissues collected from different quail groups. JQbl – Japanese quail black; JQbr – Japanese quail brown; JQwh – Japanese quail white; EQ – European quail. No significant differences were observed.

The expression levels of the pigmentation related genes, *Dct and EdnrB2*, were the highest (P<0.01) in the JQbl compared to the other JQ lines and the EQ (Fig. 3 ab). Moreover, the expression levels of the *Dct and EdnrB2* genes were higher (P<0.05) in JQbr and EQ compared to JQwh.

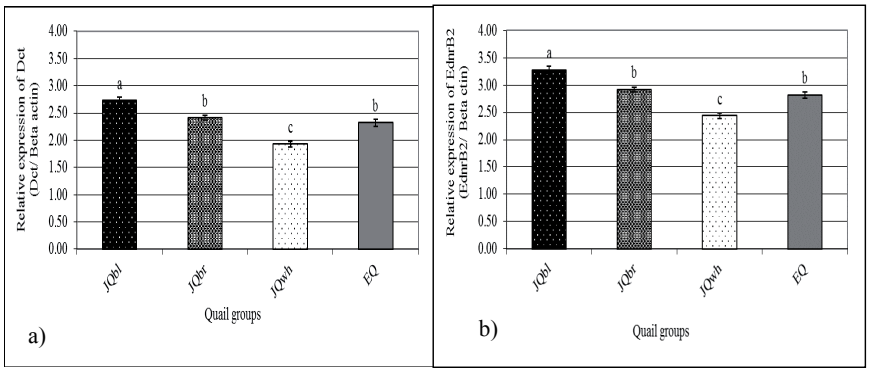

Fig. 3. Quantitative RT-PCR analysis of a) Dct-mRNAs and b) EdnrB2-mRNAs in feather cells bulb collected from different quail groups. Columns with different letters are significantly different (P≤0.05) from each others. JQbl – Japanese quail black; JQbr – Japanese quail brown; JQwh – Japanese quail white; EQ – European quail.

Restriction fragment length polymorphism (PCR-RFLP) analysis

In the current study, primers were used to amplify the common fragment (456 bp in size) of the mitochondrial 12S r RNA gene from different quail groups. DNA samples from each group was separately extracted and subjected to PCR amplification for use in the PCR-RFLP trials. PCR products were successfully amplified to the expected 456 bp fragment within the 12S r RNA gene (Fig. 4a). PCR-RFLP analysis using Acil restriction enzyme of the 12S r RNA gene from different quail groups is presented in (Tab. 2). Mitochondrial 12S rRNA partial sequences along with the *Acil* restriction site are shown in (Fig. 4b).

Table 2. PCR-RFLP bp analysis using the *Acil* restriction enzyme of the 12S r RNA gene from different quail species

Groups	Samples	456 pb	Fragment size in bp				
JQbl	5	+	-	210	100	96	50
JQbr	5	+	-	210	100	96	50
JQwh	5	+	-	218	108	85	45
EQ	5	+	225	206	-	-	25

JQbl – Japanese quail; black; JQbr – Japanese quail, brown; JQwh – Japanese quail, white; EQ – European quail.

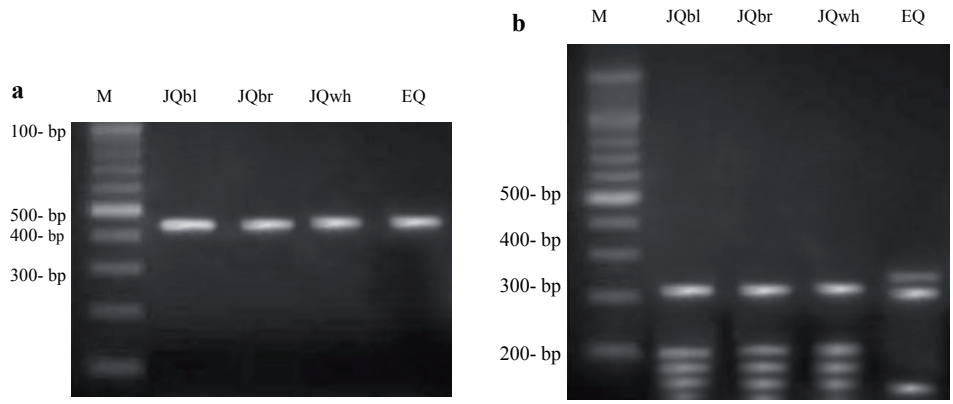

Fig. 4. Restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene from different species and lines of quail. PCR amplification (a: 456bp before digestion) were subjected to restriction analysis with *Acil* (b: 456 after digestion) resulting in the fragments presented in Table 1. M:100 bpDNA ladder. JQbl – Japanese quail black; JQbr – Japanese quail brown; JQwh – Japanese quail white; EQ – European quail.

The *Acil* enzyme was selected for the PCR-RFLP technique to detect and differentiate quail meat groups. The *Acil* enzyme generated fragments of 210, 100, 96 and 50 bp in both JQbl and JQbr (Tab. 2). However, the JQwh line revealed *Acil* enzyme generated fragments of 218, 108, 85 and 45 bp. On the other hand, the EQ species exhibited only 3 fragments (225, 206 and 25 bp) generated by the *Acil* enzyme. The bp values represent the molecular weight of DNA bands that resulted from PCR-RFLP. These bp values were calculated by a lab image software programme based on the DNA marker (Tab. 2).

This study aimed to investigate differences and similarities between different Japanese quail colour lines and the European quail. We used gene expression analysis using the RT-PCR method to determine expression of the growth (GH, IGF-I), immune (IL-1β, IFN-α) and pigmentation (Dct, EdnrB2) genes in several quail tissues.

The expression of the GH and IGF-I genes revealed that JQbl, JQbr and JQwh exhibited higher expression levels than the EQ. The results for the GH and IGF genes reveal that JQbl, JQbr and JQwh showed higher expression levels of both genes than EQ". The GH and IGF-1 gene expression had been influenced by changes in the environmental factor (heat stress) and feed supplements (methionine levels) during their lifetime; the higher expression was associated with higher levels of methionine [Del Vasco *et al*., 2015]. In our experiment, the expression of GH and IGF-1 was measured in different tissues: GH in the brain and IGF – in the liver, as GH is secreted mainly in somatotrophic cells of the anterior pituitary gland in the brain of mammals and birds [Daude *et al*., 2015], and the liver is the main source of circulating IGF-1 [Pankov, 1999; Li *et al*. 2004].

In the present study, quail were kept under the same environmental conditions, fed standard quail diets and both water and feed were available *ad libitum*. Thus, the

observed differences in GH and IGF-I gene expression levels between the JQ and EQ species may have been due to the differences in the genetic composition of each species rather than the other environmental factors.

In mammals, ELISA is usually used to measure interferon and interleukin levels in the serum; however, this method is not convenient for birds because of a lack of specific avian cytokines kits [Giotis *et al.,* 2015]. Therefore, the differences between JQ and EQ were investigated using the qRT-PCR method to quantify immune gene expression of the quail groups, because it is rapid, more accurate [Giotis et al., 2015] and requires only partial sequence information for the target genes.

The specificity of RT-PCR was validated for each gene by generating a single PCR product from quail bursa cells, which was confirmed by analysing the melt curve. The melting curves showed only one peak for each gene. These results indicate that nonspecific amplification and primer-dimer formation did not occur during PCR and guarantee the specificity of the assay. A standard curve may be used for absolute quantification. These results validate the RT-PCR assay to quantify the expressions of immune related genes in quail.

In the current study, the results of RT-PCR methods revealed that there were no significant differences in the expression of immune genes (IL-1β, IFN-α) between JQbl, JQbr, JQwh and EQ. These results may be due to the fact that the birds were not subjected to stress or infection, which could enhance the immune system resulting in an over-expression of the immune genes (IL-1β, IFN-α). Even though no differences had been noticed, the results showed that the immune genes in both species have the same pattern in the absence of stress, challenge or infection. The response of the quail species to challenges with different antigens may be investigated in future studies.

Wild-type quail embryos have longitudinal black and brown stripes formed by coloured feather buds (FBs) on their back, in which epidermal melanocytes (MCs) produce black (eumelanin) and brown (pheomelanin) pigments. However, the pigmentation pattern is disrupted by the autosomal dominant mutation of the black at hatch (Bh) locus [Minezawa and Wakasugi 1977]. In this study we examined the expression patterns of genes relating to MCs development (EdnrB2) and pigment production (Dct) in feathers of different quail species and lines (JQbl, JQbr, JQwh and EQ). Results revealed that expression levels of the EdnrB2 and Dct genes in JQbl, JQbr and EQ were higher than those in JQwh. Endothelin receptor B (DdnrB) is a G-protein-coupled receptor that is required for the development of MCs [Shin *et al.,* 1999]. They found that mice with a null mutation in the Ednrb gene are almost completely white, which partially agrees with the present results.

Dopachrome tautomerase (Dct) acts as an enzyme in the eumelanin synthetic pathway in mice [Kobayashi *et al*. 1995, Hearing 2000], and Dct mRNAs and their proteins are expressed during eumelanogenesis [Kobayashi *et al.,* 1995]. Melanosomal matrix protein 115 kDa (Mmp115) is a 115 kDa protein that was first identified in chicks using the MC/1 monoclonal antibody raised against chick melanosomes [Mochii *et al.* 1992]. Niwa *et al.* [2002] found that expression of Dct in the black MCs of quail was

stronger than in the brown MCs. They also stated that the melanogenic genes (Dct, Tyrp1, Tyr and Mmp115) were also expressed in dermal MCs of quail. Moreover, in the mouse mRNA expression of Dct depends on the type of the melanin pigment produced; Dct mRNAs are specifically expressed in eumelanogenic MCs [Kobayashi *et al.,* 1995]. These findings explain the variation in the gene expression within the JQ groups as well as the differences between JQ (bl and wh) and EQ.

The PCR-RFLP technique is more efficient in detecting species-specific DNA using mitochondrial DNA (mtDNA). That is because mtDNA expressed in each species has their evolution specificities. In addition, there are 10^4 available copies of mtDNA per cell and just one copy for genomic DNA [Cheng *et al.* 2003].

Therefore, PCR-RFLP for the 12S rRNA gene was used in this study to differentiate the closely related Japanese and European quail species. The present results also indicated that it is not possible to detect fragments (<50 bp in size) by the conventional PCR-RFLP gel-based method due to the low resolution of the agarose gel.

The results showed that each quail species exhibited a unique specific PCR-RFLP pattern that allowed its identification and discrimination from others. The PCR-RFLP pattern of 12S rRNA indicated that JQbl and JQbr shared 4 DNA bands that were identical. Moreover, JQwh exhibited 4 DNA bands which were relatively different compared to JQbl and JQbr. On the contrary, EQ exhibited only 3 DNA bands which were different compared to JQbl, JQbr and JQwh. Thus, the EQ exhibited different genetic profiles based on the 12S rRNA gene and the Acil restriction enzyme used in this study, enabling easy and unambiguous interpretation of the restriction results. The PCR-RFLP of the 12S rRNA gene in this study could differentiate between meat from closely related quail species, such as JQ and EQ.

It could be concluded that JQ exhibited significant growth and pigmentation related gene expression compared to EQ. The PCR-RFLP technique is an efficient method to differentiate between the JQ and EQ quail species. The results show similarities and differences between the JQ and EQ quail species in different characteristics of interest, which is important for restocking and biodiversity studies. In addition, PCR-RFLP as a rapid and accurate method may be used to differentiate between the JQ and EQ quail species and avoid possible conflicts arising from agricultural practices.

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