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Potential mitochondrial diversity role in the productivity of three lines of Japanese quails

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Abstract. *Hussein TH, Al-Shuhaib MBS, Al-Thuwaini TM. 2020. Potential mitochondrial diversity role in the productivity of three lines of Japanese quails. Biodiversitas 21: 2258-2265.* This study was conducted to identify the mitochondrial D-loop genetic diversity in three lines of Japanese quails that differ in productive performance. A total of 223 quails consisting of 54, 84, and 85 of black (control line), white (egg-producing line), and brown (meat-producing line) quails respectively were genotyped by PCR-single strand conformation polymorphism (SSCP). The genetic and phylogenetic differences within and among quails' populations were analyzed. Three different SSCP banding patterns were observed in black and brown quails, while white quails exhibited six different SSCP-banding patterns. Sequencing reactions confirmed the presence of 12 haplotypes with 48 variations distributed among the studied birds. The white line exhibited the most diverse nucleotide variability, followed by the brown, and black lines respectively. The mean diversity for all populations was mainly due to within-population variation (71.6%), while among-population variation accounted for much less value (28.4%). Tajima's D test showed significant values for both productive white (2.45680) and brown (3.07723) lines. In conclusion, this study suggested a wide nucleic acid variation in the investigated egg productive line than the meat productive line respectively compared with the black line control, implying a positive correlation between mitochondrial variability and productive performance.

Keywords: Biodiversity, mtDNA, phylogenesis, production performance, quails

INTRODUCTION

The mitochondrial DNA (or mtDNA) is a marker of choice in many populations. It is a powerful tool for assessing the evolutionary relationships. It can be utilized to provide a highly efficient maternal discrimination tool among many species. Several reasons have made mtDNA ideal for analyzing species origins, differentiation, and evolution, such as its simple and stable structure, low molecular weight, and maternal origin of inheritance (Mariotti et al. 2013). The sequences of mtDNA are valuable information that have been widely used to evaluate the genetic diversity of various animal species (Groeneveld et al. 2010). This is due to the fact of the elevated mutations in the mtDNA sequences compared to the nuclear DNA (Ladoukakis and Zouros 2017). These mitochondrial sequences are inherited maternally and evolve five to ten times faster than nuclear genetic markers as it appears in multiple copies in the cells and the mitochondrial gene content is strongly conserved across generations (Di Lorenzo et al. 2015). Due to this largest variation and highest mutation rate, mtDNA has been regarded as an appropriate marker to study the genetic biodiversity (Yu et al. 2019). Moreover, mtDNA has become a useful marker for the study of genetic variability and population structure in livestock (Muchadeyi et al. 2008). Within the mtDNA, a well-known control region is called D-loop which has been identified with its high degree of sequence variation. Due to this hypervariable nature of the mitochondrial D-loop, it has been utilized efficiently to explore genetic variations of species among different populations (Hudson 2017). However, the mtDNA-based polymorphic studies have taken lots of consideration in many birds, such as chicken (Meydan et al. 2016), ducks (Gaur et al. 2018), guinea fowls (Murunga et al. 2018), and ostriches (Miller et al. 2011). Islam and Nishibori (2012) reported that the high genetic diversity of the mitochondrial D-loop region was highly correlated with phenotypic variation in chicken. Moreover, D-loop diversity has also been analyzed recently in several lines of quails (Nunome et al. 2017; Rifki et al. 2018). Quails are the most attractive alternative to chickens in terms of eggs and meat. These birds are characterized by their high level of adaptability in minimized places. Furthermore, they get sexual maturity in a matter of a few weeks to produce highly valuable eggs and lower cost meat (Jeke et al. 2018). However, such promising features have not been available in all lines as some lines have been characterized with higher production of eggs, whereas the other lines have been raised only for their meat characteristics. Also, other lines have not been recognized with any remarkable superiority to be raised neither for eggs nor for meat (Nasar et al. 2016). Thus, it is maybe a rationale to estimate the patterns and the intensities of mtDNA polymorphism to identify the possible correlation for such differences in productivity. This mtDNA-based comparison may provide an extraordinary beneficial tool for breeders to take a snapshot of the polymorphic status of the mtDNA in each population (Lancioni et al. 2013). However, no special emphasis has been considered for screening the mtDNA D-

loop polymorphism with quail lines that differ in eggs and meat production criteria. Therefore, the present study was devised to analyze the mitochondrial genetic diversity in three lines of quails that differ in their productive characterizations, including white (eggs producing) line, brown (meat-producing) line, and black (control) line (Al-Kafajy et al. 2018). A simultaneous genotyping of these three lines have been developed to evaluate the possible role of these highly variable D-loop mitochondrial polymorphisms in this characterized differentiation of productivity.

MATERIALS AND METHODS

Animals

The sampling and handling procedures were conducted according to the international guidelines of the Federation of Animal Science Societies (Vaughn 2012). Three lines of Japanese quails (*Coturnix japonica*) that are two weeks of age were purchased from the directorate of agricultural research/ministry of agriculture, Baghdad, Iraq. All animals were raised according to the conditions mentioned in Al-Kafajy et al. (2018). Only 223 birds sexually mature females were included in the study, namely black (n = 54), white (n = 84), and brown (n = 85). The white and brown lines are usually raised for their eggs and meat respectively, while the black line was used as a control in this study.

Genomic DNA extraction

Blood samples were withdrawn from the wing vein using a sterile syringe and placed in anticoagulation tubes. Genomic DNA was extracted from the whole blood using a rapid salting-out technique (Al-Shuhaib 2017), with its modification mentioned in (Al-Shuhaib 2018). Genomic DNA quantity and quality were evaluated by a nanodrop spectrophotometer (Biodrop – UK).

PCR Primer design and amplification

One specific PCR oligonucleotides pair was designed with the help of PCR Primer-BLAST server (Ye et al. 2012). The PCR primers pair was made to scan 332 bp that positioned in the highly variable D-loop of the mtDNA sequences. The specified primer design was based on the GenBank accession number NC_003408.1. The sequence of D-loop forward primer was 5′- CTCTTGCTCTTTT GCGCCTC-3′, while the sequence of D-loop reverse primer was 5′- CCCAATGCGATCCAAAGTGC-3′. The melting temperature of these oligonucleotides was determined empirically by a gradient PCR thermocycler (Mastercycler, Nexus, Eppendorf, Germany). Ready PCR premix sets were utilized in this study (*Accu*Power PCR premix, Bioneer, Korea). Only 100 ng of DNA template and 1µl of 10µM of both forward and reverse primers of D-loop were utilized for each reaction. PCR reactions were initiated by 5 min denaturation, followed by 30 cycles of 30 sec denaturation at 94℃, 30 sec annealing at 60.1℃, and 30 sec of *Taq* DNA polymerase extensions at 74℃. PCR reactions were finalized by further extension of *Taq* polymerase for 5 min. The integrity and specificity of PCR amplicons were assessed by 1.5% agarose gel electrophoresis.

SSCP

The post-PCR genotyping reactions were performed using the PCR-SSCP method. The main procedures were conducted as described by Al-Shuhaib et al. (2018) with minor optimizations. Briefly, an equal volume of SSCP denaturing-loading buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA pH 8) was added to each PCR amplicon. The mixtures were heat-denatured at 95℃ for 7 min, and immediately placed in a wet icebox and kept frozen for at least 10 min. Subsequently, denatured samples were loaded on a 0.1 thickness mm neutral polyacrylamide gel (Mini-wide gel format, JUNYI Electrophoresis equipment, China). Electrophoresis running conditions were optimized on 200 V and 99 mA for 5 hours at 12℃. Gels were stained by silver nitrate according to the protocol described by Byun et al. (2009).

Sequencing

Each observed SSCP banding pattern was submitted for sequencing reactions from both forward and reverse termini as recommended by sequencing laboratories (Macrogen, Korea). The sequences of all the observed SSCP-banding patterns were aligned and annotated using Editseq DNA STAR ver. 7.0 software (DNASTAR, USA). Each detected variant was manually visualized and confirmed by SnapGene viewer (http://www.snapgene.com). Only clear electropherograms were considered in the alignment of the observed variations with their retrieved references from the Japanese quails' sequences (GenBank accession number NC_003408.1).

Data analyses

A median-joining network was generated to compare among the observed haplotypes using PopART software ver. 4.8.4 (French et al. 2014). A comprehensive tree was initially generated by a Clustal Omega tool (Siever and Higgins 2014), which assess the phylogenetic associations of the detected haplotypes together with their related sequences using a neighbor-joining method with calculated distances. Subsequently, the generated preliminary tree was annotated by the iTOL server (Letunic and Bork 2019). The positions and numbers of nucleic acid variations as well as corresponding haplotypes were calculated using DnaSP software ver. 6.12.01 (Librado and Rozas 2009). The Analysis of Molecular Variance (AMOVA), including the relative haplotype frequencies, the percentage of variation within and among the studied three birds' populations and pairwise fixation index (F_{ST}), was carried out using Arlequin software ver. 3.5.2.2 (Bern, Switzerland) (Excoffier et al. 2005).

RESULTS AND DISCUSSION

Results

Due to a large number of the included mtDNA samples, a sensitive post-PCR genotyping approach was successfully conducted to identify the patterns of the unknown genetic polymorphism in large-scale screening purposes (Hashim and Al-Shuhaib 2019). PCR-SSCP reactions were indicated a total of 12 SSCP banding patterns in all three studied populations. Concerning both black and brown lines, only three SSCP-banding patterns were observed having different gel migrations and profiles characterized by each line. Meanwhile, the white line exhibited 6 forms of SSCP electrophoretic patterns (Figure 1.A). Sequencing reactions confirmed these detected patterns and indicated the presence of 12 mitochondrial haplotypes; haplotypes no. 1 –3, 4 –9, and 10 –12 for black, white, and brown lines respectively (Figure 1.B). Within the identified 12 haplotypes, sequencing reactions showed a total of 48 variations, and 30 variations were common for all the investigated populations (Table 1).

All the observed haplotypes were registered in GenBank under the accession numbers MN217537- 39, MN217540- 45, and MN217546- 49, for Black, White, and Brown lines respectively (Table 2).

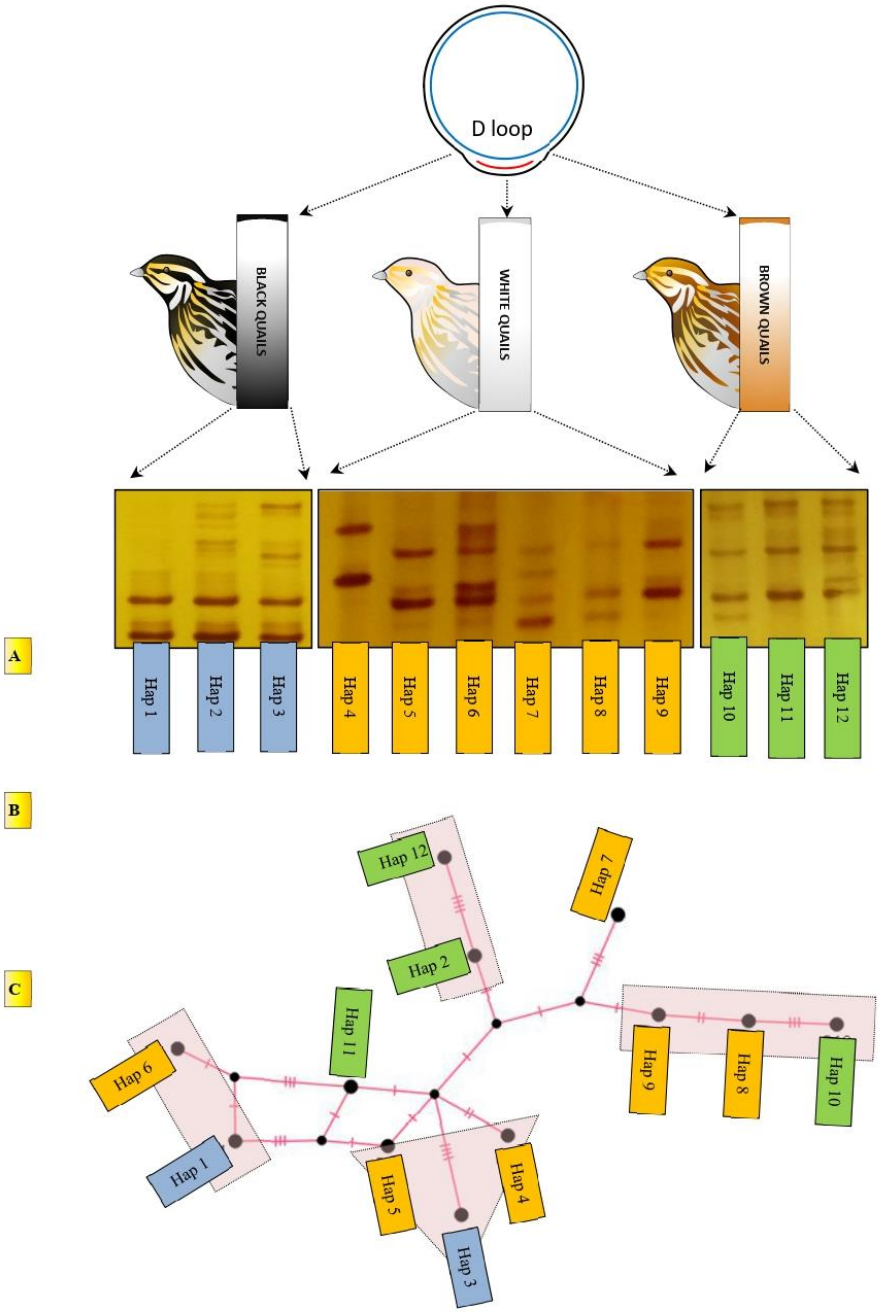

Figure 1. A workflow for the mitochondrial based genetic diversity of three lines of quails; black, white, and brown based on D-loop sequences. A. The detected PCR-SSCP banding patterns for each studied line. B. haplotyping of the involved three lines as confirmed by sequencing. C. a median-joining network for the interconnections between the observed haplotypes.

A specified median-joining network was generated to identify the accurate maternal relationship for these detected haplotypes. Two types of interconnections were revealed among the three studied lines (Figure 1.C). One of these interspecies connections was observed between both black and white lines. This is due to the detected clear vicinity of haplotypes no. 1 of black line with haplotypes no. 6 of the white line and haplotypes no. 3 of black line with haplotypes no.4 and no. 5 in the white line. Another interconnection was observed between haplotype no. 8 and no. 9 of the white line with the haplotype no. 10 of the brown counterpart.

However, the generation of a comprehensive neighbor-joining phylogenetic tree indicated close clustering of all of these observed mitochondrial haplotypes within the same quails' phylogenetic regions (Figure 2). However, other organisms other than quails were also shown to possess close homology with the investigated mitochondrial haplotypes of similar organisms, including many strains that belonged to *Alectoris chukar*, *Alectoris magna*, and *Alectoris graeca*.

Table 1. Nucleic acids variations of the *D loop* region detected in three lines of quails. The symbol "Hap" refers to the detected haplotype in the amplified portion of the mitochondrial D-loop. The symbol "ins" refers to insertion mutation.

Polymorphic	**Black line**					**White line**				**Brown line**		
site	**Hap1**	**Hap2**	**Hap3**	**Hap4**	**Hap5**	**Hap6**	**Hap7**	**Hap8**	**Hap9**	**Hap10**	**Hap11**	**Hap12**
3	T	T	T	T	T	T	T	T	T	T	T	T
7	G	.	.	.	G	.	.	G	.	G	.	.
9	T	T	T	T	T	T	T	T	T	T	T	T
15	G	.	.	.	.	G	.	.	.	.	.	.
20	T	T	T	T	T	T	T	T	T	T	T	.
53	A	.	.	A	A	A	.	.	.	A	A	.
61	A	.	.	.	.	A	.	.	.	.	A	.
65	T	T	T	T	T	T	T	T	T	T	T	T
67	C	C	C	C	C	C	C	C	C	C	C	C
74	C	C	C	C	C	C	C	C	C	C	C	C
85	T	T	T	T	T	.	T	T	T	T	T	.
90	G	.	.	.	.	G	.	.	.	.	.	.
92	T	T	T	T	T	T	T	T	T	T	T	T
96-97 ins	A	A	A	A	A	A	A	A	A	A	A	A
104	T	T	T	T	T	T	T	T	T	T	T	.
114	G	.	.	G	.	G	.	.	.	.	.	.
133	T	T	T	T	T	T	T	T	T	T	T	T
136	T	T	T	T	T	T	T	T	T	T	T	T
138	T	T	T	T	T	T	T	T	T	T	T	T
150	T	T	T	T	T	T	T	T	T	T	T	T
153	T	T	T	T	T	T	T	T	T	T	T	T
156	T	T	T	.	T	T	T	T	T	T	T	T
158	T	.	T	T	T	T	T	T	T	T	T	.
166	G	G	G	G	G	G	G	G	G	G	G	G
169	T	T	T	T	T	T	T	T	T	T	T	T
178	G	G	G	G	G	G	G	G	G	G	G	G
193	G	G	G	G	G	G	G	G	G	G	G	G
195	C	C	C	C	C	C	C	C	C	C	C	C
198	C	C	C	C	C	C	C	C	C	C	C	C
204	C	C	C	C	C	C	C	C	C	C	C	C
210	.	.	A	.	.	.	.	.	.	.	.	.
220	G	G	G	G	G	G	G	G	G	G	G	G
227	A	A	A	A	A	A	A	A	A	A	A	A
236	G	G	G	G	G	G	G	G	G	G	G	G
242	.	.	.	.	.	.	C	.	.	C	.	.
248	.	.	A	.	.	.	A	.	.	A	.	.
253	A	A	A	A	A	A	A	A	A	A	A	A
255	A	A	A	A	A	A	A	A	A	A	A	A
264	G	G	G	G	G	G	G	G	G	G	G	G
270	C	C	C	C	C	C	C	C	C	C	C	C
275	A	A	A	A	A	A	A	A	A	A	A	A
278	.	.	.	.	.	.	.	C	.	C	.	C
280	G	G	G	G	G	G	G	G	G	G	G	G
288	G	G	G	G	G	G	G	G	G	G	G	G
297	.	.	A	.	.	.	A	.	.	.	.	.
306	.	.	.	.	.	.	A	A	A	A	.	.
312	A	A	A	A	A	A	A	A	A	A	A	A
314	.	.	G	.	.	.	.	.	.	.	.	.

Table 2. Nucleic acids variations of 12 detected in three lines of quails. The symbol "N" refers to the number of quails shares the same SSCP-banding pattern, while the symbol "Hap" refers to the detected haplotype patterns in the amplified portion of the mitochondrial D-loop.

Quail line (n)	Haplotype	N	GenBank accession number
Black	Hap-1	7	MN217537
Black	Hap-2	41	MN217538
Black	Hap-3	6	MN217539
White	Hap-4	7	MN217540
White	Hap-5	10	MN217541
White	Hap-6	36	MN217542
White	Hap-7	11	MN217543
White	Hap-8	11	MN217544
White	Hap-9	9	MN217545
Brown	Hap-10	32	MN217546
Brown	Hap-11	34	MN217547
Brown	Hap-12	19	MN217548
Black/White/Brown	Hap-1-12	223	MN217537-48

Table 4. The relative haplotype frequencies of three Japanese quails' populations. the symbol "Hap-" refers to "Haplotype-D-loop

Haplotype	Black line	White line	Brown line
Hap-1	0.129630	0	0
Hap-2	0.759259	0	0
Hap-3	0.111111	0	0
Hap-4	0	0.083333	0
Hap-5	0	0.119048	0
Hap-6	0	0.428571	0
Hap-7	0	0.130952	0
Hap-8	0	0.130952	0
Hap-9	0	0.107143	0
Hap-10	0	0	0.376471
Hap-11	0	0	0.400000
Hap-12	0	0	0.223529

The genetic diversity was estimated through evaluating the number of polymorphic sites (S), the number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (Pi), the average number of nucleotide differences (K), and Tajima's D test statistic (D) for all three quail populations. The number of mitochondrial haplotypes was varied from 3, in both black and brown lines, to 6 in the white line with higher Hd values (0.758 and 0.656) of the white and brown lines than Hd values of the black line (0.402). Moreover, both Pi and K values showed higher values (0.01578 and 0.01532 for Pi, and 5.254 and 5.1437 for K) in white and brown lines respectively than the black line values (0.00811 for Pi and 2.700 for K). These higher values of Hd, Pi, and K were represented by significant deviations from neutrality as it was shown by Tajima's D test statistic (2.45680 and 3.07723) for both white and brown lines. Meanwhile, no significant deviation of Tajima's D test statistic values (0.33989) was observed in the black line (Table 3).

Relative haplotype frequencies were calculated in each included line (Table 4). Haplotypes no. 2, 6, and 11 were found to be the most frequent (0.759259, 0.428571, and 0.4) in the black, white, and brown lines respectively.

With respect to other AMOVA calculations, it was found that within-population variation value was accounted for 71.60% of the genetic variation while differences among populations generated a rather small contribution of only 28.40% to the total genetic variation of the investigated three quail's populations (Table 5).

The total F_{ST} value was 0.28397 which indicated that the investigated population differentiation was highly significant ($P < 0.01$). Likewise, the estimated individual values of pairwise F_{ST} in the three quail's populations were shown in Table 6.

The black line was most closely related to the white line (F_{ST} = 0.26508), while further apart from the brown line (F_{ST} =0.40924). Likewise, the white line was least related to the brown line (F_{ST} = 0.21549).

Table 6. Pairwise F_{ST} values between three lines of Japanese quails based on mtDNA D-loop sequence

Line	Black	White	Brown
Black	0.00000	-	-
White	0.26508	0.00000	-
Brown	0.40924	0.21549	0.00000

Table 3. Sampling size (N), number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (Pi), average number of nucleotide differences (K), and Tajima's D test statistic (D) for each black, white, and brown quail populations.

Line	N	S	H	Hd	Pi	K	D
Black	54	11	3	0.402	0.00811	2.700	0.33989 (non-significant)
White	84	14	6	0.758	0.01578	5.254	2.45680 (significant P<0.05)
Brown	85	12	3	0.656	0.01532	5.1437	3.07723 (significant P<0.01)

Table 5. Analysis of Molecular Variance (AMOVA) of the mt-DNA D-loop region analyzed in three populations of Japanese quails. The symbol "Fst" refers to the fixation index.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation	F_{ST}	p-value
Among populations	2	116.784	0.77378 Va	28.40	-	-
Within populations	220	429.243	1.95111 Vb	71.60	-	-
Total	222	546.027	2.72488	-	0.28397	0.00

Note: Va= variance among population; Vb= variance between populations

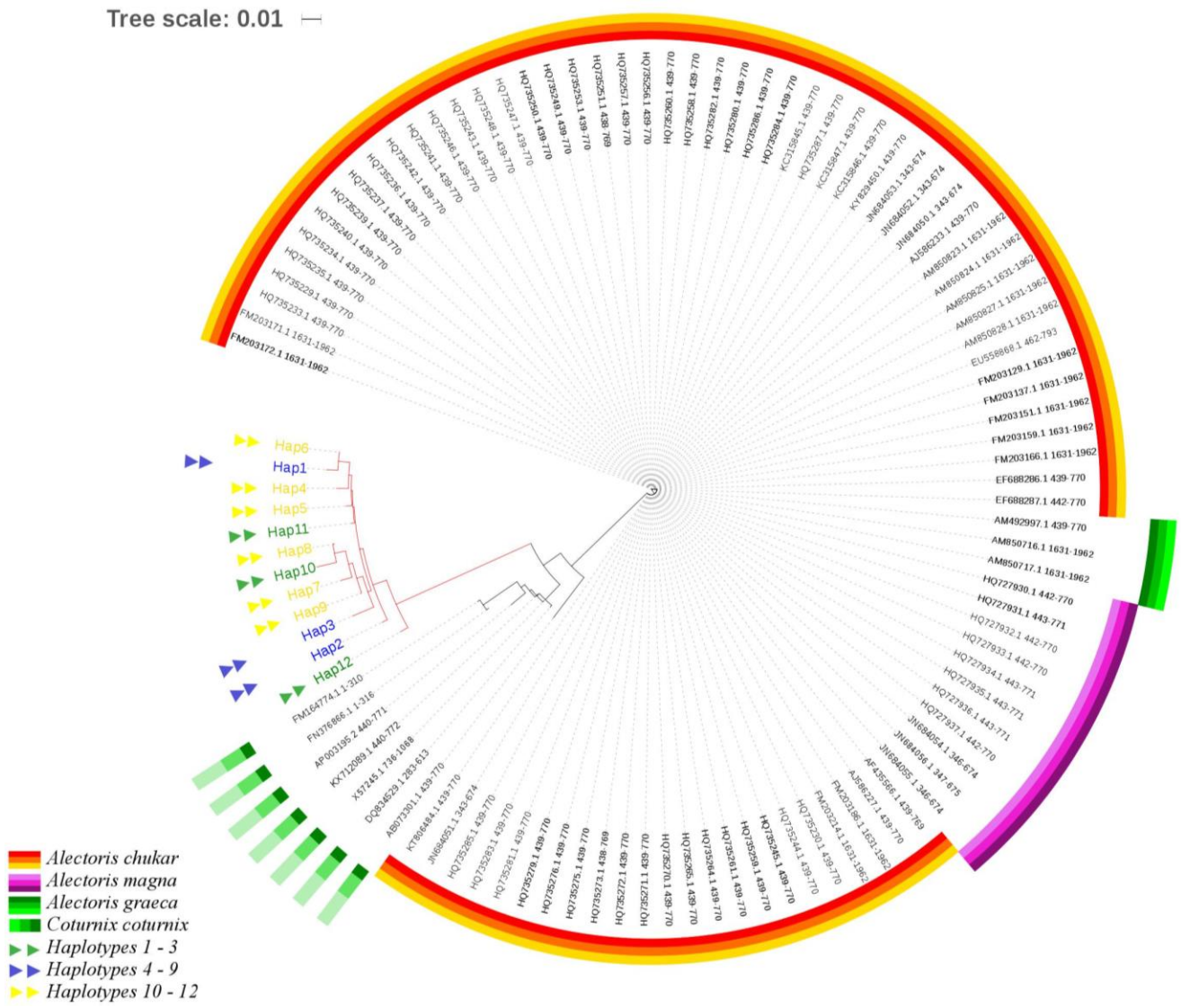

Figure 2. A mitochondrial-based neighbor-joining tree for three lines of quails. the analysis involved 106 sequences. The tree was conducted using Clustal Omega phylogeny, then annotated by the ITOL server.

Discussion

The mitochondrial diversity was evaluated among three different lines of quails. A total of 12 haplotypes were identified in this study, in which the white line showed the highest number of haplotypes. The higher ration of the mtDNA-D-loop diversity was observed in the white line that reared for its ability to produce a higher number of eggs than the other two lines. However, this finding may relate such observation to the high productivity point that is usually known in the white line in this aspect. This analysis revealed considerable genetic diversity in the white line quails, which is known to have been reared for egg production. However, long-term artificial selection programs may lead to reduced nucleotide diversity (Yu et al. 2019). This finding implies that the current selection programs for egg production have not conducted over long time. In addition to the egg producing line, this finding of the possible association between the high rate of D-loop polymorphism with productivity rate could not be eliminated in the meat producing line too. This is due to the detection of relatively higher nucleotide polymorphism in the meat producing brown line than the control black line. These findings of higher D-loop diversity in both productive white and brown lines compared with the non-productive black line may implicate the observed maternal genetic variations with the elevated productive performance of these birds. In agreement with our results, it is reported that the individual differences in birds' performance might be rooted in the mitochondrial variations (Stier et al. 2019). Moreover, the finding of this study has been aided by other findings that reported a similar contribution to D-loop in such patterns of association (Bottje 2018; Hood et al. 2018). In addition, it was found that birds display a positive correlation between

mitochondrial performance and the rate of growth (Jimenez 2018). However, the functionality of mitochondrial variations on avian species could not be excluded from this explanation (Stier et al. 2013).

The detection of many shared genetic variations in all three lines of quails refers to the fact of the presence of remarkable genetic similarity in these populations. The generation of a specified network has provided more data regarding this issue. Furthermore, clear intra-species phylogenetic associations were observed in white, brown, and black lines in this study. These associations were rooted in the presence of high sequences similarity among the involved species. Such close relations could suggest that the studied lines were derived from common maternal ancestors regardless of distinctive differences in their phenotypes and physiological/reproductive performance (Teinlek et al. 2018). Meanwhile, AMOVA calculations revealed larger population variability values within populations than values from among populations. This observation indicated that Tajima's D statistics showed significant deviations from neutrality in both white and brown lines. Both Tajima's D and Fu's F-statistics values obtained for the neutrality test revealed a significant departure from the neutrality assumption. These results support the idea that domestication of these investigated birds facilitated population increases (Wu et al. 2014).

The estimated haplotype diversity for the white line was higher than that found in brown and black lines respectively. Accordingly, it can be suggested that the white line is more polymorphic than the other two lines. This observation implies that the white line quails have desirable conservation status, as they carry high levels of genetic diversity. Thus, the white line deserved more attention since it has the highest number of haplotypes and it exhibited interesting maternal relationships with both black and brown lines respectively. These data raised a question of whether the white line high ratio of polymorphism participates in the high productivity of eggs. Mansour et al. (2010) showed that knowledge of the relationships between genetic diversity and quail phenotypes could be an invaluable tool in the improvement of production performance. However, no concrete data was provided here as there were no direct phenotype-genotype connections between both findings were confirmed. The data obtained in this study will assist future conservation management of local lines of quails and also reveals intriguing implications mtDNA diversity for both white and brown lines in further genetic breeding programs, whether in eggs or meat production.

In conclusion, this study has uncovered the population structure and the mitochondrial genetic diversity among three lines quails of different productivity. The higher ration of mitochondrial genetic diversity may be related to the highly dynamic nature of the white line with a special tendency to undergo the essential productivity functions in terms of the production of eggs. The same thing could be applied to the brown line with a less extent. On the other hand, no high mtDNA polymorphism was observed in the non-productive black line. These findings signify a particular potential correlation between elevated polymorphism of the mtDNA sequences with high productivity in quails.

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