HAPLOTYPE ANALYSIS IN CHICKEN MITOCHONDRIAL DNA D-LOOP REGION FOR BREED IDENTIFICATION

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***Abstract*-The mitochondrial (mt) DNA D-loop sequences have been widely used for identification of origin of the breeds in animals. In this study, chicken mtDNA haplotypes were investigated for identifying the mtDNA haplotyes of the commercial Korean native chicken breed and the genetic relationships were investigated by comparing with other chicken breeds. A total of 693 individual birds were used in this study. Of these, 336 mtDNA sequences were obtained from different chicken breeds. Using the mtDNA sequence information, 357 individual birds from two different commercial Korean native chicken populations were used for identifying genetic relationships with sequenced chicken breeds. The sequence data indicated that there are 19 mtDNA haplotypes and the major number of individuals was represented in haplotype 1. Haplotype analyses using Neighbor-joining phylogenetic tree indicates that the genetic diversity and relationships among the breeds. We also investigated whether the D-loop hypervariable region in chicken mtDNA found three specific single nucleotide polymorphisms (SNP) can be used for the breed identification. The results indicated that the three SNPs mtDNA in the D-loop region can be possibly used for the breed discriminating markers. The results obtained in this study can be used for designing proper breeding and conservation strategies for the commercial Korean native chicken populations, as well as development of breed identification markers in chicken.**

***Index term*- Breed identification, haplotype, mtDNA, phylogenetic analysis, Korean native chicken.**

I. INTRODUCTION

There is increasing demand from consumers for commercial Korean native chicken in Korea. Two commercial Korean native chicken populations (CCP1 and CCP2) are available in Korea and their meat prices are 2 to 3 times higher than that of the commercial broiler meat. The chicken Mitochondrial DNA (mtDNA) is widely used for the population, biogeographic and phylogenetic studies. Chicken mtDNA is 16,775 base pair long (Desjardins and Morais, 1990) and the mitochondrial genome is maternally inherited in most species and does not undergo recombination (Hayashi et al., 1985). The D (displacement)-loop region in mtDNA is the major control region for mtDNA expression and highly polymorphic compared with the nuclear DNA. The evolutionary rate is five to ten times higher than that of the nuclear genome (Brown et al., 1982). Therefore, mtDNA sequences have been used for determining whether individuals or breeds are biologically related.

Chicken is known to be domesticated from a single ancestor, mainly contributed by red jungle fowl (*Gallus gallus),* which originated in Southeast Asia (Akishinonomiya et al., 1994, 1996). The mtDNA sequences have successfully used to determine genetic diversity in Asian chicken (Niu et al., 2002; Liu et al., 2004*)* and African chicken (Mobegi and Chicken Diversity Consortium, 2005). In recent development of mtDNA sequence tag or bar-code can give the guideline for selection of animals for the breeding purpose (Hebert et al., 2003). Recently, conservation of farm animal genetic resources has been focused on maintaining minimum number of animals for each breeds/species and there is some progress for this (http://www.fao.org/dad-is/). Therefore, hypervariable D-loop region of mtDNA can be used to detect ancient population structures, interspecies variability, archaeological inference about the origins and relationships between populations or species, identification of maternal lineages and post natal growth (Bradley et al., 1998; Troy et al., 2001; Lui et al., 2004; Malau-Aduli et al., 2004; Yoon et al., 2005; Odahara et al., 2006; Lei et al., 2007; Lee et al., 2007). Along with hypervariable D-loop nucleotide substitutions in mtDNA, breed specific markers were also investigated in chicken for delineating the breed structures and phylogenetic relationships with other breeds for the conservation perspectives (Hillel et al., 2003).

In our study, the D-loop hypervariable region of mtDNA has been used for determining the relationships of two commercial Korean chicken populations with other chicken breeds. Also the possibility of mtDNA markers for the breed traceability has been investigated.

II. MATERIALS AND METHODS

A. Experimental animals and sampling

A total of 693 blood or meat samples from individual birds were collected from Poultry Science Division, National Institute of Animal Science (NIAS), CCP1 farm and CCP2 in Korea (Table 1).

Table 1. The information of samples used in this study.

Breed	Line	Location	Sex	No. of individuals
RIR	-	NIAS	Female	95
RIR	S, s	CCP 2	Unknown	22
RIR	W, w	CCP 2	Unknown	23
Cornish	-	CCP 1	Male	24
Cornish	1	CCP 1	Male	12
Cornish	H, h	CCP 2	Unknown	23
Cornish	F, f	CCP 2	Unknown	23
KNC-B	-	NIAS	Male	28
KNC-R	-	NIAS	Male	30
KNC-Y	-	NIAS	Male	30
F1	-	-	Female	26
F2	-	-	Unknown	185
F2	-	-	Unknown	172
Total number of individuals				693

CCP means commercial chicken population

B. DNA extraction and PCR amplification

Genomic DNA was extracted from blood and liver samples using PrimePrep™ Plasmid DNA Isolation Kit (GeNet Bio, Korea) in according to the manufacturer's instruction. Due to PCR amplification, the primer pair (Forward: 5' AGGACTACGGCTTGAAAAGC- 3' and Reverse: 5'-ATGTGCCTGACCGAGGAACCAG-3') was used to amplify about 600 bp of the D-loop hypervariable region from the mtDNA. The PCR reactions were included approximately 100 ng of genomic DNA, 2.5 µL of 10× buffer [contains Tris-HCl (pH 9.0), PCR enhancers, (NH4) 2 SO4, 20 mM MgCl2], 2.0 µL of 10 mM dNTPs mixture (2.5 mM each of dATP, dCTP, dGTP and dTTP), 1 µL of 10 pM of each primer and 1 U *HS Prime Taq* (GeNet Bio, Korea) in a 25 µL reaction volume. The PCR reaction was performed in a My-Genie96 Thermal Block (Bioneer, Korea) with an initial denaturation step at 94℃ for 10 min followed by 35 cycles of 30 sec at 94℃, 30 sec at 61℃, 40 sec at 72℃ and a final extension step at 72℃ for 10 min.

C. DNA purification and sequencing

Purification of PCR products was performed using Accuprep® PCR purification kit (Bioneer, Korea) according to the manufacturer's instructions. All the PCR products were run on 1.5% agarose gels stained with ethidium bromide and DNA bands were visualized under UV light. Purified PCR products were sequenced by Genotech (www.genotech.co.kr).

D. Data analyses

The chicken mtDNA D-loop nucleotide sequences obtained in this study were aligned using the ClustalW program (Thompson et al., 1994) and saved as bioedit format. Nucleotide replacement export data were carried out in haplotype sequences and identical sequences were considered as the same haplotypes by using MEGA software version 4.0.2 (Kumar et al., 2008). Neighbor-joining phylogenetic tree was estimated by 1,000 random bootstrap resampling of the data (Kumar et al., 2008).

III. RESULTS AND DISCUSSION

MtDNA D-loop Sequence Variation

The analysis of chicken mtDNA D-loop sequences indicated a total of 31 nucleotide substitutions were identified, which classified as 19 haplotypes (Table 2). The highest number of individuals from different breeds contains in haplotype 1, which were contained majority numbers of Rhode Island Red (RIR) breed. In the D-loop region, three polymorphic positions, 225, 239 and 243, were considered for breed discrimination markers. As the description of individuals, RIR contains 7 haplotypes from 19 haplotypes. The haplotype 1 and 2 are containing RIR breed from NIAS and haplotypes 3, 4, 5, 6 and 7 also containing another strain of RIR. On the other hand, Haplotype 8 is holding Cornish and Korean native chicken and rest of the haplotypes are enclosing few numbers of birds from chicken breeds. No deletion or insertion was detected in our sequence analysis. Moreover, It might be interpreted that chicken mtDNA had gone trough an evolutionary bottleneck during the course of domestication (Niu et al., 2002).

Phylogenetic Analysis

Phylogenetic tree was constructed using neighbor-joining method with mtDNA D-loop haplotypes (Figure 1). In this analysis indicates that haplotypes belong to the four major lineages (A to D), representing these populations in diverse heredity. Where, Korean native chickens maintain genetic variability to some extent within the small

population in Korea (Lee et al., 2007). Here, it is noted that lineage A partially related with haplotypes 1, 8, 9 10, 16, and 19 and lineage B also correlated with haplotypes 2, 11, 12 and 13. However, haplotypes 3, 4, 5, 6 and 14 were linked in a lineage C and lineage D represented haplotypes 7, 15 and 17. When we look at the geographical locations, lineage A and B stand for NIAS RIR birds and lineage C and D represents another strain of RIR located in the middle of Korea that indicates divergence of RIR birds. The present findings are supported by the results that the sequence divergence among D-loop segments of Korean native chicken RIR and Cornish breeds have relationship with the major lineage of Red jungle fowl (Fumihito et al., 1996; Hoque et al., 2009).

Table 2. Mitochondrial D-loop sequence polymorphisms identified in this study.

Haplotypes	Nucleotide position in mtDNA D-loop region[1]																													
	16 67	17 78	19 89	19 92	21 12	21 72	22 22	**22 25**	**22 39**	22 42	**22 43**	22 45	22 46	25 61	26 61	26 65	28 51	29 11	30 16	31 03	31 13	31 15	33 02	34 23	36 37	37 01	39 91	39 93	44 43	44 46
hap1 (145)[2]	C	A	C	T	G	T	A	T	A	G	T	C	C	T	C	C	A	A	T	C	C	C	C	A	C	T	T	C	G	T	C
hap2 (41)	T	-	-	-	A	-	-	C	-	-	-	-	T	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
hap3 (36)	T	-	-	C	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T
hap4 (8)	T	-	-	-	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	-	-	-	-	-	A	-	-	T
hap5 (16)	T	-	-	-	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	T	-	-	-	-	-	-	-	T
hap6 (13)	T	-	-	-	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T
hap7 (5)	T	-	-	-	-	-	-	C	-	A	C	-	-	C	T	-	G	-	-	T	-	-	-	-	T	C	-	-	-	-	-
hap8 (41)	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
hap9 (3)	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-
hap10 (1)	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
hap11 (3)	T	-	-	-	A	-	-	C	-	-	-	-	T	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-
hap12 (1)	T	-	A	-	A	-	-	C	-	-	-	T	T	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
hap13 (1)	T	-	-	-	A	-	-	C	-	-	-	T	T	-	-	-	-	-	-	-	-	T	A	-	-	-	-	-	-	-	-
hap14 (4)	T	-	-	-	-	C	-	C	-	-	C	-	-	C	-	-	-	-	-	T	-	-	T	-	-	-	-	-	-		T
hap15 (7)	T	T	-	-	-	-	-	C	-	-	C	-	-	C	T	T	G	-	C	T	-	-	-	G	T	-	-	-	-	-	-
hap16 (5)	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	T	-	-	-	-	C	C	-	-	-	-
hap17 (2)	T	-	-	-	-	-	-	C	-	A	C	-	-	C	-	-	G	-	-	T	-	-	-	G	T	C	-	-	-	-	-
hap18 (1)	T	-	-	-	-	C	G	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	T	-	-	-	-	-	-	-	T
hap19 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	T	-	-	-	-	C	C	-	-	-	-
Total=336																															

[1]Numbers indicate nucleotide base position in mitochondrial D-loop region and hypen represents the identical nucleotide with the type 1 sequence.
[2]Numbers in parentheses indicate the observed number of chickens.

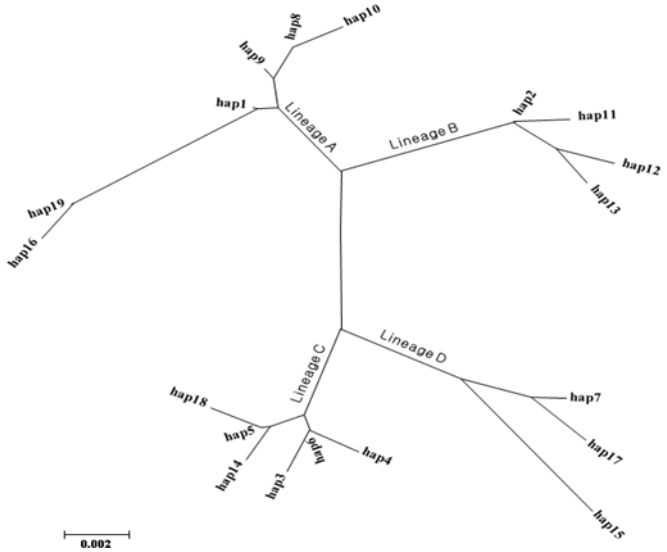

Figure 1. Neighbor-joining phylogenetic tree for the chicken breeds as all haplotypes. Haplotypes in Table 2 are also indicated.

Table 3. Chicken mtDNA D-loop region polymorphisms in RIR and two commercial chicken populations.

SNP-no.	Allele	RIR		CCP 1		CCP 2	
		n	%	n	%	n	%
SNP-225	T	98	70	158	83.6	25	14.53
	C	42	30	31	16.4	147	85.47
Total		140	100	189	100	172	100
SNP-239	G	0	00	47	24.74	148	86.05
	A	140	100	143	75.26	24	13.95
Total		140	100	190	100	172	100
SNP-243	C	34	24.29	20	10.75	140	81.4
	T	106	75.71	166	89.25	32	18.6
Total		140	100	186	100	172	100

Specific Polymorphisms and Haplotype Analysis

The chicken mtDNA D-loop region sequence analysis was confirmed three specific polymorphisms in position of 225, 239 and 243 (Table 3). As a maternal source of RIR were compared with two commercial populations.

Comparison with two commercial chicken populations by the three specific polymorphisms consists of a TAT haplotype. In breed of RIR were considered with 70% individuals of TAT haplotype. On the other hand, two commercial chicken populations were genotyped by restriction enzymes on the same position of those three polymorphisms (not shown). The results of these genotyping were confirmed 71.9% individuals in CCP1 as inherited from RIR. Whereas, CCP2 were contained only 14%, which indicated that CCP2 was not inherited from maternal lineage of RIR.

IV. CONCLUSION

Due to our study, haplotype 1 was represented in a maternal origin of CCP1 population mostly inherited from this maternal lineage by the breed discrimination markers of TAT haplotype. Whereas, CCP2 has been indicating different maternal origin. The results presented here can give the broad idea for the phylogenetic relationship of chicken breeds and provide opportunities for the development of breed specific markers. In order to maintain valuable genetic materials, more detailed studies have to be conducted along with the appropriate breeding programs.

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