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Discrimination of Korean Native Chicken Populations Using SNPs from mtDNA and MHC Polymorphisms

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ABSTRACT : Korean native chickens are a very valuable chicken population in Korea and their prices are higher than that of commercial broilers. In order to discriminate two commercial Korean native chicken populations (CCP1 and CCP2), single nucleotide polymorphisms (SNPs) from mitochondrial (mt) DNA D-loop sequences and LEI0258 marker polymorphisms in the major histocompatibility complex (MHC) region were investigated. A total of 718 birds from nine populations were sampled and 432 mtDNA sequences were obtained. Of these, two commercial Korean native chicken populations (363 birds) were used for investigation of their genetic relationship and breed differentiation. The sequence data classified the chickens into 20 clades, with the largest number of birds represented in clade 1. Analysis of the clade distribution indicated the genetic diversity and relation among the populations. Based on the mtDNA sequence analysis, three selected SNPs from mtDNA polymorphisms were used for the breed identification. The combination of identification probability (Pi) between CCP1 and CCP2 using SNPs from mtDNA and LEI0258 marker polymorphisms was 86.9% and 86.1%, respectively, indicating the utility of these markers for breed identification. The results will be applicable in designing breeding and conservation strategies for the Korean native chicken populations and also used for the development of breed identification markers.
(**Key Words :** mtDNA, LEI0258 Marker, Discrimination, Korean Native Chicken)

INTRODUCTION

With the burgeoning global population, techniques for the identification of highly productive food sources are needed. Many native livestock breeds, which have less productivity and are mostly found in developing countries, have diminished in number or become extinct. Recently, the United Nations called for the identification of all the native livestock breeds in the world in order to develop conservation strategies (http://dad.fao.org).

In Korea, there are increasing demands from consumers for Korean native chickens. For this reason, two commercial Korean native chicken populations (CCP1 and CCP2) were recently developed at the National Institute of Animal Science (NIAS) and from a commercial company in Korea, respectively, by crossing Korean native chickens with meat-type chicken breeds. Their meat prices are 2-3 times more expensive, compared with commercial broilers. However, the recent marketplace availability of CCP1 and CCP2 has proved confusing to consumers.

To discriminate CCP1 and CCP2 from their meat-type breed counterparts at the molecular level, variations in their mitochondrial genome were initially investigated. The mitochondrial genome is maternally inherited and the sequences of mitochondrial DNA (mtDNA) have been extensively used in biodiversity studies of vertebrates (Baker and Marshall, 1997; Mindel et al., 1997; Moore and Defilippis, 1997; Wayne et al., 2002) including chickens and domestic animals (Komiyama et al., 2003, 2004; Liu et al., 2006; Odahara et al., 2006; Sasazaki et al., 2006; Lei et al., 2007; Wang et al., 2007; Li et al., 2008). Typically, coding genes of the mitochondrial genome are used for phylogenetic studies to distinguish species (Moore and Defilippis, 1997), whereas the control region is considered more suitable for inter-specific population studies (Baker

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and Marshall, 1997). Advantages of the mtDNA control region as molecular markers include the maternal inheritance of the mtDNA, rapid nucleotide substitutions compared with the nuclear genome, and the absence of recombination (Aquadro and Greenberg, 1983; Lansman et al., 1983; Cann et al., 1984).

In the nuclear genome, the major histocompatibility complex (MHC) has important biological functions associated with immunity, design of effective vaccines, reproductive success and production traits of domestic animals (Bernatchez and Landry, 2003; Piertney and Oliver, 2006). Parasites and sexual selection have been documented to sustain the unusually high levels of MHC polymorphism (Piertney and Oliver, 2006). Particularly, the microsatellite marker LEI0258 of domestic chickens is widely-used for the discrimination of MHC alleles. There are large numbers of alleles identified in this marker, which is located in the MHC region (McConnell et al., 1999). Also, LEI0258 marker amplicons have repeat number variations, indels (insertion or deletion) and single nucleotide polymorphisms (SNPs), which make easy identification of each allele using direct polymerase chain reaction (PCR) and sequencing (Fulton et al., 2006).

In this study, SNPs in mtDNA and LEI0258 polymorphisms of Korean native chicken populations were investigated to develop molecular markers for breed identification.

MATERIALS AND METHODS

Experimental animals

A total of 718 chicken samples from nine populations were collected from NIAS, and commercial chicken farms. They comprised 140 Rhode Island Red (RIR) chickens, 82 Cornish chickens, 28 black Korean native chickens (BKNC), 30 red Korean native chickens (RKNC), 30 yellow Korean native chickens (YKNC), 26 parent stocks (PS1) of CCP1, 19 parent stocks (PS2) of CCP2, 191 CCP1 chickens and 172 CCP2 chickens.

DNA extraction and PCR amplification

Genomic DNAs were extracted from blood and liver samples using PrimePrep™ Genomic DNA Isolation Kit (GeNet Bio, Korea) according to the manufacturer's instructions. The primers forward: 5'- AGGACTACGGCTT GAAAAGC -3' and reverse: 5'-ATGTGCCTGACCGAGG AACCAG -3') were used to PCR amplify a 591 bp fragment of the D-loop hypervariable region in mtDNA. Also a new primer set (forward: 5'- TCAGTGATGTCATC GGGAAA -3' and reverse: 5'- TTTTCAGATCGCGTTCC TCT-3') was designed to amplify the LEI0258 marker to investigate the MHC alleles. Both PCR reactions included approximately 100 ng of genomic DNA, 2.5 μl of 10× buffer [Tris-HCl (pH 9.0), PCR enhancers, $(NH_4)_2SO_4$, 20 mM $MgCl_2$], 2.0 μl of 10 mM dNTPs mixture (2.5 mM each of dATP, dCTP, dGTP and dTTP), 1 μl of 10 pmol of each primer and 1 U *HS Prime Taq* (GeNet Bio, Korea) in a 25 μl reaction volume. PCR was performed in a My-Genie96 Thermal Block (Bioneer) with an initial denaturation step at 94°C for 10 min followed by 35 cycles of 30 s at 94°C, 30 s at 61°C, 40 s at 72°C and a final extension step at 72°C for 10 min. The PCR products for mtDNA and LEI0258 markers were electrophoresed on 1.5% and 4% agarose gels stained with ethidium bromide, respectively, and DNA fragments were visualized under ultraviolet light.

DNA purification and sequencing

Purification of PCR products was performed using an Accuprep® PCR purification kit (Bioneer) according to the manufacturer's instructions. Purified PCR products were also confirmed by using agarose gels for sequencing. In case of MHC alleles, homozygous alleles were considered for sequencing. All the purified PCR products were sequenced by Genotech (www.genotech.co.kr).

Data analysis

The chicken mtDNA D-loop nucleotide sequences and MHC alleles were aligned using the ClustalW program (Thompson et al., 1994) and saved as bioedit format. Nucleotide replacement export data from mtDNA were carried out in haplotype sequences and identical sequences for MHC alleles were considered as the same haplotypes by using MEGA software version 4.0.2 (Kumar et al., 2008). To calculate the identification probabilities between CCP1 and CCP2, allele frequencies of these two populations were investigated by PCR-restriction fragment length polymorphism (PCR-RFLP). The RFLP reaction consisted of 10× buffer, 3-5 units of each enzyme and 15 μl PCR product in a total volume of 20 μl. Three selected SNPs at position 225, 239 and 243 were identified by digestion of the mtDNA PCR products with *Hph*I, *Hpy*CH4III and *Alu*I restriction enzymes, respectively. The obtained RFLP fragments were separated on 4% agarose gels stained with ethidium bromide (Figure 1). Also, haplotype frequencies were investigated using the algorithm from Haploview program developed by the Broad Institute (Haploview, USA).

RESULTS AND DISCUSSION

Analysis of mtDNA SNP

Based on the analysis of 432 chicken mtDNA D-loop sequences, a total of 32 nucleotide substitutions were identified and classified in 20 clades (Table 1). Indel (insertion or deletion) mutations were not detected. The

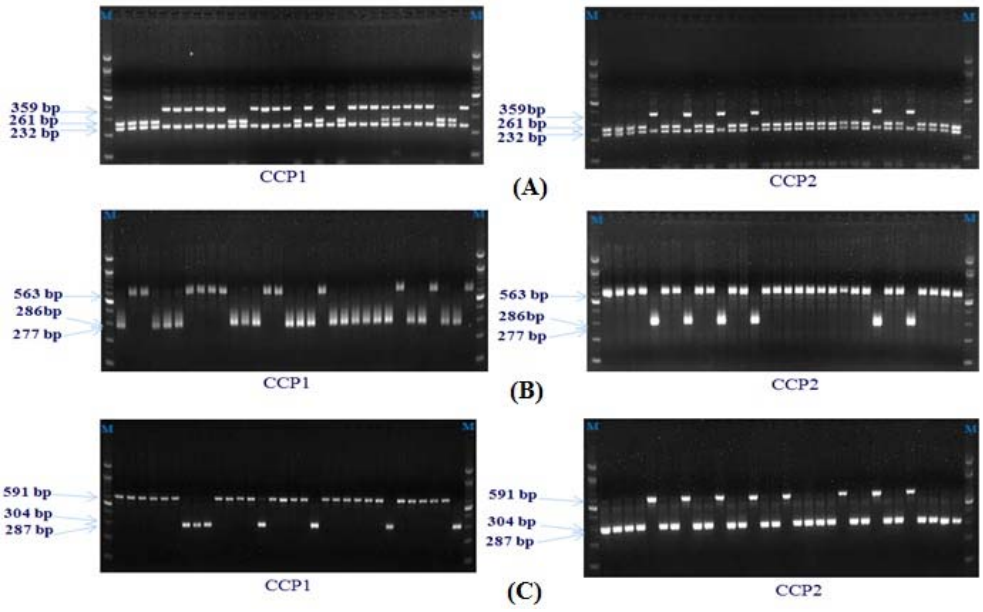

Figure 1. PCR-RFLP patterns for SNP-225 (A), SNP-239 (B) and SNP-243 (C) between CCP1 and CCP2.

Table 1. Mitochondrial D-loop sequence polymorphisms identified in the chicken populations used in this study

Clades	Nucleotide position in mtDNA D-loop region[1]																														
	112	167	177	198	199	212	217	222	225	239	242	243	245	246	256	261	265	281	291	306	310	313	315	330	342	363	367	370	391	399	443	446
Clade 1	A	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
Clade 2	-	T	-	-	-	A	-	-	C	-	-	-	-	T	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
Clade 3	-	T	-	-	C	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T
Clade 4	-	T	-	-	-	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	-	-	-	-	-	A	-	-	T
Clade 5	-	T	-	-	-	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	T	-	-	-	-	-	-	-	T
Clade 6	-	T	-	-	-	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T
Clade 7	-	T	-	-	-	-	-	-	C	-	A	C	-	-	C	T	-	G	-	-	T	-	-	-	-	T	C	-	-	-	-	-
Clade 8	-	T	-	-	-	-	C	-	C	-	-	C	-	-	C	-	-	-	-	-	T	-	-	T	-	-	-	-	-	-	-	T
Clade 9	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
Clade 10	-	T	-	-	-	A	-	-	C	-	-	-	-	T	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-
Clade 11	-	T	-	A	-	A	-	-	C	-	-	-	T	T	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
Clade 12	-	T	-	-	-	A	-	-	C	-	-	-	T	T	-	-	-	-	-	-	-	-	T	A	-	-	-	-	-	-	-	-
Clade 13	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-
Clade 14	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clade 15	-	T	-	-	-	-	C	G	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	T	-	-	-	-	-	-	-	T
Clade 16	-	T	-	-	-	-	-	-	C	-	A	C	-	-	C	-	-	G	-	-	T	-	-	-	G	T	C	-	-	-	-	-
Clade 17	-	T	T	-	-	-	-	-	C	-	-	C	-	-	C	T	T	G	-	C	T	-	-	-	G	T	-	-	-	-	-	-
Clade 18	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	T	-	-	-	-	C	C	-	-	-	-
Clade 19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	T	-	-	-	-	C	C	-	-	-	-
Clade 20	G	T	-	-	C	-	C	-	C	-	-	C	-	-	C	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T

[1] Numbers indicate nucleotide base position in mitochondrial D-loop region and hypen (-) represents the identical nucleotide with the type 1 sequence.

identified average percentage of polymorphic sites was 5.86% for the 546 bp mtDNA sequences. In Chinese native chicken breeds, the average percentages of polymorphic sites were previously determined as 6.4% and 4.45%, respectively (Fu et al., 2001; Liu et al., 2004). In the D-loop region, three polymorphic positions, 225, 239 and 243, were considered for breed discrimination markers because of the large differences in allele frequencies between the CCP1 and CCP2. Of these three SNPs, five haplotypes were obtained from two commercial populations (Table 2). Four haplotypes (TAT, CGC, TGT and CAT) were found in CCP1 and the highest frequency (71.9%) was the TAT haplotype. In CCP2, TGT and CAT haplotypes were not identified while TAT, CGC and CGT haplotypes were identified. The CGC haplotype had the highest frequency (81.4%) among the three haplotypes.

Based on the 432 mtDNA sequence analysis from nine populations, 20 clades were observed (Table 3). In each population, seven RIR, eleven Cornish, nine BKNC, six RKNC, four YKNC, one PS1, five PS2, one CCP1 and five CCP2 haplotypes were identified. Among the clades, PS1 and CCP1 populations were fixed in clade 1. Also, RIR (70%), Cornish (1.2%), BKNC (10.7%), RKNC (56.6%), PS2 (10.5%) and CCP2 (17.5%) populations were present in clade 1. Clade 2 contained a large number of the Cornish (40.2%) population with a minimum number of RIR (5.7%) individuals. On the other hand, the highest proportion of CCP2 (32.5%) individuals were in clade 3, followed by PS2 (31.6%), YKNC (23.3%), BKNC (39.3%), Cornish (13.4%) and RIR (5%) populations. Also, the 25% CCP2 population in clade 4 was shared with PS2 (36.8%) and RIR (5.7%) populations. Clade 5 contained 20% of CCP2, 10% of RIR and 7.1% of BKNC populations. However, Cornish (28%) and YKNC (60%) populations were included in clade 14. Based on these mtDNA haplotypes, it is reasonable to suppose that the CCP2 population is closely related with the Cornish, BKNC and YKNC populations.

Table 2. Haplotype frequencies in CCP1 and CCP2 based on RFLP genotyping

Haplotype ID	Haplotype	CCP1	CCP2
Hap 1	TAT	0.719	0.14
Hap 2	CGC	0.103	0.814
Hap 3	TGT	0.124	-
Hap 4	CAT	0.049	-
Hap 5	CGT	-	0.041

MHC allele investigation

Polymorphisms and repetitive sequences were identified for the LEI0258 alleles in CCP1 and CCP2 populations by sequencing for homozygous animals. The alleles having Genbank accession numbers were related with serologically identified MHC-B haplotypes (Table 4). The identified

Table 3. Distribution of clades in each chicken population based on sequencing

Clades	Total individuals		RIR Individuals		Cornish Individuals		BKNC Individuals		RKNC Individuals		YKNC Individuals		PS1 Individuals		PS2 Individuals		CCP1 Individuals		CCP2 Individuals	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Clade 1	191	44.2	98	70.0	1	1.2	3	10.7	17	56.6	-	-	26	100	2	10.5	37	100	7	17.5
Clade 2	41	9.5	8	5.7	33	40.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clade 3	55	12.7	7	5.0	11	13.4	11	39.3	-	-	7	23.3	-	-	6	31.6	-	-	13	32.5
Clade 4	25	5.8	8	5.7	-	-	-	-	-	-	-	-	-	-	7	36.8	-	-	10	25.0
Clade 5	24	5.5	14	10.0	-	-	2	7.1	-	-	-	-	-	-	-	-	-	-	8	20.0
Clade 6	14	3.2	4	2.9	3	3.7	1	3.6	2	6.7	3	10.0	-	-	1	5.3	-	-	-	-
Clade 7	7	1.6	1	0.7	-	-	2	7.1	2	6.7	-	-	-	-	-	-	-	-	2	5.0
Clade 8	4	0.9	-	-	4	4.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clade 9	1	0.2	-	-	1	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clade 10	3	0.7	-	-	3	3.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clade 11	1	0.2	-	-	1	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clade 12	1	0.2	-	-	1	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clade 13	3	0.7	-	-	1	1.2	-	-	-	-	2	6.7	-	-	-	-	-	-	-	-
Clade 14	41	9.5	-	-	23	28.0	-	-	-	-	18	60.0	-	-	-	-	-	-	-	-
Clade 15	1	0.2	-	-	-	-	1	3.6	-	-	-	-	-	-	-	-	-	-	-	-
Clade 16	2	0.5	-	-	-	-	2	7.1	-	-	-	-	-	-	-	-	-	-	-	-
Clade 17	7	1.6	-	-	-	-	4	14.3	3	10.0	-	-	-	-	-	-	-	-	-	-
Clade 18	5	1.2	-	-	-	-	2	7.1	3	10.0	-	-	-	-	-	-	-	-	-	-
Clade 19	3	0.7	-	-	-	-	-	-	3	10.0	-	-	-	-	-	-	-	-	-	-
Clade 20	3	0.7	-	-	-	-	-	-	-	-	-	-	-	-	3	15.8	-	-	-	-
Total	432		140		82		28		30		30		26		19		37		40	

RIR = Rhode Island Red, BKNC = Black Korean native chicken, RKNC = Red Korean native chicken, YKNC = Yellow Korean native chicken, PS1 = CCP1 parent stock, PS2 = CCP2 parent stock, CCP1 = Commercial chicken population 1 and CCP2 = Commercial chicken population 2.

Table 4. Polymorphisms identified for the LEI0258 alleles in CCP1 and CCP2 populations by sequencing

Breed	Primer size (bp)	LEI0258 Consensus size (bp)	Position -30-29 TT	Position -28 G	Position -10 G	R13	R12	Position 11-----18 ATTTTGAG	Position 26 A	Position 28 △	Position 34 T	Position 65-66 CT	Position 93 T	Genbank accession no	B haplotype
CCP1	319	194	-	-	A	1	3	Δ	-	A	-	-	G	DQ239561	BW3
	330	205	-	-	-	1	4	Δ	-	-	-	-	-	DQ239505 DQ239514 DQ239560	13.2 17 BW11
	420	295	Δ	A	-	1	11	-	-	-	-	-	-	DQ239496	11.1
	420	295	Δ	-	-	1	11	-	-	-	-	-	-	DQ239541	5
	434	309	-	-	-	1	12	-	T	-	-	-	-	DQ239494 DQ239533 DQ239537 DQ239554	10 24 26 76
	470	345	-	-	-	1	15	-	T	-	-	GC	-	DQ239508	14
	545	420	-	-	-	16	5	-	-	-	-	-	-	DQ239548	62
	568	443	-	-	-	15	8	-	-	-	-	-	-	DQ239544	6
	599	474	-	-	-	22	3	-	-	-	-	-	-	DQ239499	12.2
CCP2	374	249	-	-	-	1	7	-	-	-	A	-	-	DQ239513 DQ239531 DQ239551	15.2 22 73
	384	259	Δ	-	-	1	8	-	-	-	A	-	-	DQ239523 DQ239539	2 29
	420	295	Δ	-	-	1	11	-		-	-	-	-	DQ239541	5
	444	319	Δ	A	-	1	13	-		-	-	-	-	DQ239552	74
	482	357	-	-	-	1	16	-	T	-	-	-	-	DQ239506 DQ239507	130 131
	545	420	-	-	-	16	5	-		-	-	-	-	DQ239548	62
	568	443	-	-	-	15	8	-		-	-	-	-	DQ239544	6

MHC alleles in CCP1 and CCP2 had 13 and 10 alleles, respectively (Figure 2). To calculate breed identification between CCP1 and CCP2, the sum (64.4%) of three alleles (330, 420 and 434) having high allele frequencies in CCP1 and the sum (62.2%) of three alleles (482, 545 and 568) having high allele frequencies in CCP2 were considered.

Calculation of discrimination probability

Three mtDNA markers (SNP225, SNP239 and SNP243) and the LEI0258 marker were evaluated for discriminating

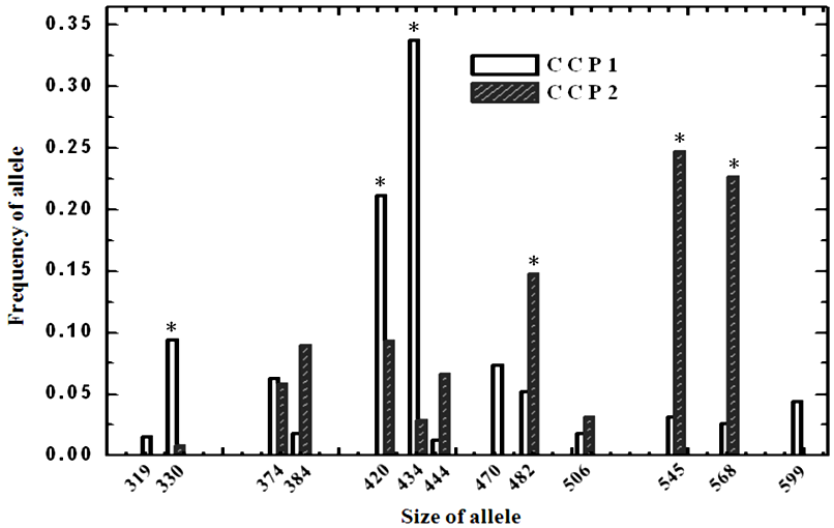

Figure 2. Comparison of allele frequencies for LEI0258 marker between CCP1 and CCP2. Major alleles in CCP1 and CCP2 are indicated by asterisks (*).

Table 5. Allele frequencies and identification probabilities (Pi) for four effective markers in CCP1 and CCP2

Marker	CCP1				CCP2			
	Allele frequencies		Individuals number	Pi value	Allele frequencies		Individuals number	Pi value
	Major	Minor			Major	Minor		
SNP225	0.84(T)	0.16(C)	189	0.30	0.85(C)	0.15(T)	172	0.27
SNP239	0.75(A)	0.25(G)	190	0.43	0.86(G)	0.14(A)	172	0.26
SNP243	0.89(T)	0.11(C)	186	0.20	0.81(C)	0.19(T)	172	0.34
Sub total				0.685				0.642
LEI0258	0.644	0.356	191	0.585	0.622	0.378	172	0.613
Total				0.869				0.861

CCP1 and CCP2 populations. These probabilities were calculated based on the estimated allele frequency for each marker (Table 5). The probability of identification within the breeds was estimated by considering minor allele frequencies. Based on the combination of three mtDNA markers in CCP1 and CCP2, the obtained probability of identifications were 68.5% and 64.2% respectively. When the MHC allele was included in the analysis, the probability of identification increased to 86.9% and 86.1% for CCP1 and CCP2, respectively. Previous results indicated that more than 0.8 of identification probability (Pi) were considered as effective for breed identification in cattle (Alves et al., 2002; Sasazaki et al., 2004, 2006). Presently, the combination of four markers effectively discriminated these two commercial Korean native chicken populations.

In this study, we investigated the probabilities of breed identification using molecular markers derived from mtDNA and MHC alleles for the discrimination of chicken populations. To maintain the valuable native chicken population, molecular markers for breed discrimination would be very useful for an appropriate conservation breeding program as well as for the establishment of molecular markers for chicken breeds in the marketplace. However, more investigation of molecular studies is required to further improve the breed discrimination.

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