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Research Article
The Development of Multiplex PCR Microsatellite Marker Sets for Korean Chicken Breeds

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Abstract

Background and Objective: Korea's National Institute of Animal Science (NIAS) has preserved two types of purebred chicken strains and the Hanhyup Company has maintained several purebred chicken stains. This study aimed to facilitate the analyses of genetic diversity, parentage and population structure through the isolation and characterization of novel multiplex PCR microsatellite (MS) marker sets for Korean chicken breeds. **Materials and Methods:** Genotyping of all 27 markers was performed for 469 purebred chicken samples from 20 Korean breeds. Of these, 16 highly polymorphic MS markers were selected based on the number of alleles, expected heterozygosity (H_{exp}) and polymorphic information content (PIC). The selected markers were classified into two sets of 16 markers each. **Results:** The expected probability of identity values for the 16 MS markers in random individuals (PI), random half-sib ($PI_{half\text{-}sibs}$) and random sibs (PI_{sibs}) were estimated at 1.79×10^{-21}, 6.76×10^{-16} and 5.70×10^{-8} respectively. **Conclusion:** Our multiplex PCR marker sets were observed to have favorable applications in the conservation, planning of breeding and traceability systems in the Korean chicken breeds. Additionally, the marker sets will be of considerable use in small- and large-scale population genetic structure analyses.

Key words: Breeding, chicken, Hanhyup, Korean native chicken, microsatellite, multiplex PCR marker, polymorphisms

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Korea's National Institute of Animal Science (NIAS) has preserved two types of purebred chicken strains: purebred Korean native chickens (KNCs), which include five breeds with different feather colors (red-brown, yellow-brown, gray-brown, black and white) and the "imported and adapted chickens", which includes two Rhode Island Red breeds, two Cornish breeds and two Leghorn breeds. The latter type were imported in the 1960s for industrialization purposes, adapted to the Korean habitat and maintained as purebred strains[1,2]. The KNC breeds, owing to their dismal commercial performance, were ignored for many years. Consequently, the populations of these native animal breeds have declined markedly[3]. The private native chicken breeding-stock company (Hanhyup) is responsible for more than 80% of the native chicken distribution in Korea. The company has maintained purebred chicken strains for commercial purposes for the past 60 years. These are known as the Hanhyup H, F, G, V, S, W, Y, A, 3 (HH, HF, HG, HV, HS, HW, HY, HA and HHC, respectively) and Woorimatdag breeds[1]. At present, sufficient detailed information about these Korean chicken breeds is not available. Evaluation of the genetic diversity and relationship between local breeds is an important factor in the identification of unique and valuable genetic resources. In addition, in recent times native animals have attracted attention due to the recognition of the importance of breed diversity in the face of ongoing climate change and as potential sources of economically important traits[1,4,5]. Therefore, it is necessary to be able to investigate the genetic diversity of Korean chicken breeds and classify the breeds accordingly. Genetic marker polymorphisms provide a reliable method to assess the biodiversity within and among chicken breeds. Microsatellites are being used in diversity studies because of their codominant, highly polymorphic nature and availability throughout the genome. Thus, microsatellites have been identified as reliable markers in chickens[3,6,7]. The identification of these specific markers could aid the selection process for the development of native chickens that are more suitable for the chicken industry in Korea. Therefore, in this study, to facilitate the analyses of genetic diversity, parentage and population structure, novel multiplex PCR microsatellite marker sets for Korean chicken breeds were isolated and characterized.

MATERIALS AND METHODS

Sample collection: A total of 469 purebred chicken samples from 20 breeds were acquired in Korea from Hanhyup and the NIAS. Eight commercial breeds of chickens (HH, HF, HG, HV, HS, HW, HY and HA) were collected from Hanhyup. Samples from 12 purebred breeds were obtained from the NIAS as follows: Red KNC (NR), Yellow KNC (NY), Gray KNC (NG), Black KNC (NL), White KNC (NW), Ogye (NO), Leghorn F (NF), Leghon K (NK), Black Cornish (NH), Brown Cornish (NS), Rhode Island Red C (NC) and Rhode Island Red D (ND) (Table 1). Multiplex

Table 1: Description of the 20 chicken breeds used in this study with 27 microsatellite markers

	Codes	Population	No. of sample
Hanhyup	HH	Hanhyup H	24
	HF	Hanhyup F	24
	HG	Hanhyup G	24
	HV	Hanhyup V	24
	HS	Hanhyup S	24
	HW	Hanhyup W	24
	HY	Hanhyup Y	24
	HA	Hanhyup A	13
Sub-total			181
NIAS	NR	Red Korea native chicken	24
	NY	Yellow Korea native chicken	24
	NG	Gray Korea native chicken	24
	NL	Black Korea native chicken	24
	NW	White Korea native chicken	24
	NO	Ogye	24
	NF	Leghorn F	24
	NK	Leghorn K	24
	NH	Black cornish	24
	NS	Brown cornish	24
	NC	Rhode Island red C	24
	ND	Rhode Island red D	24
Sub-total			288
Total			469

Table 2: Description of the 7 chicken breeds used in the case study with multiplex-PCR marker sets

Population	No. of sample
Hanhyup H (HH)	19
Hanhyup F (HF)	34
Hanhyup S (HS)	43
Hanhyup W (HW)	56
Hanhyup Y (HY)	40
Woorimatdag (WM)	50
Hanhyup 3(HCC)	97
Total	339

PCR was used on 339 commercial chickens (Table 2). Genomic DNA (g DNA) was extracted from blood samples collected from the wing veins. DNA from the blood was extracted according to the standard protocol provided with the PrimePrep Genomic DNA isolation kit for blood (GeNetbio, Korea).

Marker selection and multiplexing: For the analysis of genetic diversity, 27 microsatellite markers from the FAO/ISAG recommended diversity panel were used. (ISAG/FAO Standing Committee, 2004; Table 3). From these results, 16 highly polymorphic MS markers were selected based on the number of alleles, expected heterozygosity (H_{exp}) and polymorphic information content (PIC). The selected markers were classified into two sets of 16 markers each (SET1 and SET2). Size fractionation of all 16 markers in each group was made possible by capillary electrophoresis using a combination of fluorescent-labeled primers (Table 6). The possibility of multiplexing the size fractionation by capillary electrophoresis was also considered in the selection of suitable markers.

PCR: All 469 DNA samples were amplified using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The amplifications were carried out using 15 μL reaction mixtures containing genomic DNA (5-20 ng μL^{-1}), primer (10 pM) and 1.5 U Thermo Scientific Maxima Hot Start Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA), which were then subjected to 30 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C. The multiplex PCR program was the same as the program described above.

Genotyping and statistical analysis: Genotyping of the amplified DNA was performed using an automated Genetic Analyzer 3130xl (Applied Biosystems, USA). The results were obtained using GeneMapper V 4.1 (Applied Biosystems, USA). The genotyped data were analyzed using the Cervus V 3.0 program[8] and Excel MS toolkit version 3.1[9] to calculate the allele frequencies at each locus for each population, the H_{exp}, the observed heterozygosity (H_{obs}) and the PIC values. The levels of inbreeding-like effects within subpopulations (genetic distance [F_{st}]), among subpopulations (within inbreeding [F_{is}]) and within the entire population (total inbreeding [F_{it}]) were analyzed by F-statistics[10]. The STRUCTURE program[11] was used to analyze the proportion of the membership within the studied breeds. The expected probability of identity values among genotypes of random individuals (PI), random half sibs ($PI_{half\text{-}sibs}$) and random sibs (PI_{sibs}) were calculated using API-CALC (ver 1.0)[12]. The neighbor-joining method[13] was used to construct a phylogenetic tree. Principal coordinates analysis (PCoA) was performed on 7 breeds using the GenAlEx 6.4 program. A correspondence analysis, which is a weighted principal component analysis (PCA), was performed using the allele frequency data for the individuals of all 7 breeds (Table 2) and 16 MS markers with GENETIX software[14].

This study was approved by the research Ethics Committee of Hankyung National University 2018-2.

RESULTS AND DISCUSSION

Microsatellite polymorphisms, within and between populations: The microsatellite polymorphism, evaluated by the number of alleles (NA) per locus, mean heterozygosity, PIC and F-statistics values for each breed are described in Table 4. A total of 316 alleles were observed at the 27 microsatellite loci distributed in 469 Korean chickens representing the 20 Korean chicken populations. All the microsatellite loci typed were polymorphic. The NA per locus ranged from 5 (ADL0268) to 20 (MCW0127), with a mean of 11.7 alleles. The mean H_{Exp} across loci was 0.797, with estimates per locus ranging from 0.688 (MCW0288) to 0.881 (MCW0104). For H_{Obs}, the mean for all loci was 0.547, with a range of 0.359 (GCT0016) to 0.677 (MCW0145, MCW0029). In this study, all the loci had high PIC values (PIC>0.5). Estimation of the genotypic diversity of heterozygosity and PIC value data of the MS markers were previously used for the determination of animal breed selection and animal traceability. Accordingly, markers with PIC>0.5 and H_{Exp}>0.6 are the most reasonably informative loci for applications in genetics[15-17]. F-statistics were estimated in a fixation index as genetic differentiation (F_{st}), the global heterozygote deficit among the 20 chicken breeds (F_{it}) and the heterozygote deficit within the breed (F_{is}) among the 27 MS markers (Table 4). Among these markers, estimation of fixation index has been discovered for F_{st}, F_{it} and F_{is}, with values ranging from 0.119 to 0.382, 0.199 to 0.565 and

Table 3: Primer information for 27 selected microsatellite markers in the study

No.	Markers		Chr	Primer	Allele range (bp)	Dye
1	ADL0268	1	F	CTCCACCCCTCTCAGAACTA	105-117	PET
			R	CAACTTCCCATCTACCTACT		
2	MCW0111	1	F	GCTCCATGTGAAGTGGTTTA	90-118	NED
			R	ATGTCCACTTGTCAATGATG		
3	MCW0145	1	F	ACTTTATTCTCCAAATTTGGCT	178-214	FAM
			R	AAACACAATGGCAACGGAAAC		
4	MCW0288	2	F	GATCTGCTTCTCTGCCCCATG	102-122	FAM
			R	GGTACTGTCACCAGAATGAGC		
5	LEI0141	2	F	CGCATTTGATGCATAACACATG	218-244	FAM
			R	AAGGCAAACTCAGCTGGAACG		
6	MCW0063	2	F	GGCTCCAAAAGCTTGTTCTTAGCT	128-150	FAM
			R	GAAAACCAGTAAAGCTTCTTAC		
7	MCW0264	2	F	CTTACTTTTCACGACAGAAGC	225-243	FAM
			R	AGACTGAGTCACACTCGTAAG		
8	MCW0039	2	F	CATTGGACTGAGATGTCACTGCAG	128-148	VIC
			R	ACATTTGTCTAATGGTACTGTTAC		
9	MCW0087	2	F	ATTTCTGCAGCCAACTTGGAG	265-289	NED
			R	CTCAGGCAGTTCTCAAGAACA		
10	MCW0127	3	F	GAGTTCAGCAGGAATGGGATG	226-274	VIC
			R	TGCAATAAGAGAAGGTAAGGTC		
11	LEI0094	4	F	GATCTCACCAGTATGAGCTGC	232-282	FAM
			R	TCTCACACTGTAACACAGTGC		
12	ADL0317	4	F	AGTTGGTTTCAGCCATCCAT	178-204	FAM
			R	CCCAGAGCACACTGTCACTG		
13	MCW0029	5	F	GTGGACACCCATTTGTACCCTATG	131-187	VIC
			R	CATGCAATTCAGGACCGTGCA		
14	ADL0292	5	F	CCAAATCAGGCAAAACTTCT	110-140	FAM
			R	AAATGGCCTAAGGATGAGGA		
15	ROS0013	5	F	TGCTGCTCCTGGRAAATTG	220-262	NED
			R	GAAAAGCCATGGAGGAATCA		
16	ROS0019	7	F	ATGTACAGGTTCCAGTGTCCG	118-150	NED
			R	CCAGTTCATACAACCTTGAGTTGG		
17	ADL0259	9	F	CTCATTGCAGAGGAAGTTCT	107-147	VIC
			R	GTAATGGAGGATGCTCAGGT		
18	GCT0016	9	F	TCCAAGGTTCTCCAGTTC	108-168	NED
			R	GGCATAAGGATAGCAACAG		
19	MCW0228	10	F	GATCTCTGCATTACAAGCATG	220-248	PET
			R	TTGCTGACCTGCTCATGCAAG		
20	MCW0104	13	F	TAGCACAACTCAAGCTGTGAG	192-232	PET
			R	AGACTTGCACAGCTGTGTACC		
21	ROS0083	13	F	CATTACAGCTCAGTGTTGGCA	108-130	VIC
			R	TTGCAAGTGCTCTCCCATC		
22	MCW0123	14	F	CCACTAGAAAAGAACATCCTC	80-90	FAM
			R	GGCTGATGTAAGAAGGGATGA		
23	MCW0330	17	F	TGGACCTCATCAGTCTGACAG	252-286	VIC
			R	AATGTTCTCATAGAGTTCCTGC		
24	ADL0293	17	F	GTAATCTAGAAACCCCATCT	107-127	PET
			R	ACATACCGCAGTCTTTGTTC		
25	ADL0304	18	F	GGGGAGGAACTCTGGAAATG	138-162	FAM
			R	CCTCATGCTTCGTGCTTTTT		
26	LEI0074	26	F	GACCTGGTCCTGACATGGGTG	132-244	VIC
			R	GTTTGCTGATTAGCCATCGCG		
27	LEI0135	28	F	CACAATGAAGGATGAATAGTGC	132-152	NED
			R	AATTCACAGTTACACCTGAGG		

-0.037-0.337, respectively. The estimated mean values of F_{st}, F_{it} and F_{is} were 0.233, 0.321 and 0.115, respectively. The breed statistics generated by the 27 microsatellite markers in nine chicken breeds are shown in Table 5. The mean number of alleles (MNA) in each breed ranged from 3.59 (NF) to 6.63 (NY). The two most diverse breeds were the HF and NL breeds, which had the highest mean H_{Exp} (0.745 and 0.734), H_{Obs} (0.705 and 0.675) and PIC (0.575 and 0.686).

Table 4: Statistical analysis of heterozygosity (H_{Obs} and H_{Exp}), polymorphic information content (PIC) and F-statistics value using 27 selected microsatellite markers among the 20 chicken breeds

Locus	No. of alleles	H_{Obs}	H_{Exp}	PIC	$F_{st}(\theta)$	$F_{it}(F)$	$F_{is}(f)$
ADL0268	5	0.515	0.768	0.731	0.272	0.338	0.091
MCW0111	10	0.494	0.735	0.688	0.130	0.333	0.234
MCW0145	11	0.677	0.838	0.817	0.156	0.199	0.051
MCW0288	9	0.507	0.688	0.646	0.284	0.273	-0.016
LEI0141	14	0.478	0.851	0.833	0.158	0.442	0.337
MCW0063	11	0.577	0.779	0.749	0.224	0.267	0.055
MCW0264	9	0.567	0.827	0.806	0.271	0.324	0.072
MCW0039	8	0.566	0.746	0.709	0.265	0.251	-0.019
MCW0087	15	0.565	0.878	0.865	0.176	0.362	0.226
MCW0127	20	0.594	0.859	0.843	0.173	0.314	0.170
LEI0094	18	0.609	0.843	0.825	0.119	0.282	0.185
ADL0317	11	0.612	0.831	0.811	0.261	0.274	0.017
MCW0029	15	0.677	0.859	0.845	0.249	0.222	-0.037
ADL0292	11	0.561	0.751	0.714	0.248	0.262	0.019
ROS0013	16	0.590	0.835	0.813	0.182	0.299	0.144
ROS0019	13	0.496	0.699	0.677	0.266	0.300	0.047
ADL0259	14	0.655	0.880	0.868	0.254	0.265	0.014
GCT0016	12	0.359	0.812	0.788	0.326	0.565	0.355
MCW0228	12	0.573	0.796	0.769	0.311	0.291	-0.029
MCW0104	17	0.629	0.881	0.869	0.251	0.295	0.059
ROS0083	8	0.560	0.750	0.716	0.260	0.264	0.006
MCW0123	6	0.451	0.729	0.683	0.382	0.394	0.020
MCW0330	12	0.481	0.746	0.715	0.151	0.361	0.248
ADL0293	10	0.499	0.779	0.746	0.271	0.368	0.133
ADL0304	9	0.508	0.788	0.757	0.232	0.364	0.171
LEI0074	13	0.537	0.846	0.826	0.150	0.370	0.259
LEI0135	7	0.443	0.728	0.681	0.307	0.400	0.135
Total/mean	316/11.7	0.547	0.797	0.770	0.233	0.321	0.115

H_{EXP}: Expected heterozygosity, H_{obs}: Observed heterozygosity, PIC: Polymorphic information content, F_{it}: Total inbreeding, F_{st}: Genetic distance, F_{is}: Within inbreeding

Table 5: Statistical analysis of the mean number of alleles (MNA), heterozygosity (H_{Obs} and H_{Exp}), polymorphic information content (PIC) and within inbreeding (F_{is}) observed across 27 microsatellite loci for each population

Population	MNA	H_{Obs}	H_{Exp}	PIC	F_{is}
HH	5.44	0.695	0.684	0.626	-0.017
HF	5.78	0.705	0.745	0.686	0.055
HG	4.70	0.539	0.593	0.531	0.091
HV	4.30	0.453	0.503	0.447	0.102
HS	4.22	0.548	0.581	0.515	0.057
HW	4.30	0.565	0.600	0.532	0.060
HY	4.59	0.507	0.583	0.523	0.132
HA	5.26	0.713	0.717	0.643	0.007
NR	6.22	0.597	0.721	0.665	0.176
NY	6.63	0.636	0.716	0.660	0.113
NG	5.44	0.561	0.646	0.592	0.134
NL	5.96	0.675	0.734	0.677	0.082
NW	4.93	0.580	0.624	0.562	0.073
NO	4.89	0.550	0.628	0.570	0.126
NF	3.59	0.416	0.478	0.417	0.131
NK	3.78	0.358	0.465	0.412	0.234
NH	4.52	0.501	0.615	0.550	0.188
NS	4.81	0.489	0.623	0.558	0.220
NC	3.63	0.440	0.555	0.481	0.211
ND	3.96	0.493	0.584	0.512	0.159
Total	4.85	0.551	0.620	0.558	0.117

MNA: Mean number of alleles, H_{EXP}: Expected heterozygosity, H_{obs}: Observed heterozygosity, PIC: Polymorphic information content, F_{is}: Within inbreeding, HH: Hanhyup H, HF: Hanhyup F, HG: Hanhyup G, HV: Hanhyup V, HS: Hanhyup S, HW: Hanhyup W, HY: Hanhyup Y, HA: Hanhyup A, NR: Red Korea native chicken, NY: Yellow Korea native chicken, NG: Gray Korea native chicken, NL: Black Korea native chicken, NW: White Korea native chicken, NO: Ogye, NF: Leghorn F, NK: Leghorn K, NH: Black cornish, NS: Brown cornish, NC: Rhode Island red C, ND: Rhode Island red D

Table 6: Characteristics of microsatellite markers flanking each sarcomere gene and their primer sequences and modifications

Marker	Allele range (bp)	Dye	Primer concentration (pmol μL^{-1})	Set no.
MCW0145	178-214	FAM	10	1
LEI0094	232-282	FAM	10	1
MCW0127	226-274	VIC	10	1
GCT0016	108-168	NED	10	1
MCW0104	192-232	PET	10	1
MCW0330	252-286	PET	10	1
ADL0293	107-127	PET	10	1
MCW0029	131-187	VIC	10	1,2
ADL0317	178-204	NED	10	1,2
MCW0264	225-243	FAM	10	2
MCW0123	80-90	FAM	10	2
ADL0304	138-162	FAM	10	2
LEI0074	132-244	VIC	10	2
MCW0087	265-289	NED	10	2
ADL0292	110-140	PET	10	2
MCW0228	220-248	PET	10	2

Table 7: Expected probability values among genotypes of random individuals (PI), random half-sib ($PI_{half\text{-}sibs}$), random sibs (PI_{sibs}) and total expected probability (PE) for the discrimination of chicken breeds using multiplex-PCR marker SET1 and SET2

Marker	PI	$PI_{half\text{-}sibs}$	PI_{sibs}	PE
SET1	4.67×10^{-10}	9.69×10^{-8}	2.50×10^{-4}	0.9892
SET2	1.50×10^{-9}	3.39×10^{-7}	9.82×10^{-4}	0.9872
SET1+SET2	1.79×10^{-21}	8.76×10^{-16}	5.70×10^{-8}	0.9888

Construction of multiplex PCR microsatellite marker sets and case study: Genotyping of all 27 markers was performed for 469 Korean chickens. Table 4 shows the characteristics of different alleles for each marker including the repeat number, frequency, mean heterozygosity (MH) and PIC. Among the 27 loci that were polymorphic in the 20 Korean chicken breeds sampled, 16 highly polymorphic MS markers were selected based on the number of alleles, H_{exp} and PIC. They were segregated into two sets for multiplex-PCR based on fragment size, genetic variation and peak interpretation (Table 6). For these chicken breeds, a satisfactory expected probability of identity among the genotypes of PI, $PI_{half\text{-}sibs}$ and PI_{sibs} was suggested to require approximately 16 markers. Thus, the expected probability of the identity values from the 16 MS markers in PI, $PI_{half\text{-}sibs}$ and PI_{sibs} was estimated as 1.79×10^{-21}, 6.76×10^{-16} and 5.70×10^{-8} respectively. Overall, the total expected probability (PE) of the identity values was 98.89% for the discrimination of Korean chickens (Table 7). Our study identified multiplex PCR marker sets in Korean chicken breeds.

Figure 1 illustrates the population relationships based on the PCoA using the individual multilocus genotypes of the 16 MS markers, with 2 types of multiplex PCR marker sets from the 7 Korean commercial chicken breeds. With the first type, the two components contributed 29.46 and 12.05%, respectively. By the first component, the HF and HH breeds were confirmed to be clearly separated from the other groups and by the second component, the HY breed was confirmed

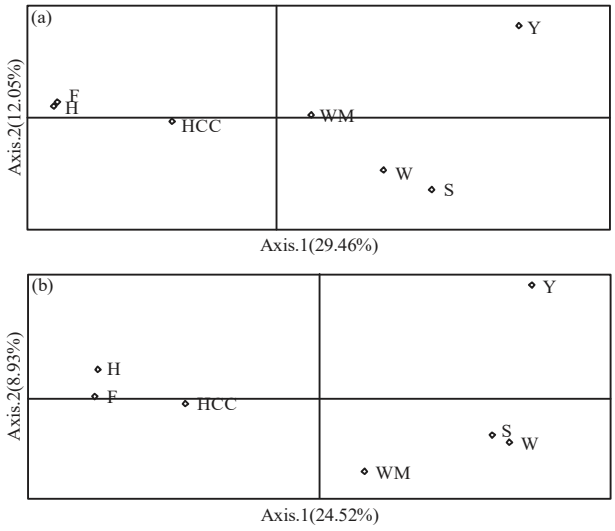

Fig. 1(a-b): Principal components analysis of allele frequencies from (a) SET1 and (b) SET2 in 7 populations using GENETIX

H: Hanhyup H, F: Hanhyup F, S: Hanhyup S, W: Hanhyup W, Y: Hanhyup Y, HCC: :Hanhyup 3, WM: Woorimatdag

to be clearly separated from the HS breed. With the second type, the two components contributed 24.52 and 8.93% respectively and the intergroup relationship showed similarity with the analysis using the first type marker set. From the multidimensional scatter (MDS) plot using 600K SNP genotypes, it can be inferred that HF and HH were classified

and maintained together until relatively recently, as their clusters overlap and are spread widely[1]. Upon comparison, the two results in this study are similar.

Our results identified multiplex PCR marker sets in Korean chicken breeds could be applied in future breeding plans and utilized as discrimination markers for these breeds. This finding suggested that, considering the scale of the domestic chicken market, the 16 identified markers are sufficient and the maintenance of the strains of Korean chickens with appropriate discrimination markers is essential for the conservation of these breeds. Our results indicated that these multiplex PCR marker sets will have considerable applications in small- and large-scale population genetic structure analyses. In addition, they can be used to aid the conservation, traceability and future improvement of these native Korean chicken breeds.

SIGNIFICANCE STATEMENT

This study identified multiplex PCR microsatellite marker sets for chicken breeds that can be beneficial for investigating the genetic diversity of Korean chicken breeds and classifying the breeds. This study will help Korean chicken breeds to be isolated and characterized. Thus, these specific markers could aid the selection process in the development of native chickens that are more suitable for the chicken industry.

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