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Characteristics of Seven Japanese Native Chicken Breeds Based on Egg White Protein Polymorphisms

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ABSTRACT : In this study, to examine genetic variability within a breed and genetic relationships between populations/breeds, we genotyped 606 birds from seven Japanese native chicken breeds at seven polymorphic loci of egg white proteins and compared those with Asian native chicken populations and commercial breeds. Genotyping of the Japanese native breeds showed that ovalbumin, two ovoglobulins and ovotransferrin were polymorphic, but ovomacroglobulin, ovoflavoprotein and lysozyme were monomorphic. The proportion of polymorphic loci (P_{poly}) and average heterozygosity ($\overline{H}$) within a population ranged from 0.286 to 0.429 and from 0.085 to 0.158, respectively. The coefficient of gene differentiation (G_{ST}) was 0.250 in the Japanese native chicken breeds. This estimate was higher than that of Asian native chicken populations (G_{ST} = 0.083) and of commercial breeds (G_{ST} = 0.169). Dendrogram and PCA plot showed that Satsuma-dori, Jitokko, Amakusa-daio and Hinai-dori were closely related to each other and grouped into Asian native chickens and that Tsushima-jidori, Nagoya and Chan (Utaichan) were ramified far from other Japanese native chicken breeds. The egg white protein polymorphisms demonstrated that the population differentiation of the seven Japanese native chicken breeds was relatively large. (**Key Words :** Egg White, Protein Polymorphism, Heterozygosity, Japanese Native Chicken)

INTRODUCTION

Eggs are a major source of biologically active compounds that are beneficial for human health and are widely used by pharmaceutical, cosmetic and food industries. Eggs have functional properties such as foaming, binding, thickening, coating and emulsifying capacity and they control crystallization (Meszaros et al., 2006). Egg white protects the egg yolk from physical impact and provides additional nutrition for embryo development, as it is rich in proteins and also of high nutritional value. The egg white contains approximately 148 different proteins (D'Ambrosio et al., 2008). Out of these, seven proteins, namely ovalbumin (O_v) (Lush, 1961, 1964; Inafuku et al., 1997), ovoglobulin G_3 (G_3) (Baker et al., 1971), ovoglobulin G_2 (G_2) (Baker et al., 1970), transferrin (Tf_{EW}) (Ogden et al., 1962; Baker, 1968a), ovo-*macroglobulin* (*Omg*) (Kimura, 1972), ovoflavoprotein (*Rd*) (Winter et al., 1967) and lysozyme (G_1) (Baker, 1968b; Inafuku et al., 1998), are found to exist in different polymorphic forms by electrophoresis. These proteins have been reported to have valuable functional properties as follows: *Ov* has been widely used as a model protein in many biochemical studies (Nichol et al., 1985; Boschetti and Coffman, 1998) and in pharmaceutical processing (Nierken et al., 2002). *Ov*, G_3 and G_2 are associated more or less intensively with high hatchability and low mortality (Harpreet and Nordskog, 1981). Tf_{EW} has several functions: to transport iron to storage cells and to impede the embryo from bacterial infections, (Seviour and Board, 1972), to be associated with the host innate immune defensive system (Valenti and Antonini, 2005) and to have antiviral activity (Giansanti et al., 2007). Kato et al. (1991) reported that *Omg* has inhibitory activity for pepsin and rennin. *Rd* is applied to several molecular species that are thought to be important in maintaining supply of the vitamin riboflavin to the developing embryo during pregnancy (White and Merrill, 1988) and has antimicrobial functions (Jasir et al., 2004). G_1 has many functions including a potent antibacterial agent with hydrolytic activity against the cell wall polysaccharide of the gram-positive bacteria inactivation of certain viruses (Hasselberger, 1978) and an important role in surveillance of membranes of mammalian cells (Osserman et al., 1974). It enhances phagocytic activity of polymorphonuclear

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leukocytes and macrophages (Thakur et al., 1999). G_1 was also used to inhibit *Clostridium butyricum* during ripening of cheeses (Maullu et al., 1999), extend shelf life of selected processed foods by inhibiting spore forming and non-spore forming spoilage organisms (Peck and Fernandez, 1995), and in wine preparation as a substitute for sulfites (Marchal et al., 2002).

A study of allele distributions of the egg white proteins in chicken populations and breeds can be used to explain genetic variability within a population or breed and genetic relationships among chicken breeds and populations. Our previous study elucidated the genetic variability of Asian native chicken populations and commercial breeds and genetic relationships among them using seven egg white protein polymorphisms (Kinoshita et al., 2002). However, allele distributions for the seven egg white proteins have not yet been analyzed in Japanese native chicken breeds.

Genetic relationships among Japanese native chicken breeds have been reported for blood protein polymorphisms (Tanabe et al., 2000), mitochondrial DNA sequences (Komiyama et al., 2003; Oka et al., 2007), microsatellite markers (Nakamura et al., 2006; Osman et al., 2006; Rikimaru and Takahashi, 2007; Tadano et al., 2007) and specific skull features (Ino et al., 2008). In the present study, we investigated allele distributions of the seven egg white protein loci in seven Japanese native chicken breeds (Hinai-dori, Nagoya, Amakusa-daio, Tsushima-jidori, Satsuma-dori, Jitokko and Chan) and compared these data with those of Asian native chicken populations and commercial breeds.

MATERIALS AND METHODS

Collection of egg whites

A total of 606 eggs from seven Japanese native chicken breeds were utilized in the present study; 108 Hinai-dori eggs from the livestock experimental station of the Akita prefectural agriculture, forestry and fisheries research centre; 120 Nagoya eggs from Aichi prefecture agricultural research center; 100 Tsushima-jidori eggs from Nagasaki prefecture livestock experiment station; 67 Amakusa-daio eggs from Kumamoto prefecture agricultural research center; 64 Satsuma-dori eggs from Kagoshima prefecture poultry experimental station; 74 Jitokko eggs from Miyazaki prefecture livestock experimental station; and 73 Chan (Utaichan) eggs from farmers of Okinawa prefecture. The eggs were rubbed with distilled water and 70% ethanol to clean their shells and to detect any cracks. After cleaning the egg shell surface, the eggs were opened and egg white albumen was collected with a syringe and kept in the plastic tube at -20°C until use.

Detection of egg white polymorphisms

For detection of *Ov*, G_2, Tf_{EW} and *Omg* loci, the egg white was diluted 8 times with 50% glycerol and electrophoresed using 8% native polyacrylamide gel (PAGE) (Davis, 1964) with an initiation step of 40 mA for 1 h and a separation step of 50 mA for 2.5-3 h. For detection of *Rd*, the egg white was diluted with an equal volume of 50% glycerol and electrophoresed using 20% native PAGE at 20 mA for 4 h. For detection of G_3, the egg white was electrophoresed using 12% starch gel (Stratil, 1968) with an initiation step of 100 V for 1 h and a separation step of 150 V for 2 h. For analyzing G_1 polymorphism, we performed Acid-PAGE (Reisfield et al., 1962). The egg white was diluted with an equal volume of 2× sampling buffer (0.28 ml acetic acid, 6.64 ml glycerol and 9.68 ml distilled water) and electrophoresed using 20% Acid-PAGE at 40 mA for 6 h. All electrophoreses were done at 4°C. After electrophoresis, the gel was stained with coomassie brilliant blue R-250 (CBB; Nacalai Tesque, Inc, Kyoto, Japan).

Data analysis

In order to assess genetic variability within a population and genetic relationships among Japanese native chickens, Asian native chicken populations and commercial breeds, we calculated allele frequencies from genotyping data and a Chi square test (x^2) for Hardy-Weinberg Equilibrium. The gene constitution within a population was calculated as proportion of polymorphic loci (P_{poly}) and average heterozygosity ($\overline{H}$). P_{poly} was calculated as a ratio of polymorphic loci to the total loci analyzed. $\overline{H}$ was estimated by the Nei's formula (Nei, 1978). Relative magnitude of gene differentiation among sub populations was estimated as the coefficient of gene differentiation (G_{ST}) (Nei, 1973). Genetic relationships among breeds was illustrated by construction of a dendrogram and principal component analysis (PCA). For construction of a dendrogram, pairwise standard genetic distance (*Ds*) (Nei, 1972) was calculated using PHYLIP ver 3.67. A dendrogram was constructed by using an unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) implemented by the MEGA software ver. 4.1 (Tamura et al., 2007). For PCA, the covariance matrix was used. A two-dimensional scatter plot of the first two principal components (Prins1 and 2) was drawn by using the princomp procedure of the SAS software ver. 9.2.

RESULTS AND DISCUSSION

Egg white protein polymorphisms and their frequencies

Allele frequencies were calculated by the direct counting method for the loci including in this study. Genotyping of the seven egg white protein loci in seven Japanese native chicken breeds showed that four loci (*Ov*, G_3, G_2, Tf_{EW}) were polymorphic while the remaining three

(*Rd*, *Omg* and G_1) were monomorphic (Figure 1 and Table 1).

For the *Ov* locus, frequencies of Ov^A allele ranged from 0.533-1.000. This allele was fixed in Tsushima-jidori, Amakusa-daio, Satsuma-dori and Jitokko breeds. For the G_3 locus, G_3^A and G_3^B alleles were observed in this study. G_3^J allele was not observed in this study although it was observed in Southeast Asian native chicken populations (Kinoshita et al., 2002). Frequencies of the G_3^A allele ranged from 0.260-0.967. For the G_2 locus, frequencies of the G_2^B allele ranged from 0.290-1.000. This allele was fixed in Hinai-dori and Satsuma-dori breeds. For the Tf_{EW} locus, frequencies of the Tf_{EW}^B allele ranged from 0.742-1.000. This allele was fixed in Nagoya, Hinai-dori, Tsushima-jidori and Chan breeds.

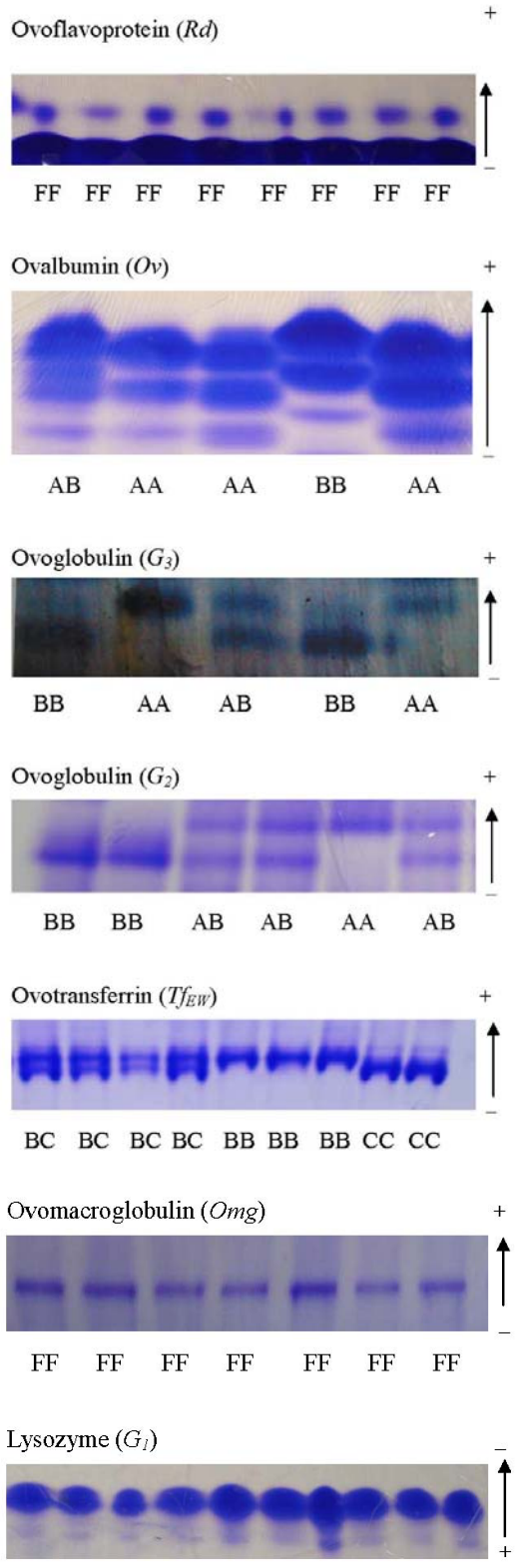

Figure 1. Electrophoretic phenotypes of five egg white protein loci (*Rd*, *Ov*, G_2, Tf_{EW} and *Omg*) in Native-PAGE, G_3 in starch gel and G_1 in Acid-PAGE.

Tests of Hardy-Weinberg equilibrium (HWE) within breeds were performed using the chi-squared goodness-of-fit test that compared expected and observed genotype numbers. These results indicated that the observed genotype frequencies of all loci except *Ov* and G_3 in Hinai-dori and Nagoya and G_2 in Chan were in HWE (Table 1).

Genetic variability and differentiation for seven Japanese native chicken breeds

The values of $P_{poly,}$ $\overline{H}$ and G_{ST} in seven Japanese native chicken breeds were computed and compared with those of Asian native chicken populations and commercial breeds (Kinoshita et al., 2002) as shown in Table 2. The resulting estimates of P_{poly} and $\overline{H}$ in these breeds ranged from 0.286-0.429 and 0.085-0.158, respectively. The range of P_{poly} and $\overline{H}$ between Japanese native chicken breeds was located within those of Asian native chicken populations (0.143-0.714 and 0.014-0.225, respectively) and was similar to those of commercial chickens (0.143-0.429 and 0.070-0.159, respectively) (Table 2). The lowest heterozygosity was observed in Hinai-dori and the highest heterozygosity was observed in Chan among the seven breeds examined here.

To evaluate the genetic subdivision among seven Japanese native chicken breeds, we calculated three parameters of the average gene diversity for the entire population ($\overline{H}_T$), the average gene diversity within subpopulations ($\overline{H}_S$) and G_{ST}. These values were 0.161, 0.120 and 0.250, respectively (Table 2). The G_{ST} value among seven Japanese native chicken breeds was higher than that of Asian native chicken populations and commercial chicken breeds (Table 2). These results suggest that the degree of gene differentiation among Japanese native breeds was higher than that of Asian native populations and commercial breeds.

Genetic relationships among seven Japanese native chicken breeds using UPGMA dendrogram

In order to evaluate genetic relationships among local populations, pairwise *Ds* values between seven Japanese

Table 1. Distribution of allele frequencies of four polymorphic loci in seven Japanese native chicken breeds

Locus	No	Genotype number			Allele frequency		x^2	p
		AA	AB	BB	Ov^A	Ov^B		
Ov								
Hinai-dori	108	91	13	4	0.903	0.097	10.668	0.005<p
Nagoya	120	37	54	29	0.533	0.467	1.106	0.75<p<0.5
Tsushima-jidori	100	100	0	0	1.000	0.000	0.000	0.995<p
Amakusa-daio	67	67	0	0	1.000	0.000	0.000	0.995<p
Satsuma-dori	64	64	0	0	1.000	0.000	0.000	0.995<p
Jitokko	74	74	0	0	1.000	0.000	0.000	0.995<p
Chan	73	48	24	1	0.822	0.178	1.076	0.75<p<0.5
G_3		AA	AB	BB	G_3^A	G_3^B		
Hinai-dori	108	61	32	15	0.713	0.287	8.232	0.025<p<0.01
Nagoya	120	113	6	1	0.967	0.033	6.029	0.05<p<0.025
Tsushima-jidori	100	8	36	56	0.260	0.740	0.415	0.9<p<0.75
Amakusa-daio	67	31	29	7	0.679	0.321	0.003	0.995<p
Satsuma-dori	64	24	29	11	0.600	0.400	0.192	0.95<p<0.9
Jitokko	74	51	20	3	0.824	0.176	0.329	0.9<p<0.75
Chan	73	48	24	1	0.822	0.178	1.076	0.75<p<0.5
G_2		AA	AB	BB	G_2^A	G_2^B		
Hinai dori	108	0	0	108	0.000	1.000	0.000	0.995<p
Nagoya	120	7	28	85	0.175	0.825	4.419	0.25<p<0.1
Tsushima-jidori	100	51	40	9	0.710	0.290	0.078	0.975<p<0.95
Amakusa-daio	67	3	22	42	0.209	0.791	0.003	0.995<p
Satsuma-dori	64	0	0	64	0.000	1.000	0.000	0.995<p
Jitokko	74	0	5	69	0.034	0.966	0.126	0.95<p<0.9
Chan	73	10	50	13	0.479	0.521	10.112	0.01<p<0.005
Tf_{EW}		BB	BC	CC	Tf_{EW}^B	Tf_{EW}^C		
Hinai dori	108	108	0	0	1.000	0.000	0.000	0.995<p
Nagoya	120	120	0	0	1.000	0.000	0.000	0.995<p
Tsushima-jidori	100	100	0	0	1.000	0.000	0.000	0.995<p
Amakusa-daio	67	45	20	2	0.821	0.179	0.015	0.995<p<0.99
Satsuma-dori	64	36	23	5	0.742	0.258	1.634	0.5<p<0.25
Jitokko	74	54	16	4	0.838	0.162	3.093	0.25<p<0.1
Chan	73	73	0	0	1.000	0.000	0.000	0.995<p

[1] P: Hardy-Weinberg Equilibrium test.

native chicken breeds were calculated using the allele frequency data. The resulting *Ds* estimates ranged from 0.011 between Amakusa-daio and Jitokko to 0.275 between Tsushima-Jidori and Nagoya (Table 3). The range of the *Ds* estimates obtained here was larger than that of Asian native chicken populations (0.000-0.063) (Kinoshita et al., 2002).

Figure 2 illustrated the dendrogram of seven Japanese native chicken breeds constructed by UPGMA clustering (Sneath and Sokal, 1973). The dendrogram grouped the seven breeds into three clades. The first clade was composed of Satsuma-dori, Jitokko, Amakusa-daio and Hinai-dori breeds; the second, Chan and Nagoya; the third, Tsushima-jidori (Figure 2). The close genetic relationships between Jitokko and Satsuma-dori and between Hinai-dori and Satsuma-dori were consistent with those obtained by microsatellites and mitochondrial DNA sequences in earlier studies (Osman et al., 2006; Oka et al., 2007). Tsushima-jidori was genetically far from other Japanese native chicken breeds in this study. The remote position of Tsushima-jidori in the dendrogram was consistent with that obtained by blood protein polymorphisms (Okabayashi et al., 1998).

Table 2. Genetic variability for seven Japanese native chicken breeds using egg white polymorphisms

Population/breed	n	P_{poly}±S.E.	$\overline{H} \pm SE$	$\overline{H}_T$	$\overline{H}_S$	G_{ST}
Japanese native chickens	606	0.286-0.429	0.085-0.158	0.161	0.120	0.250
Hinai-dori	108	0.286±0.184	0.085±0.06			
Nagoya	120	0.429±0.202	0.123±0.075			
Tsushima Jidori	100	0.429±0.202	0.122±0.071			
Amakusa-daio	67	0.429±0.202	0.153±0.074			
Satsuma-dori	64	0.286±0.184	0.125±0.081			
Jitokko	74	0.429±0.202	0.090±0.051			
Chan	73	0.429±0.202	0.158±0.079			
Asian native chickens[1]	1,112	0.143-0.714	0.014-0.225	0.140	0.129	0.083
Commercial chickens [1]	4,204	0.143-0.429	0.070-0.159	0.145	0.120	0.169

[1] Kinoshita et al. (2002).

Table 3. Pairwise *Ds* estimates for seven Japanese native chicken breeds

		[1]	[2]	[3]	[4]	[5]	[6]	[7]
[1]	Hinai-dori							
[2]	Nagoya	0.055						
[3]	Tsushima-jidori	0.182	0.275					
[4]	Amakusa-daio	0.019	0.086	0.119				
[5]	Satsuma-dori	0.020	0.115	0.180	0.014			
[6]	Jitokko	0.012	0.069	0.207	0.011	0.014		
[7]	Chan	0.060	0.050	0.103	0.041	0.098	0.062	

Genetic relationships among Japanese native chicken breeds, Asian native chicken populations and commercial breeds using PCA

In order to evaluate the genetic relationships among all chicken populations including Japanese native chickens, Asian native chickens and commercial breeds, PCA were performed using the allele frequencies of seven egg white protein loci (Figure 3 and Table 4). Contribution ratios of the first two Prins thus obtained were 49.1% and 25.3% of total variation, respectively; the sum of them was 74.4% (Table 4).

As shown in Table 4, eigenvectors in the Prin1 were -0.518 for G_3^B, 0.486 for G_3^A, -0.453 for G_2^A and 0.453 for G_2^B, indicating that G_3 and G_2 loci largely contributed to the Prin1. Of the eigenvectors in the Prin2, *Ov* (Ov^A: 0.501, Ov^B: -0.501) and G_2 (G_2^A:-0.435, G_2^B: 0.434) loci were indicated to contribute largely to the Prin2 (Table 4).

The two-dimensional scatter plot was drawn by the first two Prins (Figure 3). Relationships among Japanese native chickens and among Asian native chickens and commercial breeds in this plot were basically consistent with those of the dendrogram shown in Figure 2 and the neighbor-joining tree obtained by our previous study (Kinoshita et al., 2002), respectively. Satsuma-dori, Jitokko, Amakusa-daio and Hinai-dori were located in the center of the plot and formed into a group comprising Asian native chickens. These

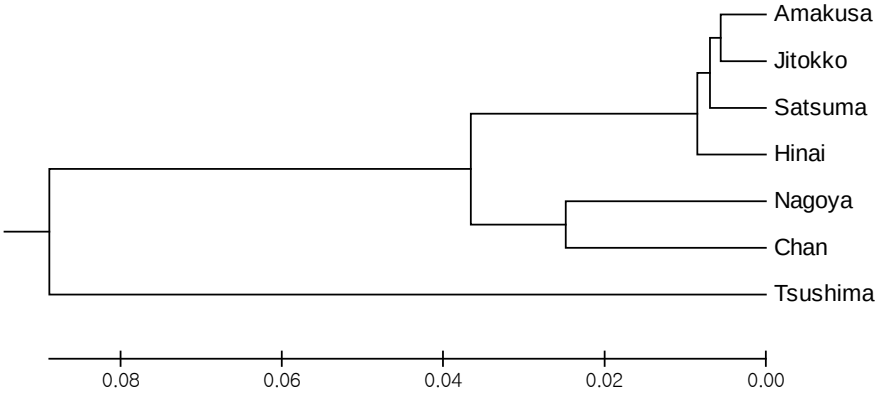

Figure 2. Genetic relationships among seven Japanese native chicken breeds by UPGMA dendrogram.

Table 4. Eigenvectors and contribution ratios of the first two principal components (Prins)

Allele	Eigenvectors	
	Prin 1	Prin 2
O_V^A	-0.193	0.501
O_V^B	0.193	-0.501
G_3^A	0.486	-0.185
G_3^B	-0.518	0.237
G_3^J	0.032	-0.051
G_2^A	-0.453	-0.435
G_2^B	0.453	0.434
G_2^L	0.001	0.001
Tf_{EW}^A	0.007	-0.040
Tf_{EW}^B	-0.071	-0.089
Tf_{EW}^C	0.064	0.130
G_1^F	-0.001	-0.002
G_1^S	0.001	0.002
Contribution ratio (%)	49.1	25.3
Cumulative (%)	49.1	74.4

results suggested that these four breeds had close relationships with Asian native chicken populations. The remaining three breeds (Tsushima-jidori, Nagoya and Chan) were in a remote position from this group (Figure 3). Tsushima-jidori was differentiated from other breeds by the Prin1 (Figure 3). This resulted from the G_3^B and G_2^A alleles, each of which was a major allele at G_3 and G_2 loci only in Tsushima-Jidori (Table 1). Nagoya and Chan were differentiated from other breeds by the Prin2 (Figure 3). The gene frequency of *Ov* in Nagoya and that of G_2 in Chan was different from those of other breeds (Table 1).

CONCLUSION

In the present study, we investigated the characteristics of seven Japanese native chicken breeds based on seven egg white protein polymorphisms. Of the seven polymorphisms, four were detected among seven Japanese native chicken breeds examined here. The $\overline{H}$ estimates within these breeds were within those of Asian native chicken populations. The G_{ST} estimate among the seven Japanese native chicken breeds was larger than that among Asian native chicken populations (Table 2). From the PCA, Satsuma-dori, Jitokko, Amakusa-daio and Hinai-dori breeds were closely related to Asian native chicken populations while Tsushima-jidori, Chan and Nagoya were genetically distant from these populations. These results suggest that genetic differentiation among the Japanese native chicken breeds has proceeded during development of each breed. Shift of the gene constitution of the egg white protein within each breed may have occurred by events such as genetic drift and artificial selection and thus lead to a high level of gene differentiation between breeds. These findings

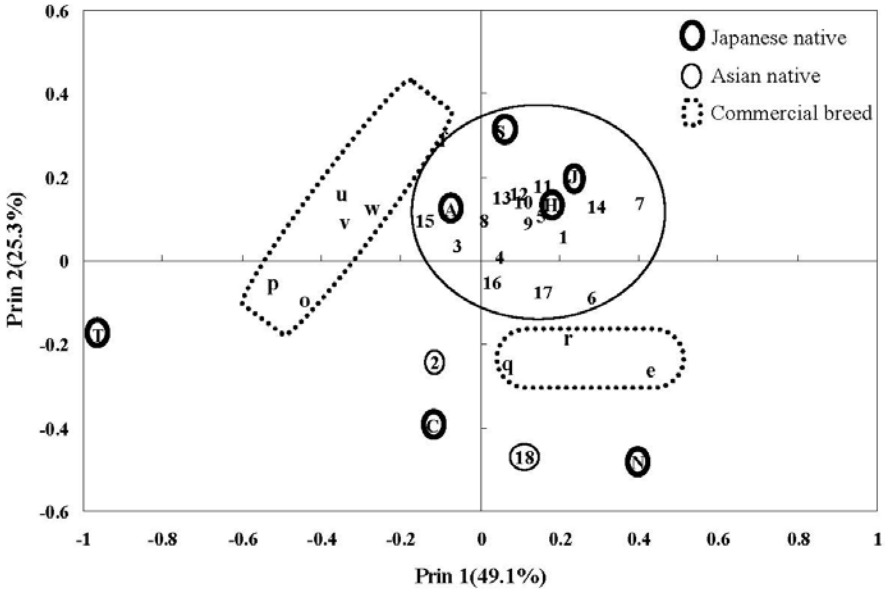

Figure 3. Two dimensional scatter plot of the first two Prins for 34 chicken populations including seven Japanese native chicken breeds. H : Hinai-dori, N : Nagoya, T : Tsushima-jidori, A : Amakusa-daio, S : Satsuma-dori, J : Jitokko, C : Chan, 1 : Vietnam North, 2 : Vietnam South, 3 : Laos North, 4 : Laos Central, 5 : Laos South, 6 : China Chahua, 7 : China Xishuangbanna game, 8 : China Xishuangbanna native, 9 : China Wuding, 10 : China Yangbi Huang, 11 : Indonesia Java island, 12 : Indonesia Bali island, 13 : Nepal West, 14 : Nepal East, 15 : Mongolia, 16 : Myanmar Yangon, 17 : Myanmar Mandalay, 18 : Thailand South, o : Boris Brown, p : Isa Brown, q : White Leghorn (WL-C36), r : White Leghorn (WL-C37), e : White Leghorn (WL-S2), f : White Leghorn (WL-S5), u : White Cornish(WC-1), v : White Cornish(WC-2), w : White Cornish(WC-3). 1-18; o-p; (Kinoshita et al., 2002), q-r; (Stratil, 1968), e-f; (Buvavendran, 1967), u-w; (Stratil, 1968).

can be used as genetic information for the preservation and further improvement of the Japanese native chicken breeds. In addition, egg traits such as antibacterial activity and hatchability may be different between Japanese native chicken breeds. Our investigation on egg protein polymorphisms was found to be a valuable tool for revealing the genetic relationships among Japanese native chicken breeds.

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