《Research Note》

**Genetic Differentiation among Populations of the Kurokashiwa
Breed of Indigenous Japanese Chickens Assessed by
Microsatellite DNA Polymorphisms**

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The Kurokashiwa breed of native Japanese chickens is primarily reared in Shimane and Yamaguchi Prefectures, Japan. To reveal the genetic diversity and differentiation among the Kurokashiwa populations in both prefectures, 29 microsatellites were analyzed. To prepare the microsatellites, 54, 55, and 24 blood samples were collected from the Kurokashiwa populations of five fancy breeders in Shimane Prefecture, three fancy breeders in Yamaguchi Prefecture, and the Livestock Technology Research Department, Yamaguchi Prefectural Agriculture and Forestry General Technology Center (LTRDY), respectively. The corrected number of alleles (allelic richness: AR) was considerably low (1.63–2.48) through all Shimane and Yamaguchi populations. However, inbreeding coefficient (F_{IS}) values were not significant in the entire Shimane and Yamaguchi ornamental populations as well as the LTRDY population. In addition, there was no statistically significant difference in expected heterozygosity (H_E) through all populations in Shimane and Yamaguchi Prefectures, although the H_E varied from 0.237 to 0.445 depending on populations. The topology of the phylogenetic tree, pairwise F_{ST} values, and STRUCTURE analysis indicated that Kurokashiwa populations were genetically separated between Shimane and Yamaguchi Prefectures. On the other hand, there was no genetic differentiation among ornamental populations within each prefecture, which seemed to have resulted from adequate random matings by exchanging individuals among fancy breeders within each conservation community, along with a proper random mating within LTRDY. The present study proposes a conservation strategy suggesting that the Kurokashiwa populations of Shimane and Yamaguchi Prefectures should be maintained separately with continuous exchange of birds within each conservation community to preserve genetic diversity. Also, the LTRDY population should be independently kept by avoiding matings with ornamental bird populations because it is a well established closed colony with a uniform genetic constitution.

Key words: conservation genetics, genetic differentiation, genetic diversity, Kurokashiwa breed, microsatellite, native Japanese chickens

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Introduction

The Kurokashiwa is one of the native Japanese chicken breeds designated as a “National Natural Treasure” of Japan (Tsudzuki, 2003) mainly reared in westernmost Honshu

particularly in Shimane and Yamaguchi Prefectures. The plumage color of adult birds is dense black in both sexes. This breed was originally raised for ornamental purpose, but the Livestock Technology Research Department, Yamaguchi Prefectural Agriculture and Forestry General Technology Center (LTRDY) is using it to produce brand meat by crossing this breed with other Japanese and foreign breeds (e.g., Oh-Shamo, Rhode Island Red, and White Plymouth Rock). Therefore, the conservation of this breed is important not only to save a rare indigenous breed as one part of

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Japanese cultural heritage, but also to contribute towards the maintenance of a genetic resource for specialty meat production.

Oana (1951) suggested that the Kurokashiwa is one of the color varieties of the Shoukoku breed based on the body shape and tail morphology. Later, however, Osman *et al.* (2006) discovered a large genetic distance between the Kurokashiwa and Shoukoku breeds based on microsatellite DNA polymorphisms, apparently different from the hypothesis of Oana (1951). The Kurokashiwa samples used in the study of Osman *et al.* (2006) were those from LTRDY, and not from fancy breeders. So far, there has been no research on the genetic differentiation between Kurokashiwa populations in Shimane and Yamaguchi Prefectures, and between those in LTRDY and other ornamental populations in Yamaguchi Prefecture.

From the viewpoint of future conservation of the Kurokashiwa breed, it is important not only to elucidate their genetic diversity but also to compare genetic uniqueness of each population to determine their effective conservation strategy. In the present study, the polymorphisms of 29 microsatellite markers were analyzed to reveal the genetic diversity and differentiation in the Kurokashiwa populations reared in Shimane and Yamaguchi Prefectures.

Materials and Methods

Samples Analyzed and Genotyping

Blood samples were collected from 133 Kurokashiwa birds reared in Shimane and Yamaguchi Prefectures. In Shimane Prefecture, blood sampling was performed in 2011 with 54 birds from five fancy breeders belonging to the same conservation community (20 from breeder A, seven from B, eight from C, nine from D, and 10 from E). In Yamaguchi Prefecture, 55 blood samples were collected in 2013 from birds kept by three fancy breeders (15 from breeder F, 20 from G, and 20 from H) belonging to the same conservation community in the prefecture. In addition, 24 blood samples were obtained in 2012 from the LTRDY population that is maintained as a closed colony for more than 30 years, with approximately 100 birds per generation per year. Based on interviews with fancy breeders, the 54 birds were all reared in Shimane Prefecture as of 2011. To our knowledge, almost all the ornamental Kurokashiwa birds in Yamaguchi Prefecture were kept by the three breeders mentioned above, and the total number of individuals reared by them was 101 as of 2013. Thus, we analyzed approximately half of the entire Yamaguchi ornamental individuals in the present study.

Genomic DNA was extracted from blood samples using a standard phenol-chloroform method (Sambrook and Russell, 2001). Twenty-nine microsatellite loci out of the 30 loci recommended by ISAG/FAO (FAO, 2004) were amplified by PCR using the GenAmp 9700 PCR System (Applied Biosystems, Foster City, CA). Of the 30 markers, marker MCW0284 was not used because of difficulty in amplification. The PCR conditions were the same as those of Oka *et al.* (2010). Genotyping was performed using the ABI PRISM 310 Genetic Analyzer and Genemapper 3.7 software

(Applied Biosystems).

Statistical Analysis

The mean number of alleles (MNA), the corrected number of alleles (allelic richness: AR), observed and expected heterozygosity (H_O and H_E), and inbreeding coefficient (F_{IS}) in each Kurokashiwa population were estimated using the FSTAT 2.9.3 program (Goudet, 1995) to compare the genetic diversity among populations. Proportion of polymorphic loci (P_{Poly}), the total number of alleles (TNA), and the number of private alleles (PA) were calculated from genotyping data. The pairwise F_{ST} among the ornamental populations in Shimane and Yamaguchi Prefectures and the LTRDY population was also estimated using the FSTAT 2.9.3 program. A phylogenetic tree was constructed using a neighbor joining (NJ) method (Saitou and Nei, 1987) based on the D_A genetic distance (Nei *et al.*, 1983) to reveal the genetic relationships among the Kurokashiwa populations. In addition, the hidden genetic structure of the Kurokashiwa populations was revealed using the STRUCTURE program (Pritchard *et al.*, 2000). Twenty independent replicates were performed for each number of the assumed cluster (K) from 1 to 10 under the same condition as reported by Tadano *et al.* (2009). Average membership coefficients for the 20 replicates were calculated using the CLUMPP program (Jakobsson and Rosenberg, 2007). The optimal K value was estimated based on ΔK values (Evanno *et al.*, 2005) calculated by the Structure Harvester program (Earl and von Holdt, 2012).

Results

P_{Poly} , TNA, MNA, AR, PA, H_O , and H_E estimated in each population are summarized in Table 1. P_{Poly} ranged from 0.552 (LTRDY population) to 0.897 (populations G and H). MNA and AR ranged from 1.66 (LTRDY) to 2.69 (G) and from 1.63 (LTRDY) to 2.48 (G), respectively. H_O and H_E ranged from 0.250 (LTRDY) to 0.436 (G) and from 0.237 (LTRDY) to 0.445 (G), respectively. MNA showed no statistically significant difference throughout all ornamental populations in both Shimane and Yamaguchi Prefectures, whereas LTRDY population showed a significantly lower MNA value than all five Shimane populations and two Yamaguchi ornamental populations. On the other hand, there was no significant difference in AR among all nine populations ($P > 0.05$, Kruskal-Wallis test with Scheffe's multiple comparison), although actual values of AR in the LTRDY and five Shimane populations showed lower tendency as compared with the three ornamental populations of Yamaguchi Prefecture. In addition, H_O and H_E in the LTRDY population and all the Shimane Prefecture populations also showed lower tendency than those of Yamaguchi ornamental populations, although there was no statistically significant differences among them. Significant and negative F_{IS} values were estimated for populations B, C, and D in Shimane Prefecture ($P < 0.01$ in breeders B and C, $P < 0.05$ in D), however the F_{IS} value estimated for the entire Shimane population comprising five populations was not significant ($P > 0.05$). The number of private alleles was 13, 16, and 1

Table 1. Genetic diversity indices estimated in each population of the Kurokashiwa breed of chickens

Populations (No. of birds)	P_{Poly}	TNA	MNA	AR	PA	H_O	H_E	F_{IS}
Shimane Prefecture								
Ornamental								
A (20)	0.621	51	1.86 (0.83) ^{ab}	1.82 (0.77) ^a	0	0.307 (0.019) ^a	0.303 (0.050) ^a	−0.013 ^{ns}
B (7)	0.586	50	1.76 (0.74) ^{ab}	1.76 (0.74) ^a	1	0.345 (0.033) ^a	0.283 (0.049) ^a	−0.241**
C (8)	0.655	54	1.72 (0.59) ^{ab}	1.72 (0.59) ^a	1	0.401 (0.032) ^a	0.306 (0.045) ^a	−0.342**
D (9)	0.621	53	1.83 (0.80) ^{ab}	1.76 (0.75) ^a	0	0.268 (0.027) ^a	0.240 (0.045) ^a	−0.126*
E (10)	0.828	63	2.17 (0.80) ^{ab}	2.08 (0.71) ^a	1	0.324 (0.028) ^a	0.346 (0.041) ^a	−0.066 ^{ns}
all (54)	0.897	72	2.48 (0.99)	—	13	0.323 (0.012)	0.332 (0.042)	0.028 ^{ns}
Yamaguchi Prefecture								
Ornamental								
F (15)	0.828	67	2.31 (0.81) ^{ab}	2.17 (0.70) ^a	2	0.382 (0.023) ^a	0.373 (0.040) ^a	−0.023 ^{ns}
G (20)	0.897	77	2.69 (1.04) ^a	2.48 (0.86) ^a	0	0.436 (0.021) ^a	0.445 (0.042) ^a	0.020 ^{ns}
H (20)	0.897	78	2.66 (0.97) ^a	2.43 (0.78) ^a	0	0.405 (0.020) ^a	0.428 (0.038) ^a	0.055 ^{ns}
all (55)	0.897	82	2.83 (1.14)	—	16	0.410 (0.012)	0.447 (0.039)	0.083 ^{ns}
Yamaguchi Prefecture								
LTRDY (24)	0.552	48	1.66 (0.67) ^b	1.63 (0.64) ^a	1	0.250 (0.016) ^a	0.237 (0.044) ^a	−0.059 ^{ns}

Twenty-nine microsatellites were analyzed. LTRDY: Livestock Technology Research Department, Yamaguchi Prefectural Agriculture and Forestry General Technology Center, P_{Poly} : proportion of polymorphic loci, TNA: total number of alleles, MNA: mean number of alleles, AR: allelic richness (corrected value of number of alleles), PA: number of private alleles, H_O : observed heterozygosity, H_E : expected heterozygosity, F_{IS} : inbreeding coefficient. Numerals in parentheses show standard deviations. MNA, AR, H_O , and H_E were compared among populations using the Kruskal-Wallis test with the Scheffe test for pairwise comparison (values with different superscripts are statistically different, $P < 0.05$). AR was calculated with the correction based on minimum population size of population B ($n = 7$). Asterisks of F_{IS} values show significant deviation from the Hardy-Weinberg equilibrium (** $P < 0.01$, * $P < 0.05$), while the superscript ns means no statistical significance ($P > 0.05$).

Table 2. Pairwise F_{ST} (above diagonal) and D_A genetic distance (below diagonal) estimated among nine populations of the Kurokashiwa breed of chickens

Populations	A	B	C	D	E	F	G	H	LTRDY
A		0.040 ^{ns}	0.126*	0.069 ^{ns}	0.132**	0.403**	0.253**	0.326**	0.530**
B	0.021		0.205**	0.149 ^{ns}	0.169*	0.410**	0.252**	0.321**	0.566**
C	0.101	0.115		0.213**	0.204**	0.411**	0.279**	0.327**	0.541**
D	0.034	0.055	0.110		0.138*	0.410**	0.260**	0.328**	0.579**
E	0.098	0.103	0.141	0.097		0.346**	0.214**	0.269**	0.505**
F	0.295	0.301	0.327	0.292	0.249		0.150**	0.091 ^{ns}	0.338**
G	0.204	0.210	0.249	0.206	0.177	0.095		0.150**	0.317**
H	0.248	0.254	0.278	0.252	0.208	0.026	0.056		0.307**
LTRDY	0.365	0.371	0.354	0.371	0.334	0.202	0.205	0.185	

A to E: ornamental populations in Shimane Prefecture. F to H: ornamental populations in Yamaguchi Prefecture. LTRDY: Livestock Technology Research Department, Yamaguchi Prefectural Agriculture and Forestry General Technology Center. Asterisks attached to the F_{ST} values show significant difference from zero (** $P < 0.01$, * $P < 0.05$), while the superscript ns means no statistical significance ($P > 0.05$).

in Shimane ornamental populations, Yamaguchi ornamental populations, and the LTRDY population, respectively.

The D_A genetic distances and F_{ST} values between all pairs of populations are shown in Table 2. In ornamental populations, the pairwise D_A distances were estimated to be 0.021–0.141 and 0.026–0.095 within Shimane and Yamaguchi Prefectures, respectively, while those were 0.177–0.327 among the populations belonging to different prefectures. In addition, the D_A distance between the whole Shimane population and the whole Yamaguchi population

was 0.196 (not shown in Table 2). The pairwise F_{ST} values were significant ($P < 0.05$) in almost all pairs with relatively high values between Shimane and Yamaguchi ornamental populations. Also, the pairwise D_A distances and F_{ST} values between LTRDY and Shimane ornamental populations were higher than those between LTRDY and Yamaguchi ornamental populations. The F_{ST} value between the whole Shimane and Yamaguchi populations was 0.268 ($P < 0.01$, not shown in Table 2).

In the D_A -NJ tree (Fig. 1), Shimane populations were

separated from Yamaguchi populations with 100 % bootstrap value. Within the Yamaguchi cluster, the LTRDY population was segregated from the other three ornamental populations. The pairwise F_{ST} values between the Shimane and Yamaguchi ornamental populations, between Shimane and LTRDY populations, and between Yamaguchi ornamental

and LTRDY populations were 0.268, 0.483, and 0.256, respectively, all of which were statistically significant ($P < 0.01$).

In the STRUCTURE analysis, $K=2$ was adopted by the ΔK value for the optimum K value ($\Delta K=583.3$), and $K=3$ was followed ($\Delta K=295.7$). In the result using $K=2$ for the STRUCTURE analysis (Fig. 2), 54 individuals of Shimane Prefecture had clearly different genetic backgrounds from the Kurokashiwa reared in Yamaguchi Prefecture. At $K=3$, 24 individuals of the LTRDY population were separated from all other Yamaguchi ornamental birds.

Discussion

Using the same microsatellite DNA markers employed in the present study, Oka *et al.* (2010, 2011a, b, c) reported that AR and H_E of native Japanese chicken breeds were 1.79–3.94 and 0.248–0.611, respectively. Osman *et al.* (2006) who used different microsatellite markers reported P_{Poly} of 0.55–1.00 and H_E of 0.21–0.67 in native Japanese chicken breeds. Judging from these genetic diversity indices, the degree of genetic diversity of the Kurokashiwa birds in Shimane and Yamaguchi ornamental populations corresponded to low or middle class diversity seen in the other Japanese breeds.

The F_{IS} value (0.028) estimated for the entire Shimane population including all five individual populations was not significant, irrespective of the negative and significant F_{IS} values observed in the small-sized populations of breeders B, C, and D. Also, F_{IS} (0.083) estimated for the entire Yamaguchi ornamental population was not significant (Table 1). These results indicated that the Kurokashiwa conservation communities of both prefectures have preserved genetic diversity in their birds, although the size of individual populations is small. According to interview information from private breeders, exchanging of birds went on among

Fig. 1. Neighbor joining tree of nine populations of the Kurokashiwa breed of chickens based on D_A genetic distance. Bootstrap values (more than 90 %) are shown in the branch. A–E: ornamental populations in Shimane Prefecture. F–H: ornamental populations in Yamaguchi Prefecture. LTRDY: population of the Livestock Technology Research Department, Yamaguchi Prefectural Agriculture and Forestry General Technology Center.

Fig. 2. Results of STRUCTURE analysis for 133 individuals of the Kurokashiwa breed of chickens ($K=2$ and 3). Each individual is represented by a vertical bar, and each color indicates one cluster. A–E: ornamental populations in Shimane Prefecture. F–H: ornamental populations in Yamaguchi Prefecture. LTRDY: population of the Livestock Technology Research Department, Yamaguchi Prefectural Agriculture and Forestry General Technology Center.

breeders within each conservation community. The non-significant F_{IS} values in the entire populations in Shimane and Yamaguchi Prefectures were thought to have resulted from such exchanging of birds among breeders. The STRUCTURE analysis (Fig. 2), which basically showed no differentiation among ornamental populations in each prefecture, also corroborated the interview information.

The topology of the NJ tree (Fig. 1), pairwise F_{ST} values, and STRUCTURE analysis (Fig. 2) clearly divided the Kurokashiwa populations of Shimane and Yamaguchi Prefectures, being in good agreement with the interview information wherein no exchanging of birds between the two prefectures took place. The considerably large number of private alleles in both the Shimane and Yamaguchi ornamental populations also supported the results of the NJ tree and STRUCTURE analyses. Within each prefecture, the genetic constitution was considerably uniform among ornamental populations as revealed in the STRUCTURE analysis. However, some birds in both prefectures (E population in Shimane Prefecture and G and H populations in Yamaguchi Prefecture) had different genetic clusters from their prefecture specific genetic background with low percentages, suggesting the possibility that there was some exchanging of birds in the past between Shimane and Yamaguchi Prefectures. Differing from the ornamental populations, the LTRDY population birds showed complete uniformity in their genetic constitution. This is thought to have resulted from a systematic mating scheme as a closed colony for more than 30 years. The genetic differentiation of the LTRDY population from the Yamaguchi ornamental populations is thought to be derived from changes of allele frequencies with the reduction of alleles.

From the results of the present study, we propose the following strategy for the conservation of the Kurokashiwa breed. The Kurokashiwa populations in Shimane and Yamaguchi Prefectures should be separately maintained without crossing with each other because they apparently showed genetic differentiation. Exchanging of birds among breeders within each conservation community should be continued to avoid decrease of genetic diversity. However, the population size of the Kurokashiwa is not so large in both Shimane (approximately 55) and Yamaguchi (approximately 100) communities. Therefore, there is a possibility that the future maintenance of the Kurokashiwa populations in Shimane and/or Yamaguchi communities becomes precarious. In such a case, exchanging birds between the two communities should be considered. According to previous studies, the D_A genetic distances between subpopulations of the same chicken breeds were 0.205, 0.203, and 0.047 in the Oh-Shamo, Satsumadori, and Chabo breeds, respectively (Oka *et al.*, 2010) and 0.103, 0.184, and 0.138 in the Oh-Shamo, Shokoku, and Yakido breeds, respectively (Osman *et al.*, 2006). Furthermore, according to a study using 28 breeds of native Japanese chickens, the D_A genetic distances between different breeds generally exceeded 0.300 (Osman *et al.*, 2006). In the present study, the D_A value between the whole Shimane and Yamaguchi populations was 0.196, suggesting

that no large genetic differentiation has occurred between the two populations for them to be recognized as different breeds and that the two populations are subpopulations within the same breed, although the two populations showed characteristic genetic constitutions as seen in the NJ-tree topology and the result of the STRUCTURE analysis. Differing from the case of ornamental birds, the LTRDY population should be independently maintained without crossbreeding with birds from ornamental populations because it is a well established closed colony with uniform genetic backgrounds. This population is also valuable as experimental and industrial resources because of its genetic uniformity, in addition to its intrinsic value as living cultural assets of Japan.

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