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Temporal Changes of Genetic Population Structure and Diversity in the Endangered Blakiston's Fish Owl (*Bubo blakistoni*) on Hokkaido Island, Japan, Revealed by Microsatellite Analysis

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The Blakiston's fish owl (*Bubo blakistoni*) population on Hokkaido Island, Japan, decreased to less than one hundred individuals over the last century due to habitat disruption by human activity. Although the ongoing conservation management has slightly restored the population, it remains endangered. In order to assess the genetic variation and population structure of the Blakiston's fish owl in Hokkaido, we genotyped eight microsatellite loci on 120 individuals sampled over the past three decades. The genotype data set showed low levels of genetic variation and gene flow among the geographically isolated five subpopulations. Comparative analysis of past and current populations indicated that some alleles shared by past individuals had been lost, and that genetic variation had declined over the last three decades. The result suggests that the genetic decline may have resulted from inbreeding and/or genetic drift due to bottlenecks in the Hokkaido population. The present study provides invaluable genetic information for the conservation and management of the endangered Blakiston's fish owl in Hokkaido.

Key words: Blakiston's fish owl, *Bubo blakistoni*, genetic diversity, microsatellite, population structure

INTRODUCTION

The Blakiston's fish owl (*Bubo blakistoni*) is the largest owl in the world, and it was formerly classified into genus *Ketupa*, which is currently included in genus *Bubo* by molecular systematics (Wink et al., 2009). Based on the geographic distribution, this species was divided into two subspecies: *B. b. doerriesi* on the Eurasian Continent and *B. b. blakistoni* on Hokkaido, Sakhalin and southern Kuril islands (Voous, 1988). The Blakiston's fish owl resides in riparian forests, and requires large amounts of fish for food and large hollow trees for nesting (Takenaka, 1998; Yamamoto, 1999). The life span of this bird is over twenty years in the wild, and lifelong breeding pair relationships are maintained in restricted territories along rivers (Hayashi, 1997; Yamamoto, 1999). The clutch size of *B. b. blakistoni* is usually two (Yamamoto, 1999).

Although the Blakiston's fish owl was widespread in forests in Hokkaido (78,000 km^2), the population markedly decreased during the 20th century due to the reduction and fragmentation of the habitat by human activity. There are no records of this owl in the southern area of Hokkaido since the 1950s, the northern area since the 1970s, and the Ishikari lowlands since the 1980s (Hayashi, 1999) (see Fig. 1 for locations on Hokkaido Island). Even in eastern Hokkaido, where the population size has been better conserved, it has declined since the 1970s (Nagata, 1972), and the minimum number in Hokkaido was estimated to be only 80–100 during this period (Takenaka, 1998; Brazil and Yamamoto, 1989). For this reason, the Blakiston's fish owl has been listed as endangered on the IUCN Red List Ver.3.1, and as a National Endangered Species under Japan's Act for the Conservation of Endangered Species of Wild Fauna and Flora. Recently, due to the conservation of artificial nesting and feeding, the population has recovered to about 140 at limited spots in mainly eastern Hokkaido: Shiretoko Peninsula where half of the owls live, northern Kushiro, Nemuro, Tokachi and Hidaka areas (T. Takenaka, unpublished data) (Fig. 1). In the past 25 years, most young owls for which nest locations were recorded have been banded with leg-rings used in ecological research. The familial relationships and immigration for several individuals have been elucidated, and inbreeding was often observed in northern Kushiro, Nemuro and a part of Tokachi (Takenaka, 1998; Yamamoto, 1999; Hayashi, 2009).

The long-term conservation management of this species will require ecological as well as genetic information, such as population structure, genetic variation among populations and the relationships among individuals. Population bottle-

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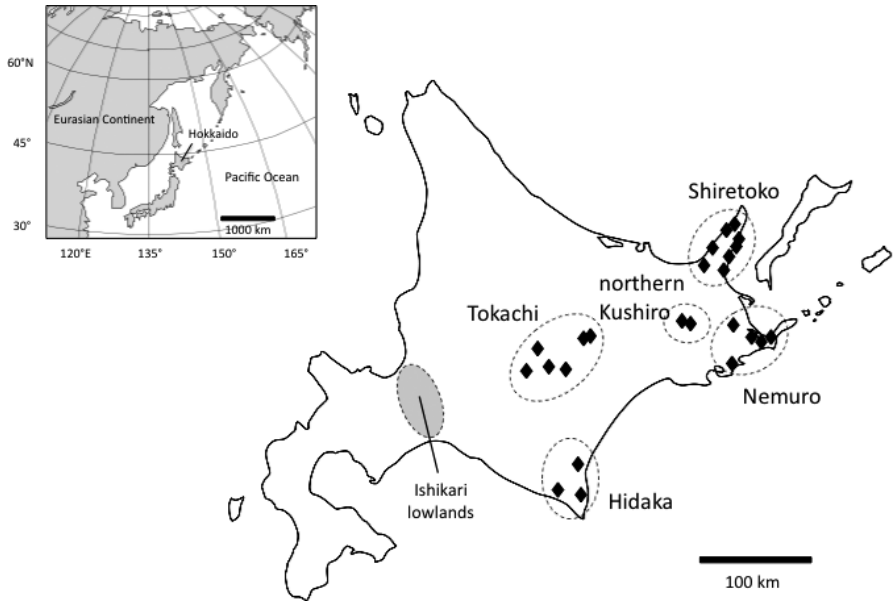

Fig. 1. Sampling locations (rhombuses) of the Blakiston's fish owls on Hokkaido Island, Japan. Broken-lined circles show the five subpopulations defined in the present study. The map in the upper left indicates the location of the Japanese islands in northeastern Eurasia.

Table 1. Sample numbers of Blakiston's fish owls at each sampling location and period.

Period	Sampling locations					Total
	Shiretoko	Northern Kushiro	Nemuro	Tokachi	Hidaka	
1986–1989	5	5	11	4	0	25
1990–1993	14	7	9	7	0	37
1997–1999	13	6	11	7	0	37
2009–2010	12	0	0	4	5	21
Total	44	18	31	22	5	120

necks generally lead to reduction of viability because of decreasing genetic variation and inbreeding depression. Based on the data of many threatened birds, Heber et al. (2010) reported that population bottlenecks increase hatching failures. Although the Blakiston's fish owl is feared to have lost genetic diversity and viability due to its small population size, no genetic characteristics are known.

In the present study, in order to assess the genetic diversity of the Blakiston's fish owl population in Hokkaido, we conducted analysis on biparentally inherited microsatellite loci, and discuss the present population structure and the temporal changes of the genetic variation over the last three decades.

MATERIALS AND METHODS

Sampling and DNA extraction

We extracted total DNA from blood or cultured fibroblasts, obtained from 120 Blakiston's fish owls (69 males and 51 females). As shown in Table 1, 25 owls were captured for banding from 1986 to 1989 (first period), 37 owls from 1990 to 1993 (second period), 37 owls from 1997 to 1999 (third period) and 21 owls from 2009 to 2010 (fourth period), at 24 locations in Hokkaido (Fig. 1). Sampling locations in the first period were fewer than in the latter periods, and some siblings and their relatives were included in the samples. Some drops of blood were dried on filter papers and frozen at –20°C until use. Fibroblasts were obtained by culturing small pieces of skin tissues, and frozen in liquid nitrogen until use. Total DNA from blood and fibroblasts was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

Selection of microsatellite markers

To select polymorphic loci suitable for genetic analysis from Blakiston's fish owl, we tested 53 microsatellite markers previously reported in seven owl species: the eagle owl (*Bubo bubo*) (Isaksson et al., 2002), Lanyu scops owl (*Otus elegans*) (Hsu et al., 2003, 2006), Mexican spotted owl (*Strix occidentalis*) (Thode et al., 2002), ferruginous pygmy-owl (*Glaucidium brasilianum*) (Proudfoot et al., 2005), boreal owl (*Aegolius funereus*) (Koopman et al., 2004), western burrowing owl (*Athene cunicularia*) (Korfanta et al., 2002; Faircloth et al., 2010) and barn owl (*Tyto alba*) (Klein et al., 2009). Using primers of the markers, several microsatellite loci from Blakiston's fish owl were amplified by polymerase chain reaction (PCR). The PCR-amplified products were then sequenced to check whether microsatellite regions were contained. First, we selected loci that included relatively long microsatellite regions with more than ten repeats of motifs. When microsatellite markers were found to be polymorphic in 10 randomly selected Blakiston's fish owl individuals in the fragment analysis as shown below, they were used for subsequent analysis in the population genetic study.

Analysis of microsatellite makers

The PCR for fragment analysis was performed in 10 μl of a reaction mixture consisting of 1.0 μl of 10 PCR buffer (Takara), 0.8 μl of dNTP mixture (Takara), 0.1 μl of *Taq* DNA polymerase (5 units/μl, Takara), 0.1 μl of a forward primer non-labeled and a reverse primer Texas-Red-labeled at the 5' end (5 pmol/μl each), 1.0 μl of DNA extract, and 6.9 μl of distilled water. PCR amplifications were carried out in a PCR thermal cycler Takara TP-600 using the following conditions: one cycle at 94°C for 3 min and 30 or 35 cycles of 94°C for 1 min; 52 or 60°C for 1 min; 72°C for 1 min; and followed by one cycle at 72°C for 10 min. The molecular size of each PCR product was determined using an autosequencer Hitachi SQ-5500 and FRAGLYS 3 software (Hitachi).

The population structure was estimated using STRUCTURE 2.3 software (Pritchard et al., 2000; Falush et al., 2003). We performed five runs at each value of K (1–6: number of subpopulations) with 100,000 burn-in and 100,000 Markov Chain Monte Carlo replicates after burn-in. To assess the patterns of genetic relationships, three-dimensional factorial correspondence analysis (FCA) plots were generated based on allele frequency data, using the program GENETIX 4.05 (Belkhir et al., 1996–2004). Observed (H_o) and expected (H_e) heterozygosities and pairwise F_{ST} values were calculated using ARLEQUIN 3.1.1 (Excoffier et al., 2005). Departures from Hardy-Weinberg equilibrium and linkage equilibrium were tested for each microsatellite locus using ARLEQUIN. Allelic richness was calculated using FSTAT 2.9.3 (Goudet, 2001).

RESULTS

Characteristics of selected microsatellite loci

Of the 53 microsatellite loci tested, 33 loci (62%) were PCR-amplified from the Blakiston's fish owl genome, and eight loci were found to be polymorphic (Table 2): loci Oe053, Oe128 and Oe129 from *Otus elegans* (Hsu et al.,

Table 2. Charactrization of eight microsatellite loci on 120 Blakiston's fish owls.

Locus*	Primer sequence (5'-3')**	Annealing temperature (°C)	Repeat motif	Range of allele size (bp)	No. of alleles	Heterozygosities	
						Ho	*He*
Oe053	F: CTCTGCATCTTAACGCACAGGAC R: CCTCCAAGTGGACAGGAAAAGC	52	$(GATA)_n$	217–241	7	0.655	0.769***
Oe128	F: CGTTGTAAATGATGAATCGCCTAGTGC R: ATGCATGTATACATACAAACCTGG	52	$(GATA)_n$	296–300	2	0.052	0.067
Oe129	F: GTCACCTCTTGACATCCGAGTAGC R: GCTAAGAGTCCATTTGCCCATCTG	52	$(GATA)_n$	246–254	3	0.492	0.496
13D8	F: CTATATCATATCGTTGCTTCCA R: CATCTGCGGTACATCATATAA	52	$(GATA)_n$	191–195	2	0.513	0.466
4E10.2	F: GTCTTCTGTAGGTCTGGG R: GAGGCTATGCTGCAAATG	52	$(ATTTT)_n$	205–235	7	0.742	0.769***
FEPO5	F: GGAGATGAATCAGCAAACCTGT R: AAATTTAAACTAGCCTAGAGTCAGC	52	$(GATA)_n$	363–383	6	0.608	0.619
FEPO43	F: CGTGAAGGTAAGAGGAGCTGG R: GGAGGGAGCCTGGAAATGG	60	$(GGAT)_n(GATA)_n$	158–178	3	0.508	0.597
BUOW-BM4-A09#	F: GCACTTAGGGACATGGTTTAGTGG R: TCCTATGAAGACCCTCAAGCCC	60	$(GATA)_n$	340–348	3	♂: 0.493 ♀: 0.000	–

* Oe053 and Oe128 from Hsu et al. (2003); Oe129 from Hsu et al. (2006); 13D8 and 4E10.2 from Thode et al. (2002); FEPO5 and FEPO43 from Proudfoot et al. (2005); BUOW-BM4-A09 from Faircloth et al. (2010).

** F, forward; R, reverse.

*** Significant (*P*-value < 0.05) in the test for departure from Hardy-Weinberg equilibrium.

That this locus is sex-linked (located on Z chromosome) was found in the present study.

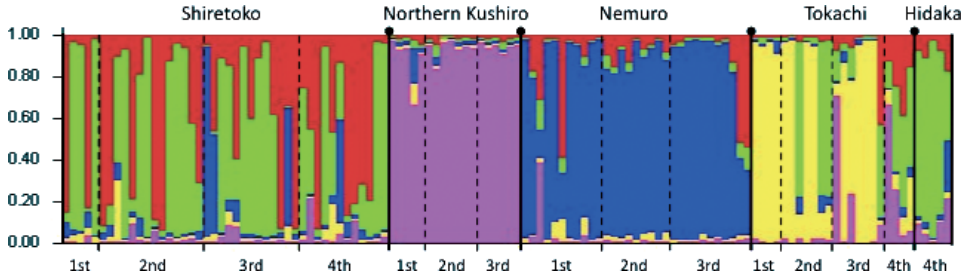

Fig. 2. Bar plot assignments of the individuals by STRUCTURE analysis of microsatellite genotypes. The number of genetic clusters was defined as five (K = 5). One vertical line for each individual is partitioned into five colored segments that represent the individual's estimated assignment probabilities to each cluster. The individuals of the five geographical subpopulations are ranged along sampling periods: 1st, 1986–1989; 2nd, 1990–1993; 3rd, 1997–1999; 4th, 2009–2010.

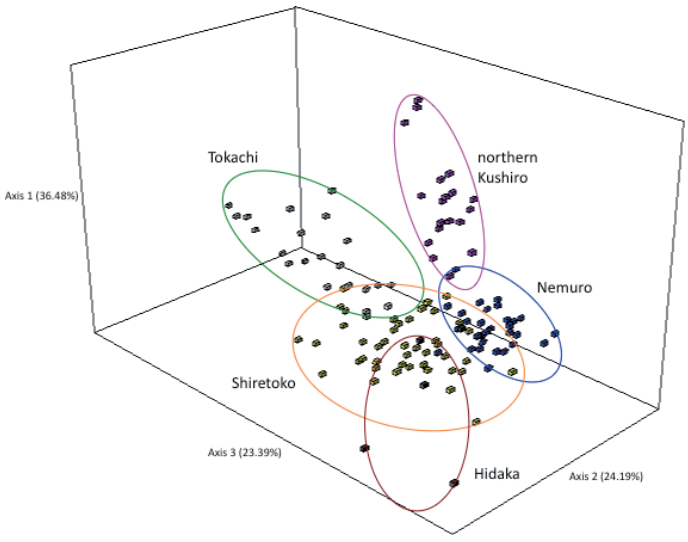

Fig. 3. Results of FCA analysis showing genetic differentiation based on microsatellite allelic frequency for individuals: yellow cubes for the Shiretoko subpopulation; purple for northern Kushiro; blue for Nemuro; white for Tokachi; black for Hidaka. One cube indicates one individual. Most individuals from each subpopulation are clustered into one group shown as a circle.

2003, 2006); 13D8 and 4E10.2 from *Strix occidentali* (Thode et al., 2002); FEPO5 and FEPO43 from *Glaucidium brasilianum* (Proudfoot et al., 2005); and BUOW-BM4-A09 from *Athene cunicularia* (Faircloth et al., 2010). The microsatellite regions of alleles consisted of four- or five-nucleotide motifs in the Blakiston's fish owls, in concordance with the other owl species above (Table 2). These markers were found to be polymorphic enough for individual test and population genetic studies of the Blakiston's fish owl.

In the analysis of locus BUOW-BM4-A09, all heterozygotes were males, and no heterozygotes were found in females (Table 2). In addition, genotyping familial individuals in some pedigrees (data not shown) showed the sex-linked inheritance pattern of the alleles. The results indicate that locus BUOW-BM4-A09 is located on the Z chromosome, whereas this locus was originally reported to be autosomal in *Athene cunicularia* by Faircloth et al. (2010); therefore, the data of BUOW-BM4-A09 were excluded, and those of the other seven loci, which were all autosomal, were used in the following analysis.

Genetic analysis of the Hokkaido population of the Blakiston's fish owl

Table 2 shows the genetic characteristics of the selected eight loci in the Hokkaido population. The number of alleles found in the Blakiston's fish owls ranged from two to seven. Observed heterozygosities (H_o) of all owls were from 0.052 to 0.742, and expected heterozygosities (H_e) were from 0.067 to 0.769: H_o and H_e at locus Oe128 were the lowest as the polymorphism at the locus was found only in the Shiretoko subpopulation. All geographical subpopulations had one or two private alleles: two private alleles (at locus Oe128 and FEPO43) in the Shiretoko subpopulation, one (at 4E10.2) in northern Kushiro, one (at Oe053) in Nemuro, two (at 4E10.2 and BUOW-BM4-A09) in Tokachi, and one (at Oe053) in Hidaka. The analysis of all owls showed statistically significant departure from Hardy-Weinberg equilibrium at two loci (Oe053 and 4E10.2) (Table 2).

STRUCTURE analysis showed $K = 5$ as the highest log-likelihood value. This indicated that the most optimal number of genetical cluster of the Blakiston's fish owl in Hokkaido was five. The five clusters mainly corresponded to the geographical subpopulations (Fig. 2). Figure 2 shows that 75% of the owls from the Shiretoko subpopulation were assigned to multiple (mainly two) clusters with more than 70% probabilities. In addition, 94% of the owls from the northern Kushiro subpopulation and 87% from the Nemuro were assigned to unique clusters with more than 70% probabilities, respectively. From the Tokachi subpopulation, all of the owls in the first period were assigned to a unique cluster (yellow in Fig. 2), but half of the owls in the second and third periods and all of the owls in the fourth period were not assigned to the cluster. From the Hidaka subpopulation, 80% of the owls were assigned to a single cluster (green in Fig. 2). FCA analysis showed that individuals from each subpopulation were clustered into one group, and the Shiretoko subpopulation had intermediate characteristics between others (Fig. 3).

In the first, second, and third periods, all pairwise F_{ST} values between the geographical subpopulations were significantly high, but the values between the Shiretoko subpopulation and other subpopulations were lower (Tables 3a, 3b and 3c). In the fourth period, the pairwise F_{ST} value between the Shiretoko and the Tokachi was too low to be significant, while that between the Shiretoko and the Hidaka was significant (Table 3d). Pairwise F_{ST} values were not correlated ($r^2 = 0.006$) with the geographical distances between

Table 3a. Pairwise F_{ST} between the subpopulations in the first period: 1986–1989.

	Shiretoko	Northern Kushiro	Nemuro	Tokachi
Shiretoko		+	+	+
Northern Kushiro	0.138		+	+
Nemuro	0.101	0.165		+
Tokachi	0.138	0.263	0.190	

+, significant (P-value < 0.05).

Table 3b. Pairwise F_{ST} between the subpopulations in the second period: 1990–1993.

	Shiretoko	Northern Kushiro	Nemuro	Tokachi
Shiretoko		+	+	+
Northern Kushiro	0.148		+	+
Nemuro	0.125	0.218		+
Tokachi	0.209	0.200	0.225	

+, significant (P-value < 0.05).

Table 3c. Pairwise F_{ST} between the subpopulations in the third period: 1997–1999.

	Shiretoko	Northern Kushiro	Nemuro	Tokachi
Shiretoko		+	+	+
Northern Kushiro	0.143		+	+
Nemuro	0.113	0.239		+
Tokachi	0.100	0.195	0.200	

+, significant (P-value < 0.05).

Table 3d. Pairwise F_{ST} between the subpopulations in the fourth period: 2009–2010.

	Shiretoko	Tokachi	Hidaka
Shiretoko		–	+
Tokachi	0.007		+
Hidaka	0.149	0.196	

+, significant (P-value < 0.05).

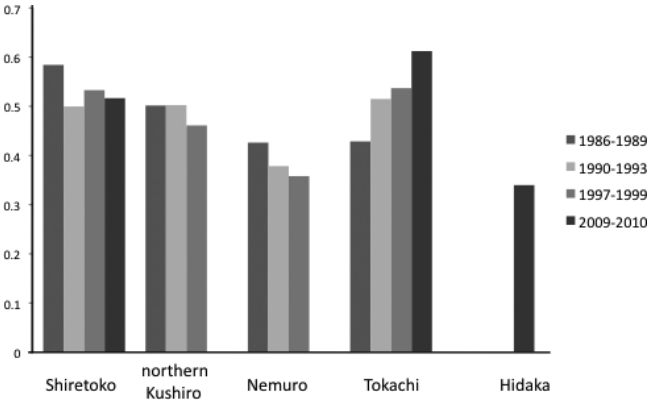

Fig. 4. Comparison of the average expected heterozygosities in the five subpopulations among four periods: 1986–1989, 1990–1993, 1997–1999 and 2009–2010.

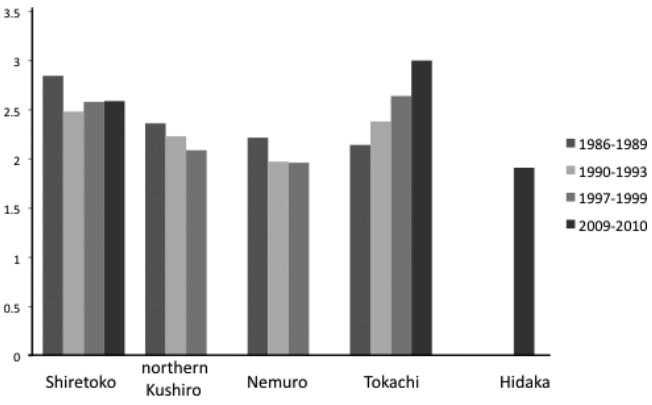

Fig. 5. Comparison of the values of the allelic richness in the five subpopulations among four periods: 1986–1989, 1990–1993, 1997–1999 and 2009–2010.

the sampling locations.

Average expected heterozygosities (H_e) of the five subpopulations in the four periods were from 0.34 to 0.61, and allelic richness ranged from 1.9 to 3.0: the highest values of the average H_e and allelic richness were found in the Tokachi subpopulation in the fourth period, and the lowest values were in the Hidaka subpopulation in the fourth period (Fig. 5). Comparison of the average H_e and allelic richness among the sampling periods showed gradual decrease in the Shiretoko, northern Kushiro and Nemuro subpopulations (Figs. 4, 5), but increase in the Tokachi subpopulation. We excluded the Hidaka subpopulation from this comparison due to lack of sampling periods.

DISCUSSION

Characteristics of the sex-linked microsatellite locus

The present study revealed that locus BUOW-BM4-A09 is located on the Z chromosome in Blakiston's fish owl, although this locus was originally reported to be one of the autosomal microsatellite markers of the western burrowing owl (*Athene cunicularia*) by Faircloth et al. (2010). Faircloth et al. (2010) reported that H_o was lower than H_e of BUOW-BM4-A09 in *A. cunicularia*, although the analyzed population did not significantly depart from Hardy-Weinberg equilibrium. These data were not discordant with locus BUOW-BM4-A09 being located on the Z chromosome, also in *A. cunicularia*. This locus represents a potentially invaluable genetic marker for future familial and individual tests of the Blakiston's fish owl.

Population structure of the Blakiston's fish owls in Hokkaido

The departure from Hardy-Weinberg equilibrium of all owls at two microsatellite loci (Oe053 and 4E10.2) suggests that Blakiston's fish owls have been geographically isolated into multiple subpopulations. Table 3a, 3b, 3c and 3d show that pairwise F_{ST} values between the subpopulations in each period were statistically significant, except for combination between the Shiretoko and Tokachi subpopulations in the fourth period as discussed later. Each subpopulation had one or two unique alleles. In addition, STRUCTURE analysis divided the owls into five genetic clusters, which mostly corresponded to the geographical subpopulations (Fig. 2), and FCA analysis also supported such grouping (Fig. 3). These results show that the five subpopulations are genetically differentiated from each other. No correlations between F_{ST} values and geographical distances of the subpopulations indicate that the genetic differentiation among subpopulations was caused by genetic drift and/or inbreeding within each subpopulation. Artificial fragmentation of the habitats may have been responsible for the isolation of the subpopulations.

By STRUCTURE analysis, the owls from the northern Kushiro, Nemuro and Hidaka subpopulations were assigned to nearly isolated single clusters, respectively (Fig. 2), indicating that these subpopulations consist of distinct genetic lineages. Although owls from the Hidaka subpopulation belonged to the cluster (green in Fig. 2) common to parts of the Shiretoko and Tokachi subpopulations, they shared one private allele (at Oe053), and F_{ST} values between the Hidaka and other subpopulations were significantly high (Table 3d). This suggests that the Hidaka subpopulation may have been isolated. By contrast, the Shiretoko subpopulation consisted of the owls assigned to multiple clusters, FCA analysis showed intermediate characteristics of the Shiretoko subpopulation between others. It is possible, therefore, that the Shiretoko subpopulation, which has the largest population size, kept its genetic variation and served as a source of individuals in Hokkaido. In the Tokachi subpopulation, one immigrant from the northern Kushiro subpopulation was found to have bred after the second period (T. Takenaka, unpublished data). STRUCTURE analysis showed some owls from the Tokachi subpopulation in the third and fourth periods assigned to the cluster unique to the northern Kushiro subpopulation (purple in Fig. 2). In addition, Fig. 2 shows that the owls from the Tokachi subpopulation in the fourth period had genetic characteristics more similar to those of the Shiretoko subpopulation rather than the Tokachi itself in the first to third periods. Pairwise F_{ST} values between the Tokachi and Shiretoko subpopulations in the fourth period were too low (Table 3d). These results further support the immigration of the owls to the Tokachi subpopulation from the Shiretoko subpopulation, and that even low levels of immigration could have contributed to the change of the genetic structure of the Blakiston's fish owl in Hokkaido.

Genetic diversity in the Hokkaido population of the Blakiston's fish owl

The present study revealed that the Blakiston's fish owl in Hokkaido had lower genetic diversity than the previously reported data of other owl species: for example, the average of H_e values was 0.54 in all owls in Hokkaido. Similar to the data of the present study, that of H_e was 0.60 in the Scandinavian population of the eagle owl (*Bubo bubo*), which passed a bottleneck below a few hundreds of individuals (Isaksson et al., 2002). The Lanyu scops owl (*Otus elegans botelensis*) living only on Lanyu Island in southeastern Taiwan and listed as endangered in the Red Date Book, has 0.80 for the average H_e (Hsu et al., 2003).

Populations that have passed a bottleneck often lose genetic diversity. Bellinger et al. (2003) investigated microsatellites in the great prairie chicken (*Tympanuchus cupido*) in Wisconsin, the United States, which had declined and fragmented their habitat over the last century, and reported loss of 29% alleles, a significant decline of allelic richness (from 9.03 to 6.55) and heterozygosity (from 0.71 to 0.56) over the last five decades. In the present study, in Blakiston's fish owl in Hokkaido, 6% (2/31) of alleles found in the first and second periods were lost in the following periods. The average H_e (Fig. 4) and allelic richness (Fig. 5) has decreased for the last decades in the Shiretoko, northern Kushiro and Nemuro subpopulations. This suggests that genetic diversity of the closed subpopulations declined due to genetic drift and/or inbreeding. The lowest values of the average H_e and allelic richness found in the Hidaka subpopulation may have resulted from isolation from other subpopulations and inbreeding.

In the Tokachi subpopulation, however, values of the allelic richness and the average H_e had greatly increased. Keller et al. (2001) showed that a natural population that experiences a bottleneck rapidly recovers allelic variation

and heterozygosity due to low levels of immigration. STRUCTURE analysis showed immigrations to the Tokachi subpopulation from the northern Kushiro (purple in Fig. 2) and possibly the Shiretoko subpopulations (red and green in Fig. 2) after the early periods. This indicates that low levels of immigration could have increased genetic diversity in the small population of Tokachi.

Based on research into many threatened bird species, Heber et al. (2010) reported that population bottlenecks increase hatching failure due to loss of genetic diversity and inbreeding, particularly when the population experienced severe bottlenecks below 100–150 individuals. Inbreeding depression often remarkably affects birth weight, survival, reproduction and resistance to disease, predation and environmental stress (Keller and Waller, 2002). Blakiston's fish owl in Hokkaido experienced a bottleneck below 100 individuals (Brazil and Yamamoto, 1989; Takenaka, 1998), and some inbreeding pairs were observed (Takenaka, 1998; Yamamoto, 1999; Hayashi, 2009). Further genetic analysis with ecological information provides insights to conservation management of this owl, such as preservation of natural populations as well as breeding and reintroduction plans of captured individuals.

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