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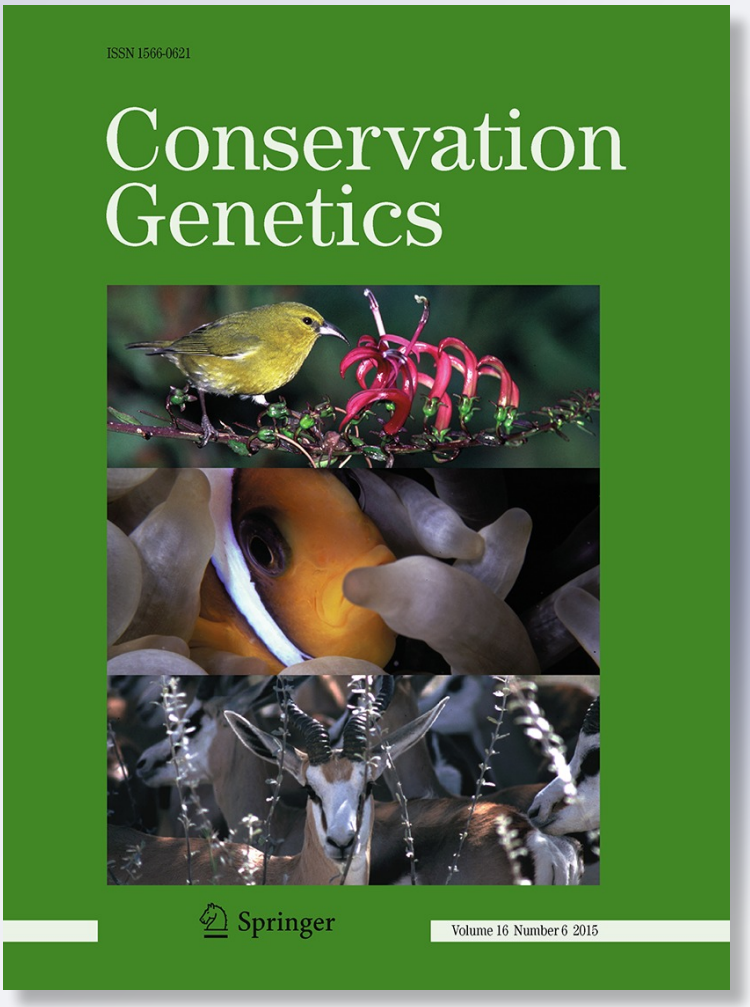

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RESEARCH ARTICLE

Genetic structure of the endangered red-crowned cranes in Hokkaido, Japan and conservation implications

Taro Sugimoto[1] · Osamu Hasegawa[2] · Noriko Azuma[3] · Hiroyuki Masatomi[4] · Fumio Sato[5] · Fumio Matsumoto[6] · Yoshiyuki Masatomi[7] · Hiroe Izumi[8] · Syuiti Abe[9]

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Abstract The red-crowned crane in Japan was once considered extinct due to hunting and habitat destruction in late nineteenth century; however, in 1926, a small group of cranes was rediscovered in the Kushiro Mire in eastern Hokkaido. Since then, various conservation efforts, including artificial feeding during winter, hunting prohibition, and habitat conservation, have increased the population size to >1400 by 2012. Despite such a successful population recovery, the genetic characteristics of the population have not been fully explored. To ensure the long-term persistence and evolutionary potential of cranes, accurate knowledge of the spatial distribution of genetic variation and its underlying causes are necessary. We assessed their genetic structure using 12 polymorphic microsatellite loci and inferred the mechanisms shaping the observed structure. Among the three regional groups in Hokkaido, we found generally low pairwise F_{ST} values and no significant differences in genetic diversity, probably because of the population expansion in the recent past. In contrast, spatial autocorrelation analysis revealed a significant positive kinship at the short distance (0–15 km) and negative kinship at the long distance (155–205 km), showing a pattern of isolation by distance. The presence of isolation by distance on a small spatial scale despite the species' strong flight ability is probably explained by the recolonization process and restricted dispersal due to natal philopatry in a non-equilibrium condition. Cranes in Hokkaido do not appear to be a panmictic (random mating) population; however they can be considered a single population without genetic discontinuity (i.e. a single management unit). Our findings confirm the importance of considering natal philopatry when developing management strategies such as dispersing cranes into unoccupied areas.

Keywords Conservation · Genetic structure · *Grus japonensis* · Isolation by distance · Red-crowned crane · Philopatry

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Introduction

Understanding the genetic structure and its underlying causes is essential for determining appropriate management interventions. Life history and behavioral traits such as migration, dispersal patterns (e.g. philopatry), and the degree of tolerance to geographic or anthropogenic barriers affect the spatial distribution of genetic variation, sometimes leading to significant genetic differentiation. Genetic structure analysis provides vital insights into such species-

specific biological characteristics (Frantz et al. 2012), upon which an effective management strategy can be developed for ensuring the long-term persistence and evolutionary potential of the endangered species in question.

The red-crowned crane (Japanese crane or Tancho), *Grus japonensis*, one of the most endangered crane species (Meine and Archibald 1996), is distributed in northeastern Asia. There are two populations, thought to have no genetic exchange between them: a continental population (breeding in China and Russia and wintering in the Korean peninsula and east coast of China) and a non-migratory population on the island of Hokkaido, Japan (Masatomi 2000). Cranes in Japan used to be observed in mainland Honshu as well, most of which were likely to be migrants from northern Japan. However, hunting and habitat destruction greatly reduced their distribution by the late nineteenth century, and eventually, the red-crowned cranes were considered extinct in Japan. However, in 1926, a small group of cranes (approximately 20 individuals) was rediscovered in the Kushiro Mire in Hokkaido (Masatomi 2000). Given the limited capability for conducting extensive field surveys at the time, it is possible that there were several tens of other individuals in Hokkaido (Masatomi et al. 2007). Since then, conservation efforts, including habitat protection and hunting prohibition, have been initiated (Masatomi 2000). In particular, artificial feeding during winter initiated in the 1950s has greatly contributed to population recovery from <100 individuals in 1952 to >1400 by 2012 (Masatomi 2000; Masatomi et al. 2014).

Cranes currently breed across the eastern part of Hokkaido; however, how their geographical distribution has expanded from its state in 1926 to its current range is unclear because of the lack of reliable field surveys during the early stage of the population recovery. In 1973, the first wide-range aerial survey conducted during the breeding season found at least 28, 28, and 2 breeding pairs in Nemuro, Kushiro, and Tokachi regions, respectively (Fig. 1, Masatomi 2000). The number of breeding pairs in Tokachi has been the smallest; however, some information indicates that cranes could have been already established there during the 1940s (Masatomi 2000). In Nemuro, local sources suggest that cranes may have persisted there since the 1920s (Masatomi 2000). Recent surveys observed population recovery in all three regions. In 1999, an aerial survey recorded 84, 104, and 26 breeding pairs in Nemuro, Kushiro, and Tokachi regions, respectively (Masatomi 2000).

Cranes in Hokkaido are non-migratory; therefore, they mainly stay in the eastern part of Hokkaido during the non-breeding season as well. During fall, cranes start moving from their breeding sites to wintering sites. It is obvious that distances between the two sites are short, as this movement occurs within the eastern part of Hokkaido.

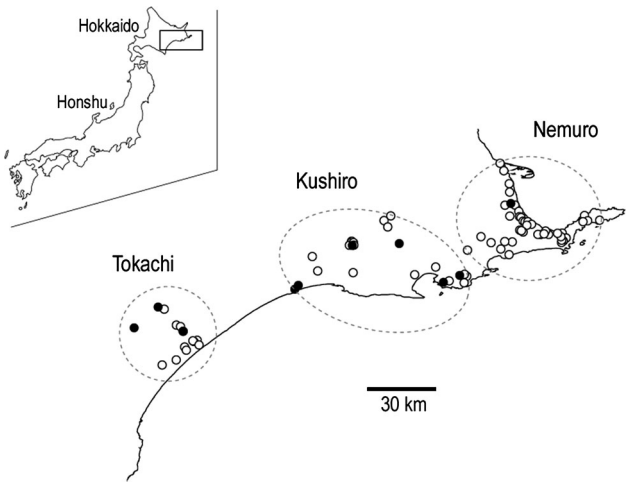

Fig. 1 Location of samples collected within the three regions Nemuro, Kushiro, and Tokachi in eastern Hokkaido. *White circles* indicate samples with haplotype Gj2, and *black circles* indicate those with haplotype Gj1, according to Hasegawa et al. (1999)

Most birds congregate around five major feeding sites in the Kushiro region (Masatomi et al. 2012), and they stay over winter depending mostly on foods (e.g. corns) supplied by people.

Although the genetic consequences of population reduction from the late 19th to early 20th centuries remain unknown, the genetic diversity of cranes in Hokkaido has been reported to be lower than that in the continental population, as inferred from nucleotide sequence analysis of mitochondrial DNA (mtDNA; Hasegawa et al. 1999; Miura et al. 2013) and microsatellite DNA variation (Hasegawa et al. 2000). Despite the reportedly low genetic diversity, the observed population growth, along with the limited accidental-death rate, suggested that the immediate extinction risk for the Hokkaido population was low (Masatomi et al. 2007). Therefore, from a demographic point of view, conservation efforts for more than half a century have greatly improved the situation of cranes in Japan. Nonetheless, accurate knowledge of the spatial distribution of genetic variation and its underlying causes is necessary for securing the long-term persistence and evolutionary potential of the red-crowned cranes. Previous genetic studies on cranes in Hokkaido mainly focused on the mtDNA sequence diversity (Hasegawa et al. 1999; Miura et al. 2013); hence, little has been done to explore the population genetic structure.

The present study aims to assess the genetic structure of the red-crowned cranes using the samples collected from Hokkaido population across their entire breeding range. Our findings will be useful for developing conservation and management strategies for cranes in Hokkaido and will be relevant to the scarcely studied cranes of the continental population. Moreover, investigation of the genetic

variability of the wild cranes will provide important reference data to assist in the effective genetic management of the captive cranes in Japan.

Materials and methods

Sampling and DNA analyses

Blood samples were collected from captured juvenile cranes within the framework of the annual banding project operated by the Ministry of the Environment normally from June to July, when juveniles are flightless (at the age of approximately 1.5–3.0 months) and remain with their parents around the nests. The locations where the juveniles were captured were recorded. In this study, we used 80 blood samples collected from 1995 to 2006 across the entire breeding range in eastern Hokkaido (approximately 70 % of them were collected between 1999 and 2005). Because an adult pair bond usually lasts for years until the partner dies, and because a breeding pair shows strong fidelity to the nesting territory (Masatomi 2000), all the samples were derived from different nest sites to avoid the inclusion of closely related individuals. The collected samples were grouped to Nemuro (n = 42), Kushiro (n = 24), and Tokachi (n = 14), according to the geographic locations of the juvenile capture sites (Fig. 1). This allows us to assess the genetic differences between Kushiro and the other regions where other genetically distinct groups may have persisted when the Kushiro group was rediscovered. A few nests were found in northern Hokkaido in recent years (Masatomi et al. 2004), but their number is too small to be considered a functional population; thus, we did not include them in this study.

The blood samples were preserved in 100 % ethanol and stored at −20 °C until DNA extraction. DNA was extracted using the QIAamp DNA mini kit (Qiagen, Tokyo, Japan) following the manufacturer's protocol. Eighteen microsatellite loci were used for multilocus genotyping (Gj-M8, Gj-M11a, Gj-M13, Gj-M15, Gj-M34, Gj-M40, and Gj-M48b in Hasegawa et al. 2000; Gram6, Gram11, Gram17, Gram20, Gram22, Gram24, Gram25, Gram32a, Gram41, Gram42, and Gram45 in Jones et al. 2010). PCR condition and cycling profile for each locus followed Hasegawa et al. (2000) and Jones et al. (2010). The PCR products were analyzed on an ABI PRISM® 3100 genetic analyzer (Applied Biosystems, Foster City, California) and the genotyping data were collected using the program GeneScan (Applied Biosystems). We sequenced a part of the mtDNA control region (418 bp) to visualize the spatial distribution of mtDNA haplotypes in Hokkaido. PCR amplification and sequencing were performed following Hasegawa et al. (1999).

Data analyses

The observed number of alleles per locus (A) and both expected heterozygosity (H_E) and observed heterozygosity (H_O) were calculated using the program GENEPOP 3.4 (Raymond and Rousset 1995). Allelic richness (AR), which was corrected by the smallest sample size, was calculated by the program FSTAT ver. 2.9.3.2 (Goudet 2001). Linkage disequilibrium between all pairs of loci and Hardy–Weinberg equilibrium for each locus in each group and overall were assessed by the program GENEPOP 3.4, where the sequential Bonferroni correction was used for multiple tests (Rice 1989). The program MICRO-CHECKER (Van Oosterhout et al. 2004) was used to check the occurrence of null alleles and large allele dropout.

We assessed the genetic structure by several approaches. First, the degree of genetic differentiation between each pair of groups was evaluated by the fixation index, F_{ST} (θ; Weir and Cockerham 1984), and tested by the log-likelihood G-test using the program FSTAT ver. 2.9.3.2. Overall F_{ST} was estimated by the analysis of molecular variance and tested for a significant difference from zero by 10,100 permutations using the program Arlequin ver. 3.11 (Excoffier et al. 2005). Differences in the genetic diversity (i.e. H_E and AR) among the three groups were assessed by the analysis of variance (ANOVA), where the loci served as error. Prior to ANOVA, the data were confirmed not to violate the assumption of normality and equality of variance by Shapilo–Wilk test and Bartlett's test, respectively. To test for a pattern of isolation by distance as a potential cause of the genetic structure, we conducted spatial autocorrelation analysis. We used Nason's estimator of kinship coefficient (F_{ij}; Loiselle et al. 1995) as a measure of pairwise genetic similarity between individuals. Negative values are possible and are interpreted as individuals being less similar than expected by chance. We calculated the average F_{ij} between pairs of individuals, whose geographic separation falls within a specified distance interval, by the program SPAGeDi ver. 1.4 (Hardy and Vekemans 2002). The seven distance intervals were defined by maintaining similar numbers of pairwise comparisons. The significance of the average kinship coefficients in each distance interval was tested by permuting the spatial locations of individuals 10,000 times, which is equivalent to a Mantel test (Hardy and Vekemans 2002), using the program SPAGeDi ver. 1.4. We repeated the analysis using another two distance intervals (five and ten intervals) to ensure consistency of the results. If a pattern of isolation by distance is present, the average kinship coefficient will decrease as the distance interval increases. Finally, Bayesian clustering analysis implemented in the program STRUCTURE ver. 2.3.3 (Pritchard et al. 2000; Falush et al. 2003) was used to infer the genetic structure without an a priori definition of

groups. We used the admixture model with correlated allele frequencies. Five independent runs were performed for each K value (number of clusters) from 1 to 6 with 200,000 MCMC steps and 100,000 burn-in steps. We determined the most likely number of clusters based on the average log-likelihood value across the 5 runs.

Results

Of the 18 loci analyzed, 12 were polymorphic (Table 1). Significant linkage disequilibrium was not detected in any combination of the 12 loci. A significant deviation from Hardy–Weinberg equilibrium was not detected in any of the three groups and the overall. Furthermore, the program MICROCHEKER did not detect any evidence of null alleles or large allele dropout; therefore, we used the 12 polymorphic loci for genetic structure analysis.

The overall F_{ST} value among the three groups was 0.022, which is low but significantly different from zero ($P < 0.001$). Pairwise F_{ST} values were in the range of 0.0200–0.0287, and a significant differentiation was detected between the most distant groups, i.e. Nemuro and Tokachi ($P = 0.001$, Table 2). The genetic diversity was not significantly different among the three groups ($F_{2,22} = 1.574$, $P = 0.230$ for H_E; $F_{2,22} = 0.606$, $P = 0.555$ for AR). A pattern of isolation by distance was detected by the spatial autocorrelation analysis, where a significant positive autocorrelation was observed in the first distance interval (0–15 km, $P = 0.016$), while a significant negative autocorrelation in the last distance interval (155–205 km, $P = 0.010$) (Fig. 2). The decline in average F_{ij} values with distance indicated a pattern of isolation by distance. Modification of the distance intervals from seven to five or ten did not significantly change the results (data not shown). Although there was weak but significant genetic differentiation according to the F_{ST} values, the Bayesian clustering analysis indicated that the most likely K was one.

Sequencing a part of the mtDNA control region revealed that there were only two haplotypes, Gj1 and Gj2, with predominance of the latter (88 %) (Fig. 1). This result was consistent with the previously reported findings (Hasegawa et al. 1999). It appears that the two haplotypes were distributed across the whole range without any clear spatial pattern (Fig. 1).

Table 1 Genetic variation at 12 microsatellite loci of 80 cranes in Hokkaido

Locus	*A*	H_O	H_E
Gj-M8	2	0.29	0.28
Gj-M11a	4	0.65	0.64
Gj-M13	3	0.66	0.61
Gj-M15	3	0.45	0.48
Gj-M34	2	0.55	0.50
Gram6	4	0.61	0.59
Gram11	2	0.03	0.02
Gram17	5	0.69	0.66
Gram20	4	0.78	0.72
Gram24	2	0.13	0.12
Gram25	2	0.05	0.07
Gram32a	2	0.24	0.25
Average	2.92	0.43	0.41

A observed number of alleles, H_O observed heterozygosity, H_E expected heterozygosity

Table 2 Pairwise F_{ST} values (θ, below the diagonal) and *P* values from the G-test for genetic differentiation (above the diagonal) among the three groups (*NM* Nemuro, *KS* Kushiro, and *TK* Tokachi)

	NM	KS	TK
NM	–	0.0187	**0.0013**
KS	0.0200	–	0.0217
TK	0.0287	0.0207	–

Bold indicates a significant *P* value after the sequential Bonferroni correction for multiple tests

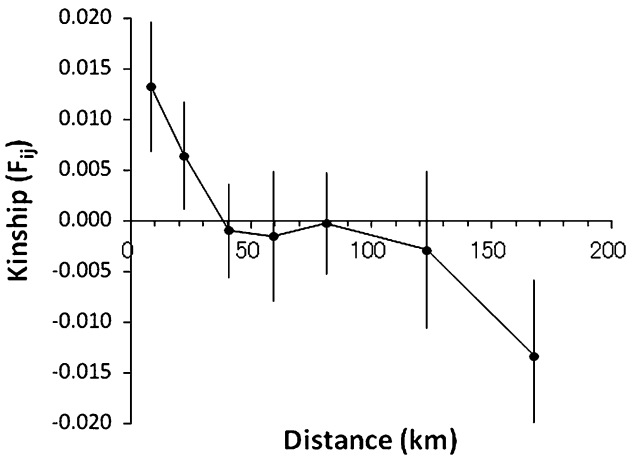

Fig. 2 Spatial autocorrelation among individual kinship estimates (F_{ij}, Loiselle et al. 1995) in seven distance classes (0–15, 15–30, 30–50, 50–70, 70–95, 95–155, and 155–205 km). CIs for F_{ij} are twice the SE obtained by jackknifing over the loci. The data points were plotted at the average pairwise distance for each distance class

Discussion

The group-level analyses found low pairwise F_{ST} values and no significant differences in genetic diversity, probably because of the population expansion in the recent past; however, the spatial autocorrelation analysis indicates a pattern of isolation by distance. This finding is unexpected

for the following two reasons: First, cranes are capable of flying long distances as demonstrated by the migratory cranes in the continental population. It is commonly recognized that species in the crane (Gruidae) family are capable of flying hundreds of kilometers in a day (e.g. Melvin and Temple 1982). This means that it must be easy for them to fly more than 204 km (the greatest distance between any two nest sites in this study), particularly in the current range that contains few geographic barriers such as high mountains. This may result in a genetically homogeneous population. Second, most individuals (e.g. approximately 96 % in the winter of 1999; Masatomi et al. 2000) congregated at the five major feeding sites in the Kushiro region for wintering, and some were observed to use multiple feeding sites within a winter (e.g. Masatomi et al. 2012, 2014). It is thought that pair formation generally occurs at these wintering grounds; thus, this situation may promote random mating throughout the distribution range.

The cause of the isolation by distance observed herein is probably related to both the recolonization process and some behavioral trait of cranes. The dispersal pattern of cranes is largely unknown, but limited observational data are suggestive of natal philopatry in Hokkaido, with males being more philopatric than females (Masatomi 2000). Other crane species, e.g. Sandhill cranes (*G. canadensis*), also show philopatric behavior (Nesbitt et al. 2002). This characteristic probably causes restricted dispersal despite their strong flight ability. Hence, although we are unsure how their distribution has expanded, recolonization through population recovery and restricted dispersal due to natal philopatry are probably the main causes of the pattern of isolation by distance. In addition, a near-linear distribution of cranes in Hokkaido (Fig. 1), which appears being in one-dimensional habitats, may contribute to the observed genetic structure, because restricted dispersal tends to cause more apparent patterns of isolation by distance in one-dimensional habitats rather than two-dimensional habitats (Slatkin 1993). We cannot rule out the possibility of secondary contact between isolated populations to explain the observed genetic structure. Nonetheless, this possibility is unlikely because of the low overall F_{ST} value and the absence of significant differences in genetic diversity among the groups.

We considered the genetic structure of cranes in Hokkaido as the pattern of isolation by distance; however, this does not mean the population is under migration–drift equilibrium. This pattern has probably arisen in a non-equilibrium condition given the recent population expansion. This notion may be consistent with the analytical theory that restricted dispersal can lead to isolation by distance on a smaller spatial scale in a non-equilibrium population (Slatkin 1993). Overall, our results suggest that cranes in Hokkaido should be considered a single population without genetic discontinuity (i.e. a single management unit). It is reasonable to attribute the significant genetic differentiation observed between Nemuro and Tokachi to a byproduct of the forces causing this pattern of isolation by distance (Meirmans 2012).

Because there have been only a few studies on the genetic structure of crane species (Jones et al. 2005, 2006), it is uncertain that our study illustrates a characteristic of the genetic structure of non-migratory crane species; however, similar results can be seen in other bird species such as seabirds (Friesen et al. 2007). Seabirds are capable of flying hundreds or thousands of kilometers on a single foraging trip (Hyrenbach et al. 2002; Croxall et al. 2005) and probably have similar or even stronger flight abilities than crane species; however, some species exhibit a pattern of isolation by distance due to natal philopatry (e.g. Northern fulmars *Fulmarus glacialis*, Burg et al. 2003). Northern fulmars establish their nests in or near natal colonies and their distribution range increases in a stepping stone manner (Burg et al. 2003). Empirical studies on seabirds (Friesen et al. 2007) and our study suggest that strong flight abilities alone cannot predict patterns of genetic structure.

It is important to note that cranes in Hokkaido are a recently recovered population and the number of individuals still increases (Masatomi et al. 2014); therefore, the observed genetic structure is probably not stable. Jones et al. (2005) investigated genetic structure of migratory sandhill cranes and found low genetic differentiation among populations on a much larger geographical scale than our study. They suggested that male-mediated gene flow mitigates genetic differentiation. Observational data, however, indicates that males are more philopatric than females (Nesbitt et al. 2002), and thus, we assume that a low frequency of long distance dispersal by males plays an important role for shaping the genetic structure. In Hokkaido, the current extent of male-mediated gene flow is unclear but is probably infrequent enough that it does not homogenize the genetic structure. However, if available habitat for breeding is reduced due to the population recovery, the frequency of long distance dispersal by males may increase, which could lead to little genetic structure.

One of the important management goals for the red-crowned cranes in Hokkaido is to expand the current restricted distribution because a major threat for them is infectious diseases, given the genetically single cluster with low genetic diversity and congregation at restricted wintering grounds (i.e. artificial feeding sites). The avian influenza virus (H5N1) was detected in a dead Whooper swan (*Cygnus cygnus*) in eastern Hokkaido in 2008, but fortunately there have been no reports that this virus can cause a serious or fatal infection in cranes. In 2013, a management strategy intending to establish additional

breeding and wintering grounds was announced by the Ministry of the Environment, and the ultimate aim was to extend their distribution to their former habitats on the Honshu Island. The successful establishment of several wintering grounds will effectively mitigate the risk of disease spread.

Achieving this goal implies that additional artificial feeding sites will be needed in other areas because of the lack of appropriate natural foraging sites during winter; however, this is not concordant with another important management goal of enabling cranes to survive without artificial feeding during winter. The artificial feeding, initiated in the 1950s, has greatly contributed to population recovery; however, now wildlife management must consider how this population will persist without human intervention (Masatomi and Masatomi 2009). This task will be a significant challenge because extensive and long-term management efforts are required such as restoration of the wetland ecosystem, creation of natural foraging sites, and development of adequate measures for the prevention of agricultural damage caused by cranes (Masatomi and Masatomi 2009; Masatomi et al. 2007). Dispersing cranes into unoccupied areas along with improving and restoring their foraging habitat will be crucial for the persistence of cranes in Japan.

This study is the first to investigate the genetic structure of the non-migratory red-crowned cranes in Hokkaido. Our findings suggest that natal philopatry plays an important role in shaping the genetic structure, which should be considered when developing management strategies. It is possible that a few newly built nests in northern Hokkaido may be comprised of closely related individuals due to natal philopatry. It is needed to monitor the individual exchange between northern and eastern Hokkaido, and if the northern group is demographically isolated, management actions that facilitate gene flow between the two regions will be needed. The present study highlights the need for further research into the mechanism underlying pair formation and the dispersal patterns of a greater number of individuals. First-time pair formation and natal dispersal are probably important for the genetic structure, considering the high adult survival rates (Masatomi et al. 2007), strong pair bonds, and strong fidelity to nest sites exhibited by the breeding pairs. The accumulation of observational data on banded individuals or GPS tracking data (Masatomi et al. 2008) will help to better understand the mechanisms shaping the genetic structure and predict population expansion into unoccupied areas.

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