



NOTE

Wildlife Science

Origin of a pair of red-crowned cranes (*Grus japonensis*) found in Sarobetsu Wetland, northwestern Hokkaido, Japan: a possible crossbreeding between the island and the mainland population

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ABSTRACT. Red-crowned cranes *Grus japonensis*, which are an endangered species, have two separate populations, a mainland population in the Eurasian continent and an island population in eastern Hokkaido, Japan. Island cranes showed three haplotypes (Gj1, Gj2 and Gj13), whereas ten haplotypes (Gj3–Gj12) were confirmed in captive cranes and stray cranes. We found Gj5 haplotype in feathers of two cranes as well as four new haplotypes in seven wild crane feathers collected in South Korea. We also found feathers in the nest in Sarobetsu Wetland in northwestern Hokkaido. While the haplotype of female-derived feathers was Gj2, that of male-derived feathers was Gj5. The results suggest that there has been crossbreeding between cranes in the island population and cranes in the mainland population.

KEY WORDS: east Eurasia, genetic diversity, *Grus japonensis*, Hokkaido, Red-crowned crane

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The red-crowned (or Japanese) crane *Grus japonensis* is an endangered species in the world with an estimated total population of about 3,000 [11]. There are two independent populations of red-crowned cranes, a migratory population in the east Eurasian continent (continental population) and a non-migratory population in eastern Hokkaido, Japan (island population), based on the difference of these geographical distributions and historical changes in the number of individuals and distribution area of the island population [8, 11]. The island population had become nearly extinct at the end of the 19th century. Their territory had been limited to southeastern Hokkaido as an isolated small group for a long time [8]. After starting the supply of dent corn in some places in southeastern Hokkaido in winter, the number of cranes dramatically increased to almost 1,000 in the early 21st century [NPO Red-crowned Crane Conservancy (RCC), <http://www6.marimo.or.jp/tancho1213/sosutyosa.html>]. In the early 21st century, some cranes moved from their original habitat in eastern Hokkaido to Abashiri area and Sarobetsu Wetland in Souya area due to an abrupt increase in their population [13]. Three pairs in Sarobetsu Wetland, two pairs in Kuccharo Lake and one pair in Onuma, Wakkanai were observed by 2015 [10]. On the other, most of the cranes in the continental population, which raise their offspring in summer along the Amur River basin in Russia and China, migrate to the east coast of middle China and wetland in the Korean Demilitarized Zone (DMZ) between North Korea and South Korea for wintering [11]. Many cranes arrive in Gyeonggi Province (Gyeonggi-do) and Gangwon Province (Gangwon-do) in the northern part of South Korea from the DMZ to feed on crops left after the harvest.

Mitochondrial DNA (mtDNA) is well known as a maternal inheritance. Thus, mtDNA is much more well conserved without recombination than the nuclear genome in birds as well as other vertebrates [3]. The D-loop (control region) of mtDNA is a noncoding DNA with a high mutation frequency and well characterized for genetic diversity within a species or subspecies including red-crowned cranes [2, 5]. Thirteen haplotypes for the D-loop {(control region 2 (CR2)} in red-crowned cranes have been reported so far [1]. Only two major haplotypes (Gj1 and Gj2) and one minor haplotype (Gj13) have been identified in wild

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cranes in the island population possibly due to bottleneck effect [1, 5, 12]. We previously reported that the haplotypes of female parent cranes of the first pair found in Sarobetsu Wetland in this century and a family in Kuccharo lakeside in 2009 were all Gj2 [13]. On the other hand, study of tissues from captive cranes in zoos and aquariums in Japan, which were possibly of continental origin, provided many haplotypes (Gj3–Gj9) [1, 5]. Although records of their origin were not available for most cases, the origin of these captive cranes might be the continental population since red-crowned cranes in Japan were regarded as being extinct for a long time. Three additional haplotypes (Gj10–Gj12) were determined from feathers of three male subadult cranes that appeared in northern Honshu and southwestern and central parts of Hokkaido along the Sea of Japan in the late 2000s [13]. One of those three cranes first appeared in Ishikari, Hokkaido in March 2008 and moved to Ogata grassland in Akita in May 2008 and stayed there as a base (named Akita No. 1) (Gj10). One of the other two cranes was first found in Miyagi in January 2010 (Gj11) and the other was first found in Akita in March 2009 (Akita No. 2) (Gj12) [13]. Akita No. 2 crane temporarily arrived in southwestern Hokkaido but returned to Akita in June 2010 [13]. All of those three cranes have since disappeared. Akita No. 1 and No. 2 cranes showed characteristic feather patterns. From many visual confirmations and photographs in a time series, it was unlikely that these cranes came from northern and eastern Hokkaido. Thus, it was suggested that these three cranes could be of continental origin [13].

The appearance of unidentified cranes in the central Hokkaido in the 2000s reminded us of the possible crossbreeding of red-crowned cranes from the continental population and the island population. The purpose of this study was to investigate this possibility by comparing haplotypes of wild cranes in Hokkaido and the continent.

Seven flight feathers of wild red-crowned cranes were collected in Gyeonggi-do and Gangwon-do in the northern part of South Korea in the winters of 2009–2015 (Fig. 1, Supplementary Table 1). Four flight feathers were collected from the nest of a red-crowned crane south of Hitomi-numa Pond in Sarobetsu Wetland in Toyotomi, Hokkaido, Japan in July 2018 (45.07905 141.69648) (Fig. 2). Mitochondrial DNA was extracted from several pieces of feather follicles or shafts as previously described [12]. The extracted DNA was used as a template for polymerase chain reaction (PCR) with a primer set (905F and 905R, Supplementary Table 2) to produce 1,423-bp bands including the whole length of the D-loop (1,165 bp) [2]. PCR products of the D-loop were directly sequenced by the dideoxy method basically following our reported method [12] with primers shown in Supplementary Table 2. Sexing was performed with two sets of new primers (Supplementary Table 2) using the PCR conditions shown in our previous paper [12]. A parsimony haplotype network was drawn by SplitsTree 5 on the D-loop (1,000 bp) [6].

Four of the seven feathers found in the northern part of South Korea (Fig. 1) showed novel haplotypes. They were designated as Gj14–Gj17 (DDBJ Accession Nos. LC647194, LC647431–LC647433) (Table 1). Since Gj6 sequence (partial) was the same as that in Gj18, Gj18 (LC647434) might be a complete form of Gj6 (Table 1). The other adult and juvenile feathers found in Cheorwon-gun, Gangwon-do showed Gj5. This is the first haplotype study on the continental population with samples that were definitely from wild red-crowned cranes. Six different haplotypes including four novel types were identified with only seven feathers, suggesting very high genetic diversity of red-crowned cranes in the continent.

We found by chance four flight feathers in the nest of a red-crowned crane south of Hitomi-numa Pond in Sarobetsu Wetland (Fig. 2). At that time, we did not see any cranes or hear any calls from cranes. It is likely that these four feathers were from a nesting pair of cranes. Cranes including chicks and nests had been found several times from 2003 to 2015 in this area [10]. Female- and male-derived feathers were included in the feathers we found. The haplotype of the two female feathers was Gj2, the most major haplotype in the island population of red-crowned cranes (Table 1) [1, 12]. Surprisingly, however, the haplotype of both of



Fig. 1. Collection sites of feathers in the northern part of South Korea. (a) indicates Wangjing-myeon, Yeoncheon-gun in Gyeonggi-do. (b) and (c) indicate Cheorwon-eup and Dongsong-eup, Cheorwon-gun in Gangwon-do.

the male-derived feathers was Gj5, which is considered to be of continental origin [5] and was found in two wild cranes in South Korea in this study.

We constructed a haplotype network of previously reported Gj1–Gj13 (Gj6/18) and newly determined Gj14–Gj17 (Fig. 3). Gj1 and Gj2 are situated in opposite ends of the network in different clades and the other haplotypes are between them. Gj13, the other

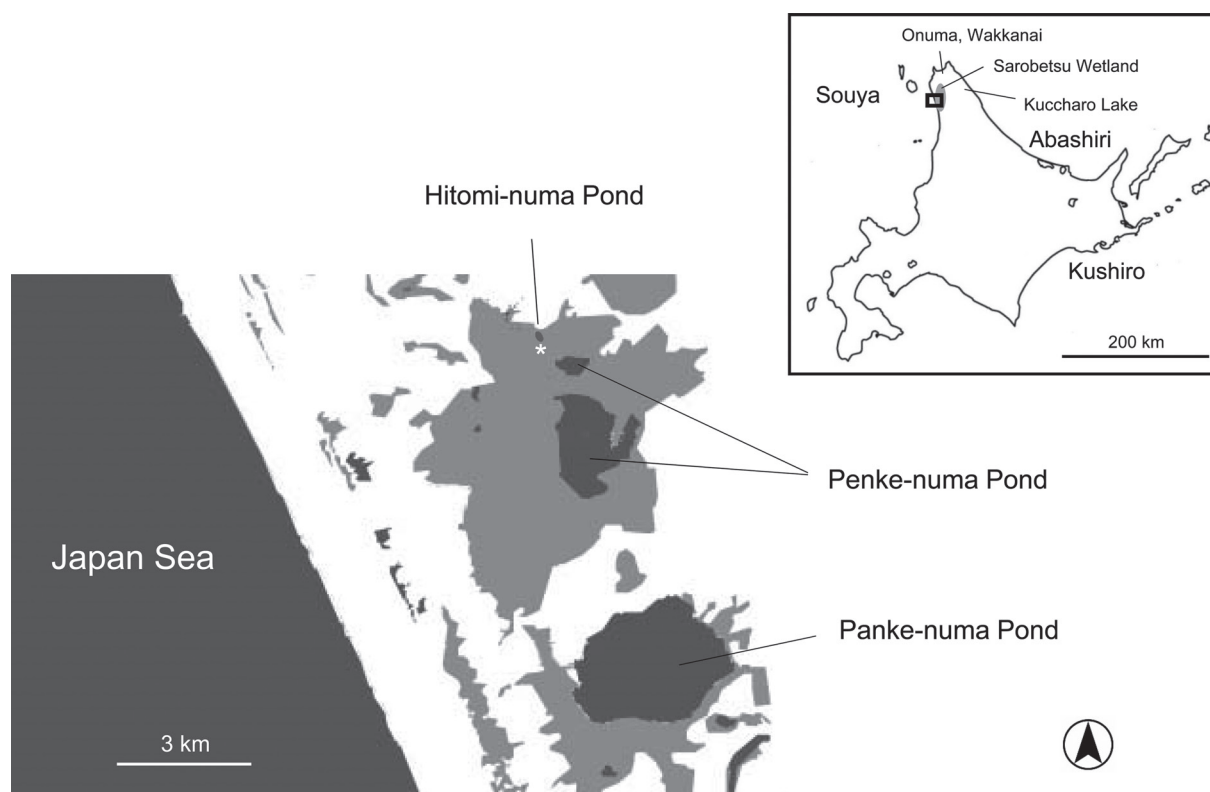


Fig. 2. Collection sites of feathers in Sarobetsu Wetland, Hokkaido, Japan. The main map of the collection area is an enlargement of the bold square in the upper-right inset map of Hokkaido.

Table 1. Sequences of the D-loop determined in northwest Hokkaido and northernmost South Korea

| Position | 23 | 24 | 25 | 49 | 74 | 106 | 130 | 166 | 190 | 237 | 239 | 247 | 252 | 257 | 274 | 318 | 323 | 402 | 407 | 440 | 539 | 550 | 562 | 622 | 629 | 761 | 788 | 791 | 808 | 836 | 1000 | | |
|----------|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|---|---|
| Gj1 | T | A | C | A | A | T | T | C | C | C | G | G | C | A | C | T | G | A | C | C | T | T | C | C | T | G | C | G | C | G | A | | |
| Gj2 | - | - | - | - | - | - | - | T | - | T | A | A | - | - | - | - | A | G | T | T | - | C | T | - | - | - | - | - | T | A | G | | |
| Gj3 | - | - | - | - | - | - | C | T | - | - | - | A | - | G | - | C | - | - | - | T | - | C | - | T | - | - | - | - | - | T | - | G | |
| Gj4 | - | - | - | G | - | - | - | T | - | - | - | A | - | - | - | - | - | - | - | T | - | C | - | - | - | - | - | - | - | - | - | G | |
| Gj5 | - | - | - | - | - | - | - | T | - | - | - | A | - | - | - | - | - | G | - | T | - | C | - | - | - | - | - | - | - | T | - | G | |
| Gj6 | - | - | - | - | - | - | - | T | - | - | - | A | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | |
| Gj7 | - | - | - | - | - | - | - | T | - | - | A | A | - | - | - | - | A | G | - | T | - | - | - | - | - | - | - | - | - | - | - | - | |
| Gj8 | - | - | - | - | - | - | - | T | - | - | - | A | - | - | T | - | - | G | - | T | - | - | - | - | - | - | - | - | - | - | - | - | |
| Gj9 | - | - | - | - | - | - | - | T | - | - | - | A | - | G | - | C | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | |
| Gj10 | C | T | A | - | - | - | - | T | - | - | - | A | - | - | - | - | - | G | - | T | - | C | - | - | - | - | - | - | - | T | - | G | |
| Gj11 | - | - | - | - | - | - | - | T | - | - | - | A | - | - | - | - | - | G | - | T | - | C | T | - | - | - | - | - | - | T | - | G | |
| Gj12 | - | - | - | - | G | - | - | T | - | - | A | A | - | - | - | - | A | G | - | T | - | C | T | - | - | - | - | - | - | T | - | G | |
| Gj13 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | - | C | - | - | - | - | - | - | - | - | - | - | - |
| Gj14 | - | - | - | - | - | C | - | T | - | - | - | A | - | - | - | - | - | G | - | T | - | C | - | - | - | - | - | - | - | T | - | G | |
| Gj15 | - | - | - | - | - | - | - | T | - | - | - | A | - | - | - | - | - | - | - | T | - | C | - | - | C | - | T | - | T | - | G | | |
| Gj16 | - | - | - | - | - | - | - | T | - | - | A | A | T | - | - | - | - | - | - | T | - | C | - | - | C | A | - | A | - | - | G | | |
| Gj17 | - | - | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | G | - | T | C | C | - | - | - | - | - | - | - | T | - | G | |
| *Gj18 | - | - | - | - | - | - | - | T | - | - | - | A | - | - | - | - | - | - | - | T | - | C | - | - | - | - | - | - | - | T | - | G | |

Position means the nucleotide position from the start of the D-loop in the mitochondrial genome. The same nucleotide as Gj1 is indicated as (-). Latter sequences of Gj6–Gj9 are not available. Gj2 (female crane) and Gj5 (male crane) were confirmed in south Hitomi-numa Pond in Sarobetsu Wetland, Hokkaido, Japan in this study. Gj5 and newly identified Gj14–Gj18 were found in northernmost South Korea in this study. Asterisk indicates that Gj18 might be a complete form of Gj6. Gj13 was quoted by Akiyama *et al.* [1]. Other haplotypes were quoted from previous publications (Hasegawa *et al.*, [5]; Miura *et al.*, [13]).

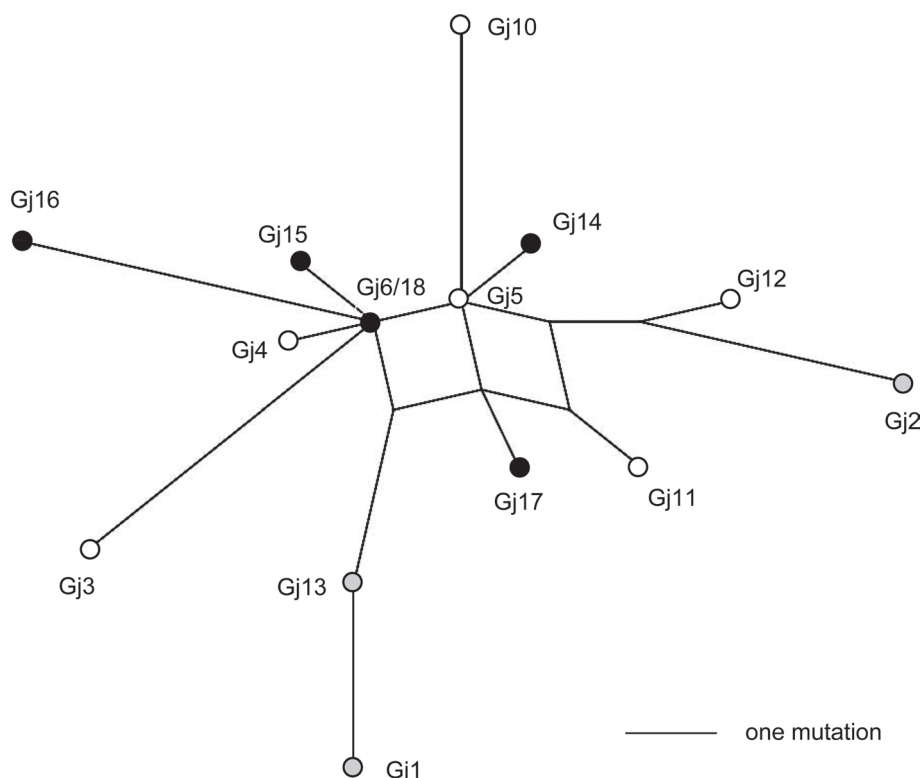


Fig. 3. Parsimony network of the control region of red-crowned cranes. Haplotype network of the CR2 region (1,000 bp) of Gj1–Gj18 was drawn by SplitsTree 5 (Consensus network). Gray circles (Gj1, Gj2 and Gj13) indicate haplotypes in the island population and open circles indicate previously reported continental haplotypes. Closed circles indicate haplotypes that were identified in wild cranes in the continental population in this study. Scale bar represents nucleotide substitutions per site. The scale bar represents a nucleotide difference. Gj6/18 means that Gj18 might be a complete form of Gj6.

haplotype in the island population is near Gj1 as previously reported [1]. A phylogenetic tree was also constructed with Siberian crane *Grus leucogeranus* served as an outgroup and is shown in Supplementary Fig. 1. Three island haplotypes are not situated in the same clade in the phylogenetic tree. Gj2 and three continental haplotypes (Gj7, Gj12, Gj16) are in the same clade. A clade of Gj1 and Gj13 and a clade of Gj2, Gj7, Gj12 and Gj16 are paraphyletic for each other. Thus, the island population seems not be extensively differentiated from the continental population, although there might have been some divergences or missing of some haplotypes that existed [1]. It is thought that many haplotypes had been lost by a bottleneck effect in the island population [5]. All of these suggestions were originally made by Akiyama *et al.* [1] and Hasegawa *et al.* [5] and the present results support these suggestions. Extensive studies on haplotypes and genomes with a sufficient number of wild cranes in the continental population are required to determine the origin of the island population.

In the 2000s, red-crowned cranes were frequently observed in the southwestern and central parts of Hokkaido along the Sea of Japan [13]. However, there was no evidence that these unidentified cranes made nests and raised offspring. No conclusive evidence has yet been obtained; however, we would like to suggest that the female crane (Gj2) and the male crane (Gj5) found in a nest in Sarobetsu Wetland were from the island population and the continental population, respectively. Only Gj1, Gj2 and Gj13 (two cranes) were found in hundreds of samples of red-crowned cranes including whole blood from banded chicks, cranes found dead in the countryside of eastern Hokkaido and stuffed cranes of island origin [1, 5, 12]. Gj2 accounted for 90% or more of the haplotypes in the island population. Since the estimated number of cranes in Kushiro Wetland in the early 20th century was only 20–40 [9], it is difficult to imagine that many haplotypes other than Gj1 and Gj2 remained in major descendants of the small surviving group until now. Akiyama *et al.* [1] suggested that Gj13 could have disappeared due to inbreeding or genetic drift after the bottleneck, since Gj13 was not found in any samples after 2007. Gj5 was found several times in a wild crane (this study) and cranes of continental origin [5, 13]. Collectively, the male crane found in Sarobetsu Wetland (Gj5) is most likely to have been from the continental population.

Gj2 type is predominant in the island population and was never found in wild cranes other than cranes in the island population, although the number of continental wild cranes studied was far from enough. All of the cranes that were possibly of continental origin (captive cranes in some facilities and unidentified cranes) showed as many as ten haplotypes but not Gj2 [1, 5, 12]. Thus, it is most likely that the origin of the female crane found in Sarobetsu Wetland (Gj2) was the island population.

The record of confirmations of a few banding red-crowned cranes suggested a tendency of a male-specific homing property to their birthplaces for nesting, while some females nested near their birthplaces [7]. It is speculated that a pair of a male crane (Gj5)

and a female crane (Gj2) found an appropriate place for nesting after pairing somewhere in Souya area or other areas in Hokkaido.

It is not known whether the offspring of a pair found in Sarobetsu Wetland have grown into adults and raised their own offspring. However, we found a pair of cranes and their chicks near Hitomi-numa Pond in 2019 and 2020. The introduction of genes from the continental population could be expected to increase the genetic diversity in the island population of red-crowned cranes. Extremely low genetic diversity of the island population had been considered a serious problem for a long time [4, 13, 17] since poor genetic diversity can lead to lower resistance to environmental changes and various infectious diseases [16].

Recently, a hooded crane *Grus monacha* and a (or a few) common crane *G. grus* were found in eastern Hokkaido in feeding places in winter as well as in summer [14, 15]. Breeding and wintering areas including stopover areas for these cranes are close to or overlapping partially with those of red-crowned cranes in the continental population, but Hokkaido is not an original habitat for hooded cranes and common cranes [Red List of Threatened Species 2016 by International Union for Conservation of Nature: <https://www.iucnredlist.org>]. Although Izumi Plain, Kyushu in Japan is the largest wintering place in the world for hooded cranes, common cranes and red-crowned cranes also arrived there (Izumi City Crane Museum Crane Park Izumi, <https://www.city.kagoshima-izumi.lg.jp/cranepark/>). The possibility that a few male red-crowned cranes have already arrived in eastern Hokkaido cannot be ruled out. If this is true, it is possible that some male cranes in the continental population had already mated with female cranes in the island population without affecting the haplotype. Further analyses of the genome with microsatellite or high-throughput sequencing as well as haplotyping of red-crowned cranes from the island population and the continental population are needed.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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