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Large-Scale Survey of Mitochondrial D-Loop of the Red-Crowned Crane *Grus japonensis* in Hokkaido, Japan by Convenient Genotyping Method

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ABSTRACT. The Red-crowned Crane, *Grus japonensis*, is an endangered species of crane that has two separate breeding populations, one in the Amur River basin (continental population) and the other in eastern or northern Hokkaido, Japan (island population). So far, only two haplotypes (Gj1 and Gj2) have been identified in the mitochondrial D-loop in island population, whereas seven haplotypes have been found in continental population (Gj3–Gj9). We developed a rapid and inexpensive method of extensive genotyping of D-loop haplotypes in Red-crowned Cranes, based on amplification refractory mutation system (ARMS) PCR assay. Two hundred and three cranes in eastern Hokkaido were studied with this method and supplemental DNA sequencing. Only two haplotypes, Gj1 and Gj2, were confirmed in eastern Hokkaido with Gj2 as a major haplotype. Additionally, only Gj2 was identified in twelve feathers from both sexes found in northern Hokkaido. These results suggest scarce genetic diversity in island population of Red-crowned Cranes in Hokkaido, Japan.
KEY WORDS: *Grus japonensis,* Hokkaido, Japan, mitochondria, Red-crowned Crane.

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The Red-crowned Crane (*Grus japonensis*) is one of the most endangered species in the world, with an estimated total population of 2,800 [6, 8, 11]. There are two main breeding populations. One is a northeast Asian continental population that migrates from the Amur River basin to the east coast of China and the Demilitarized Zone in the Korean Peninsula ('continental population', about 1,500 individuals), and the other is in the eastern area of Hokkaido Island in the northern part of Japan as a resident population ('island population', about 1,300 individuals). The island population was originally distributed throughout Hokkaido. However, the population had decreased to near extinction by the end of the 19th century, and their territory became limited to Tokachi (Fig. 1a), Kushiro (Fig. 1b) and Nemuro (Fig. 1c) in eastern Hokkaido because of extensive industrial and agricultural development [7]. Some pairs began to nest in the Abashiri region (Fig. 1d) from 1999 [7] and northern Hokkaido from 2004 (Fig. 1e and 1f) [10]. Their origin and genetic background are totally unknown.

Mitochondrial DNA (mtDNA) markers have been widely used for phylogenetic analysis in birds, including Gruinae. All mtDNA are passed from mother to offspring without recombination in birds as well as other vertebrates [1]. Among mtDNA, the D-loop (control region) is a noncoding DNA with a high mutation frequency and best characterized for genetic diversity within a species or subspecies, including some cranes [3, 13, 14]. Nine haplotypes for the D-loop in Red-crowned Cranes have been reported so far [5]. Although the cause was unknown, only two haplotypes (Gj1 and Gj2) were identified in wild cranes from the island population, while the other seven haplotypes (Gj3–Gj9) were detected in captive cranes in zoos, birds that originated from the continental population. The finding of extremely low genetic diversity in island population was also supported by a pilot study with microsatellites [4].

Red-crowned Cranes in Hokkaido increased its popula-

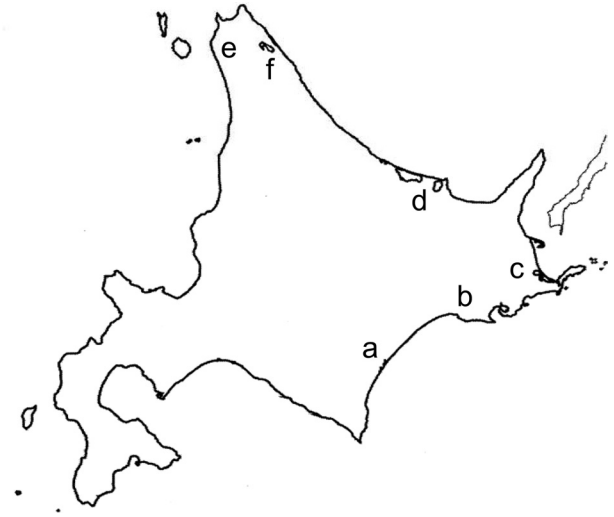

Fig. 1. Places cited in Hokkaido Island (Hokkaido Prefecture), Japan. a: Tokachi, b: Kushiro: c: Nemuro, d: Abashiri, e: Sarobetsu wetland, f: Kuccharo lake.

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tion size almost twice compared to that in the late 1990s, in which Hasegawa and his colleagues studied relatively small number of individuals in restricted area of eastern Hokkaido [4, 5]. In this study, we developed a rapid and inexpensive method of extensive genotyping of some D-loop haplotypes in Red-crowned Cranes in Hokkaido, based on amplification refractory mutation system (ARMS) PCR assay [12]. The results suggest scarce genetic diversity in Red-crowned Cranes in Hokkaido, Japan.

MATERIALS AND METHODS

Samples: With permission from the Japanese Ministry of the Environment (Tokyo), we collected whole blood samples (0.5–1 m*l*) from 98 conscious crane chicks, which were captured by hand when a banding survey was conducted by the Nonprofit Organization (NPO) Tancho Protection Group in eastern Hokkaido, including areas of Tokachi, Kushiro, Nemuro and Abashiri (Fig. 1a–1d), from 2005 to 2010. Primary flight feathers were obtained from the bodies in the freezer of Kushiro zoo, Kushiro, Hokkaido prefecture (n=87). Body feathers on the ground were also collected in the countryside in eastern Hokkaido (n=18). One body feather on the ground was found in Sarobetsu wetland, and eleven body feathers on the ground were obtained from a family in Kuccharo lakeside in 2009 (Fig. 1e and 1f). Additionally, body feathers were also obtained from 52 Red-crowned Cranes kept in feeding facilities in Okayama Prefecture. Most of them originated from cranes imported from China. Origin of the other eight cranes was Hokkaido.

Nucleotide sequencing of the mitochondrial D-loop: Mitochondrial DNA was extracted from several pieces of feather follicles (usually about 25 mg) with Isohair (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Mitochondrial DNA was also extracted from about 5 *µl* of whole blood added by ethylenediaminetetraacetic acid (EDTA) using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and Wizard SV Genomic DNA Purification System (Promega, Madison, WI, U.S.A.).

Extracted DNA was used as a template for polymerase chain reaction (PCR) with Phusion Hot Start DNA Polymerase (Finnzymes, Espoo, Finland), following the manufacturer's instructions to obtain the whole length of the D-loop, using the forward primer 5'-ACCCCATAATACGGTGAAGG-3' and the reverse primer 5'-ACGGTAAGGTTAGGACTAAG-3'. Step down PCR was carried out, consisting of an initial denaturing step at 98°C for 30 sec and three cycles of denaturation at 98°C for 10 sec, annealing at 65°C for 30 sec and extension at 72°C for 45 sec, followed by three cycles of the same conditions, except with annealing at 59°C, then 30 cycles with 52°C for annealing in a thermal cycler. After gel electrophoresis, the PCR product was extracted with QIAquick Gel extraction kit (Qiagen) and then was subcloned into T-vector (pGEM-T Easy Vector Systems, Promega). The ligation products were transformed into competent cells to obtain plasmid with a whole length of D-loop. D-loop insert was sequenced using an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.), with a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). The primers used for sequencing were SEQDLF1 (5'-GGCTTTTCTCCAAAACTCGC-3': forward), SEQDLF2 (5'-TCACGTGAAATCAGCAACCG-3': forward) and SEQDLR (5'-ATCTTCAGTGCCATGCTTTG-3': reverse).

We repeated these procedures from DNA extraction step at least twice for each sample.

Amplification refractory mutation system (ARMS) PCR assay for D-loop typing: For large-scale typing of D-loop in Hokkaido population, we developed convenient discrimination method of Gj1, Gj2 and others [5], based on Amplification refractory mutation system (ARMS) PCR originally reported by Newton *et al*. [12]. ARMS is an extremely high-fidelity PCR, which only amplifies specific alleles with a mismatch primer near to 3'-termial that pairs with only specific alleles. Gj1F (5'-ATCAGCAACCGGGTGTTATC-3': forward) and GjallR (5'-TCCTTGGGTGTTTATGGGAC-3': reverse), and GjallF (5'-CGACCCTCCCAATACAAAAC-3': forward) and Gj2R (5'-GGGTGTTGGTGTTGGTCTAA-3': reverse) were used for identification of Gj1 and Gj2, respectively (816 and 382 bp). Target sequences of GjallF and GjallR were NADH dehydrogenase subunit 6 and border region between tRNA-Phe and 12S rRNA, respectively. GjallF and GjallR were also used for positive control (1,362 bp). Extracted DNA was amplified by 3 cycles of denaturation at 94°C for 45 sec, annealing at 65°C for 45 sec, extension at 72°C for 1 min, followed by 3 cycles of the same condition except annealing at 59°C and 30 cycles with 52°C for annealing following the instructions for Go Taq Green Master Mix (Promega).

Sex identification: Crane sexing was carried out according to Fridolfsson and Ellegren [2] with some modifications. We used our designed primer set, CHDF (5'-ATCGTCAGTTTCCCTTTCAG-3': forward) and CHDR (5'-GATCCAGTGCTTGTTTCCTC-3': reverse) to clarify the size differences of PCR products (537 bp for CHD-Z and 358 bp for CHD-W) and the amplification efficiency. DNA extracts from individual cranes were amplified by the same cycle pattern as described for the ARMS method.

RESULTS

Convenient typing method for the D-loop based on ARMS-PCR: As Gj1 and Gj2 possessed C in the 440th position and T in the 237th position, respectively, as unique nucleotides among the 9 haplotypes (Gj1–Gj9) (Table 1), their specific primers were targeted in these nucleotides (Fig. 2A). We obtained body feathers from 45 among 52 Red-crowned Cranes kept in Okayama Prefecture; most of their origin is China in addition to some with Hokkaido origin. Gj3 (35), Gj4 (1) and Gj5 (1), as well as Gj2 (8) were identified in 45 cranes by DNA sequencing. As shown in Fig. 2B, each primer set for Gj1 and Gj2 produced its specific single bands, respectively, while positive control bands were confirmed for all haplotypes. This method was applied to 45 cranes, and the results were perfectly coincident with those of DNA sequencing, suggesting high selectivity of this method.

Table 1. Haplotypes of mitochondrial D-loop in Red-crowned Cranes

Haplotype	49	130	166	237	239	247	257	274	318	323	402	407	440	550	562	622	808	836	1000
Gj1	A	T	C	C	G	G	A	C	T	G	A	C	C	T	C	C	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						

Whole nucleotide sequences (1–1,165 bp) of mitochondrial D-loop for nine haplotypes reported by Hasegawa *et al*. (1999) [5]. Only nucleotides different from Gj1 are shown. The symbol [-] indicates the same nucleotide as that in Gj1. Blanks appearing in Gj6–Gj9 (550–1,000 bp) were not determined. DDBJ accession numbers were as follows: AB714136 for Gj1, AB714137 for Gj2, AB714138 for Gj3, AB714139 for Gj4 and AB714140 for Gj5.

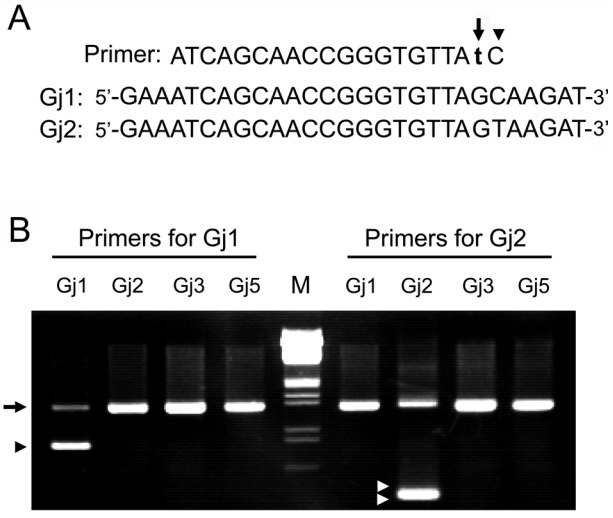

Fig. 2. Detection of Gj1 and Gj2 by ARMS-PCR. A. Illustration of the difference in the 3'-end nucleotide sequences of Gj1 and Gj2 (440th nucleotide in the D-loop) and their relation to the ARMS primer for detection of Gj1. Arrow and arrowhead indicate mismatched positions to discriminate between Gj1 and Gj2. B. Representative image of agarose gel electrophoresis of products of ARMS-PCR. Pairs of forward and reverse primers for Gj1 and for Gj2 were used for amplification with purified genomes of Gj1, Gj2, Gj3 and Gj5. Pairs of primers for all D-loop types were also used as a positive control. Arrow, arrowhead and double arrowheads indicate a band for a positive control, a band specific to Gj1 and a band specific to Gj2, respectively.

D-loop haplotypes of island population in eastern Hokkaido: We studied whole blood, flight feathers and body feathers from 203 cranes in eastern Hokkaido including the Abashiri region (Fig. 1a–1d). As the result, only two haplotypes, Gj1 and Gj2 could be identified. The number of cranes with Gj1 and Gj2 was 28 and 175, respectively. Thus, the percentage of cranes with the Gj2 haplotype in eastern Hokkaido was estimated at 86.2%. Because we could not deny the possibility that some of 18 feathers on the ground found in eastern Hokkaido could be derived from the same cranes. For most cases, however, we selected feathers found apart enough as we could. Some possible redundancies did not affect the conclusion that there were only two haplotypes, Gj1 and Gj2 in eastern Hokkaido as reported by Hasegawa *et al*. (1999) [5], with only minor impact on Gj2 percent.

Figure 3 indicates haplotype distribution for chicks, which blood samples were used for determination, and thus sampling sites were definitely in their habitats. Although these two haplotypes were almost proportionally distributed in eastern Hokkaido, the Gj1 haplotype was not found in the Nemuro region. However, one feather found in this area showed Gj1. We did not have any information on the territory of the owner. The only haplotype identified so far in the Abashiri population was Gj2.

As the results of DNA sequencing with 24 among 203 samples in island population in eastern Hokkaido, only Gj1 (4) and Gj2 (20) were confirmed again, supporting the results of large-scale typing experiment.

D-loop haplotypes of metapopulation in northern Hokkaido: The other group in island population has been recognized in northern Hokkaido since the beginning of this century. One family is in Sarobetsu wetland (one or two siblings) (Fig. 1e), and the other family is in Kuccharo lakeside (two siblings) (Fig. 1f). We could obtain one body feather in the Sarobetsu wetland and eleven body feathers in the Kuccharo lakeside. Analysis of the feather found in Sarobetsu showed that the crane was female and the haplotype was Gj2. Both sexes were identified with five (male) and six (female) feathers found in Kuccharo, and their haplotypes were all Gj2, suggesting that haplotypes of the mother crane and her juveniles were definitely Gj2. These results were confirmed by DNA sequencing.

DISCUSSION

Conventional sequencing of the D-loop is the most precise and comprehensive method for haplotype identification but is excessively time-consuming and expensive. Thus, the application is practically impossible for large-scale studies. In order to solve this problem, we developed a convenient method to identify Gj1 and Gj2 based on ARMS-PCR [12].

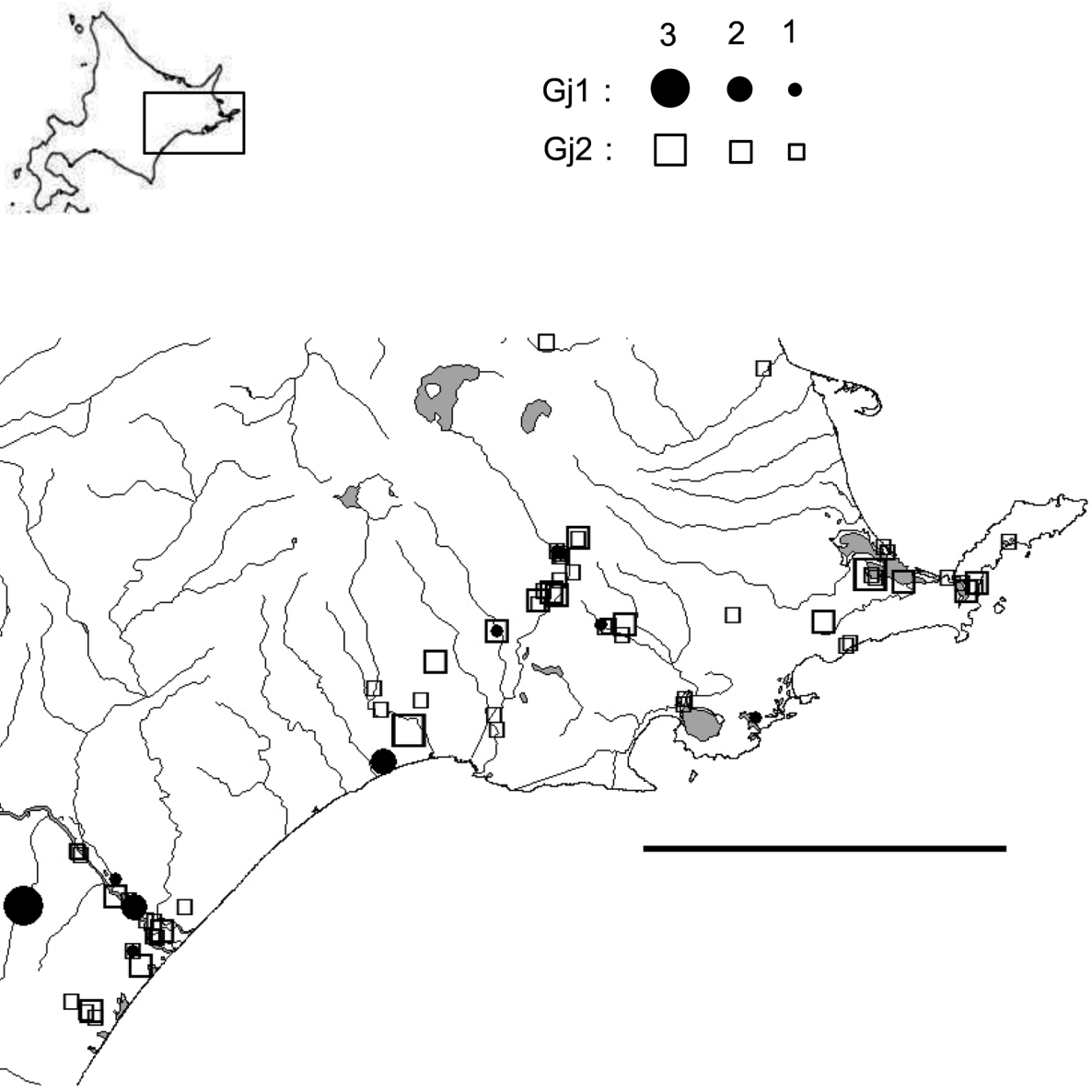

Fig. 3. Distribution of Red-crowned Cranes with Gj1 and Gj2 in eastern Hokkaido. Square in the upper map indicates the area shown in the main map. Results with whole blood samples from 99 chicks are indicated. Filled circles and open squares designate Gj1 and Gj2, respectively. Number of chicks (1, 2, 3) in each area is expressed by the symbol size as indicated by symbol legend. Bar: 100 km.

This is a kind of PCR with high-fidelity primers without the need for any other special reagents or equipment. This method could be applicable to detect other haplotypes other than Gj1 and Gj2. We determined the rest of the sequences (441–1,165 bp) of Gj1–Gj5, which were not studied in the previous study [5]. However, additional haplotypes were not found by the posterior sequences, suggesting that the anterior sequences up to 440 bp could be enough for determination of the haplotype. Application of restriction fragment length polymorphism (RFLP) analysis could be substituted for this method. Although discrimination between Gj1 and Gj2 is relatively easy using only one restriction enzyme (BsajI), the same band pattern as Gj1 should be obtained for Gj3–Gj6, Gj8 and Gj9, and the band patterns of Gj2 and Gj7 should be the same. Moreover, RFLP analysis needs one more step in enzyme digestion of PCR products in addition to ARMS-based method.

In the present study, only two known haplotypes of mitochondrial D-loop, Gj1 and Gj2, were identified for 203 cranes in eastern Hokkaido. This accounted for only about 16% of the whole population in Hokkaido (total of 1,300 cranes) [8]. This result supports the finding by Hasegawa *et al*. [5], who identified only Gj1 and Gj2 in eastern Hokkaido with DNA sequencing. As described in the following chapter, twelve

feathers found in northern Hokkaido all showed Gj2. Red-crowned Cranes in Hokkaido were in danger of extinction by the end of 19th century, but they were discovered again in Kushiro wetland in 1924 and possibly a little less than 20 cranes could live there. Therefore, it was hypothesized that the bottleneck effect could dramatically reduce the genetic diversity of this population [4, 5]. However, we have to admit that the convenient typing method we developed accidentally identifies samples other than Gj1 or Gj2, if there are other unknown haplotypes having T at the 237th position or C at the 440th position in the continental population. Taken all into consideration, it is unlikely that there is any haplotype other than Gj1 and Gj2 in island population.

Red-crowned Cranes have not nested in northern Hokkaido since the 19th century, during which time nesting was recorded around the lower reaches of the Teshio River [7]. Now, two families have been identified in this area [9, 10]. Although no information was available on their genetic background and origin, one feather found in Sarobetsu wetland and eleven feathers obtained from a family (two siblings and their parents) in Kuccharo lakeside all showed a Gj2 type in the present study. As the feather found in Sarobetsu was from a female, the mother crane and her offspring should all be Gj2, because only one family was found there. Similarly, both sexes were found among the eleven feathers, so the mother crane and her offspring should be all Gj2 for the Kuccharo family. However, we were not able to determine the haplotype of the father crane in the Kuccharo family in this study. Analysis of microsatellites is required to determine the haplotype of father crane in Kuccharo lakeside [4].

In conclusion, we developed a convenient method of extensive genotyping of D-loop haplotypes in Red-crowned Cranes in Hokkaido. This method is simpler and of far wider application for other haplotypes compared to RFLP. However, haplotypes in continental population should be extensively studied. The present results suggest scarce genetic diversity in Red-crowned Cranes in Hokkaido as reported by Hasegawa *et al*. [4, 5]. Combining this approach with individual identification methods, such as the use of microsatellites, is required in future studies, as paternal genetic information has been hardly available so far.

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