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Authors: Hasegawa, Osamu, Takada, Shigeyuki, Yoshida, Michihiro C., and Abe, Syuiti

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Variation of Mitochondrial Control Region Sequences in Three Crane Species, the Red-Crowned Crane *Grus japonensis*, the Common Crane *G. grus* and the Hooded Crane *G. monacha*

Osamu Hasegawa[1], Shigeyuki Takada[1], Michihiro C. Yoshida[1,2], and Syuiti Abe[1,2]*

[1]*Laboratory of Cytogenetics, Graduate School of Environmental Earth Science, Hokkaido University and*
[2]*Chromosome Research Unit, Faculty of Science, Hokkaido University, North 10, West 8, Kita-ku, Sappro 060-0810, Japan*

ABSTRACT—Entire nucleotide sequence of the mitochondrial control region was obtained for the red-crowned crane *Grus japonensis* and two closely related species, the common crane *G. grus* and the hooded crane *G. monacha*. Control regions of these crane species could be divided into three subdomains (I, II, III) by incidence of sequence variation. The central domain II was well conserved with C, D and F sequence motifs, while both flanking domains were variable, with greater base substitution in 5' domain I than 3' domain III with CSB-1 motif. In addition, a 51 base-pair (bp) sequence was found to be deleted in the 5' portion of domain I in *G. grus* and *G. monacha* compared with the domain I sequence of *G. japonensis*. Comparison of 418 bp sequence in variable 5' portion spanning domain I and 5' part of domain II among 29 individuals of *G. japonensis* including 14 from the East Asia mainland population and 15 from the Japan's endangered Hokkaido island population revealed seven haplotypes in the former and only two haplotypes in the latter. Small number of haplotypes in the Hokkaido population suggested less genetic variation than in the mainland population, possibly reflecting a "bottleneck" effect. Although the two studied populations shared no common haplotypes, they are likely to belong to a common lineage because of no independent branch of each population on the parsimony network and neighbor-joining tree.

INTRODUCTION

Mitochondrial DNA (mtDNA) control region (also called the D-loop region for vertebrates) is a noncoding segment that initiates replication and transcription. The control region is considered as the most variable portion of the mtDNA sequences (Fauron and Wolstenholme, 1976; Chang and Clayton, 1985). Analysis of the control region sequences therefore provides invaluable information for investigating phylogenetic relationships among closely related species and within species (e.g. reviewed by Taberlet, 1996).

The avian control region, as those in other vertebrates, is often divided into three subdomains, i.e., central (domain II) and flanking parts (domains I and III), with respective sequence variabilities. Sequence homology in the central domain II is well kept beyond species, with conserved sequence motifs such as C, D and F boxes (Brown *et al*., 1988). Base substitutions and structural changes including insertions and/or deletions, and variable number tandem repeats (VNTRs) often occur within domains I and III (Wenink *et al*., 1994; Berg *et al*., 1995), suggesting that these portions are the primary choice for analysis of inter- and intraspecific sequence variation. However, knowledge of intraspecific sequence variation in the avian control region is still meager (Quinn, 1992; Edwards, 1993; Wenink *et al*., 1994; Marshall and Baker, 1997; Masuda *et al*., 1998).

Family Gruidae (Order Gruiformes) comprises 15 species of cranes in four genera of two subfamilies, Balearicinae and Gruinae (Sibley and Monroe, 1990). However, no information on the crane mtDNA control region sequences has been available so far. The red-crowned crane *Grus japonensis* is distributed in the East Asia mainland and Japan. The Japanese population was mostly extinguished nearly a half century ago (reviewed by Swengel, 1996). Its current habitat is restricted to the eastern part of Hokkaido island. The endangered population of Hokkaido is estimated as approximately 600 in size and thought to be isolated from the population of the East Asia mainland. Thus, the Hokkaido population has long been debatable whether it is diverged from the mainland population at the level of subspecies as has been suggested

* Corresponding author: Tel. +81-11-706-2752;
FAX. +81-11-736-6304.
E-mail. sabe@ees.hokudai.ac.jp

by differences in the note structure of the female unison call (Archibald, 1976).

In the present study, the entire control region sequences of *G. japonensis* and two closely related species, the common crane *G. grus* and the hooded crane *G. monacha*, were determined in order to examine both the inter- and intraspecific sequence variations of the control region. Then, the sequences of most variable portion were applied for estimating genetic diversity between the East Asia mainland and Japanese populations of *G. japonensis* to examine whether they are genealogically isolated from each other.

MATERIALS AND METHODS

Cranes

Crane species examined in the present study comprised 29 individuals of *G. japonensis*, three individuals of *G. grus* and two individuals of *G. monacha*. *G. japonensis* included 14 individuals from the East Asia mainland and 15 individuals from Hokkaido island, Japan. In addition, one individual of the wattled crane *Bugeranus carunculatus* was used as an outgroup for nucleotide sequence comparison described below. All the specimens for DNA extraction from these species were obtained from various zoological gardens and aquariums with co-operation of the Japanese Association of Zoological Gardens and Aquariums, Tokyo.

DNA extraction

Genomic DNA was extracted from heparinized whole blood, cultured skin fibroblasts, or dissected liver tissues stored at –30°C, with the conventional phenol-chloroform method (Sambrook *et al.*, 1989) unless otherwise indicated. About 0.1 ml of whole blood was added to 10 ml STE buffer (NaCl 100 mM, Tris-HCl 10 mM, and 1 mM EDTA, pH 8.0) supplemented with 0.5 ml of 10% SDS and 0.1 ml of proteinase K (50 mg/ml) and incubated at 37°C for overnight to 3 days. DNA was extracted more than 3 times with the mixture of 10 ml of phenol and 5 ml of 24:1 chloroform:isoamyl alcohol. DNA in aqueous phase was recovered with ethanol precipitation, dried in air, and dissolved in TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.5). Frozen liver tissue was powdered in liquid nitrogen with a dismembrator (Boxy Brown) and processed for DNA extraction as above. Iso-Quick nucleic acid extraction kit (Micro Probe) was also used for cultured fibroblasts of some individuals.

PCR amplification

Symmetric PCR with equal concentration of primers shown in Table 1 was carried out to produce double-stranded DNA with LA PCR kit (TaKaRa) following the manufacturer's instruction. Control regions were amplified in 50 μl of reaction mixture containing 1.5 mM $MgCl_2$, 25 pM each primer, 2.5 mM each dNTP, 0.5 μl of template DNA, and 2.5 U of *Taq* DNA polymerase, with 30 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 1 min, and extension at 72°C for 1 min. Each amplification was accompanied by preheating at 94°C for 3 min and post-cycle extension at 72°C for 10 min.

Table 1. Primer sequences for amplification and sequencing of mitochondrial control region of cranes examined

LC16004	5' -GAGCCCTAGAAAACAAAATA- 3'
HC01342	5' -AAGAATTCTGCGGATACTTGCATGT- 3'
LC16575	5' -ACAAAAGAAACCCCCAAACTCA- 3'
GJ108	5' -TTAGATGCAACTGCCGACCCT- 3'
GJDL346	5' -AGTCATGAATGGTTCAGGTC- 3'
KB936	5' -TCTTGGCATCTTCAGTGCC- 3'

Asymmetric PCR was performed with 1 μl of symmetric PCR product as template and 1:1/100 ratio of a pair of primers in 100 μl reaction mixture containing the same amount of each component, using the same cycling profile as that of the symmetric PCR.

Nucleotide sequencing

Dideoxy sequencing was carried out directly on the purified asymmetric PCR products using the Sequenase version 2.0 sequencing kit (United States Biochemical) with [α-^{32}P]-dCTP (Amersham). Sequencing reaction products were electrophoresed on 6% polyacrylamide gel containing 6 M urea for 1.5 hr to the desired duration up to 6 hr under 30 to 35 W using TBE buffer (100 mM Tris-HCl, 90 mM boric acid and 1 mM EDTA, pH 8.0). After electrophoresis, the gel was transferred onto the filter paper (3MM, Whatman) and vacuum-dried at 80°C for 60 min. Autoradiography was performed using Fuji RX X-ray film for overnight to a week at room temperature.

For several individuals of *G. japonensis* and two other *Grus* species examined, non-RI sequencing with fluorochrome-labeled primers was carried out on either symmetric PCR products directly or those cloned with the TOPO TA cloning™ system (Invitrogen, Carlsbad, CA), using a Hitachi SQ-5500L DNA sequencer (Hitachi, Tokyo). Sequencing reactions were performed using a Thermo Sequenase™ pre-mixed cycle sequencing kit (Vistra Systems, Sunnyvale, CA), with M13 reverse, T7, and other primers (GJ108, GJDL346 and KB936 shown in Table 1) labeled with Texas-Red, according to the manufacturer's instruction.

Data analysis

Data analysis was performed with GeneWorks version 3.2 (Intelligenetics) and Clustal W (Thompson *et al.*, 1994) programs on a Macintosh computer. Phylogenetic trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987), based on genetic distance estimated by Kimura's two-parameter method (Kimura, 1980). A parsimony network connecting observed haplotypes was drawn after Bandelt (1994).

RESULTS

Comparison of the control region sequences among three *Grus* species

Complete nucleotide sequences of the mtDNA control region were obtained on the fragment successfully amplified by a primer pair of HC01342 and LC16004 or LC16575 for seven individuals of *G. japonensis*, i.e. three from the East Asia mainland population and four from the Hokkaido population, and three individuals of *G. grus* and two individuals of *G. monacha*. The same primer sets were also effective in amplifying the control region of *B. carunculatus*. The sequences have been deposited in the DDBJ/EMBL/GenBank databases with accession numbers AB017618 to AB017625 and AB023812 to AB023814. Sequencing of the adjoining regions and base homology analysis with chicken mtDNA sequence (Desjardins and Morais, 1990) revealed that the control region of *G. japonensis* is flanked by $tRNA^{glu}$ gene at the 5' end and by $tRNA^{phe}$ gene at the 3' end (accession No. AB017618). The correct folding in the secondary structure of both tRNAs was assured with the tRNAscan-SE program (Lowe and Eddy, 1997), verifying that the obtained sequences are truly from mtDNA. The tRNA gene order was also true for the control regions of the other two *Grus* species, as revealed by partially sequenced 5' and 3' flanking regions (data not shown). The

entire length of the control region was found to be 1165 base-pair (bp) for *G. japonensis* and 1102 bp for the other two species. Multiple alignment of the nucleotide sequences among three species disclosed that the observed difference is mainly caused by a deletion of 51 bp sequence near the 5' end of the control region in *G. grus* and *G. monacha*, which corresponds to 164th to 215th nucleotide in *G. japonensis* (Fig. 1A). The control region of each species had some conserved nucleotide sequence features such as a termination-associated sequence (TAS) (not shown), C, D and F boxes (Fig. 1B) and a short conserved sequence box (CSB-1) (Fig. 1C), like those reported in other avian species (Baker and Marshall, 1997).

The site and frequency of nucleotide substitution in non-overlapping 50 bp segments across the control regions of the three crane species examined are illustrated in Fig. 2. As in other vertebrates, the crane control region could be divided into three parts, tentatively designated as domains I (1–350th nucleotide), II (351–750th nucleotide) and III (751–1150th

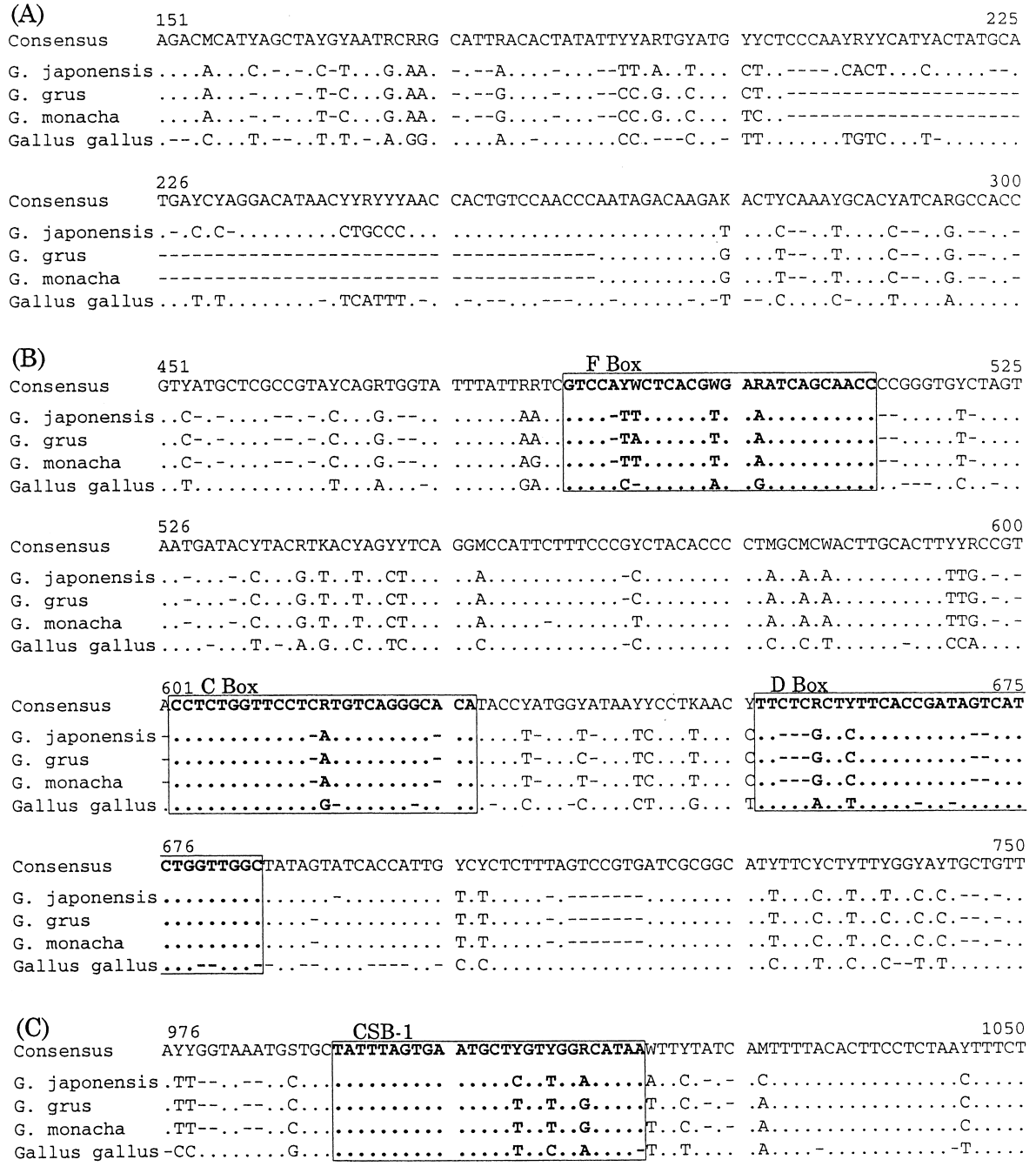

Fig. 1. Partial nucleotide sequences of mitochondrial DNA control region domain I (A), domain II (B) and domain III (C) of the three crane species, *G. japonensis*, *G. grus*, *G. monacha*, and corresponding portions of the domestic chicken *Gallus gallus*. Identical bases and deletions are indicated with dots and hyphens, respectively. The conserved C, D and F boxes, and CSB-1 motifs are shown in bold and boxed.

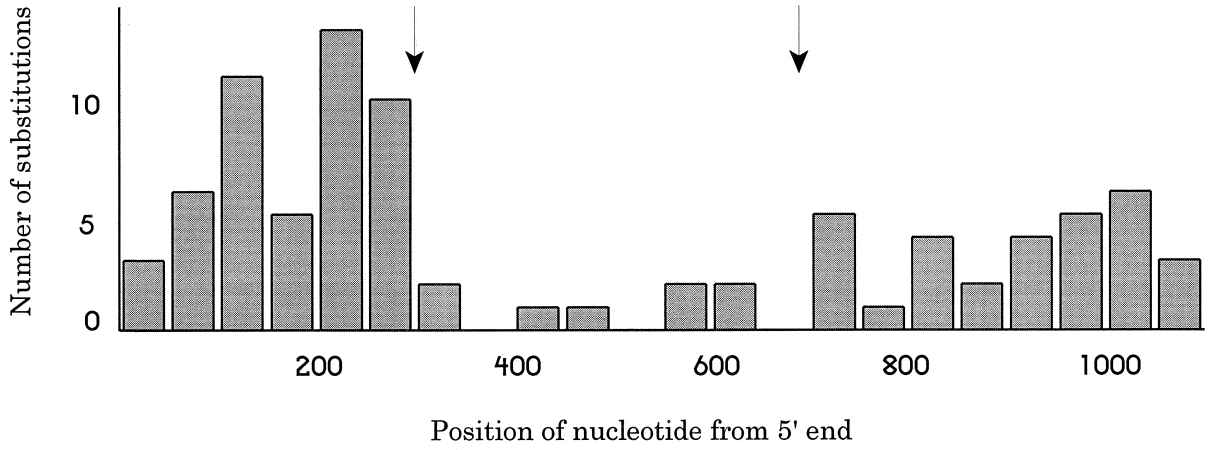

Fig. 2. Distribution of nucleotide substitutions in non-overlapping 50 bp segments across the control regions of the three crane species studied, *G. japonensis*, *G. grus* and *G. monacha*, after interspecific multiple sequence alignment. Arrows indicate the boundary of three subdomains I to III, which is given tentatively by the frequency of substitutions.

nucleotide) from 5' end to 3' end, excluding the 51 bp deletion in *G. grus* and *G. monacha*, by the frequency of base substitutions. As shown in Fig. 2, domains I and III showed a higher substitution frequency than domain II containing the conserved C, D and F boxes. Domain I with TAS, also containing 51 bp deletion in *G. grus* and *G. monacha*, showed a greater base substitution frequency than domain III with the conserved CSB-1 sequence. There were no obvious tandem repeat sequences such as those found in 3' portion of the control region in the loggerhead shrike (Mundy *et al.*, 1997) and the golden eagle (Masuda *et al.*, 1998). In the examined species, overall number of transitions, transversions and insertions/deletions varied in the different domains (51 for I, 11 for II and 25 for III), although transition-to-transversion ratios appeared to be rather constant among three domains (11.8 for I, 10 for II and 7.3 for III). Overall control region sequence homology was 95.7% for *G. grus* and *G. monacha*, 87.9% for *G. grus* and *G. japonensis* and 87.5% for *G. monacha* and *G. japonensis*, respectively.

Fig. 3 shows a NJ tree constructed on the basis of entire nucleotide sequences of the control regions in the above 12

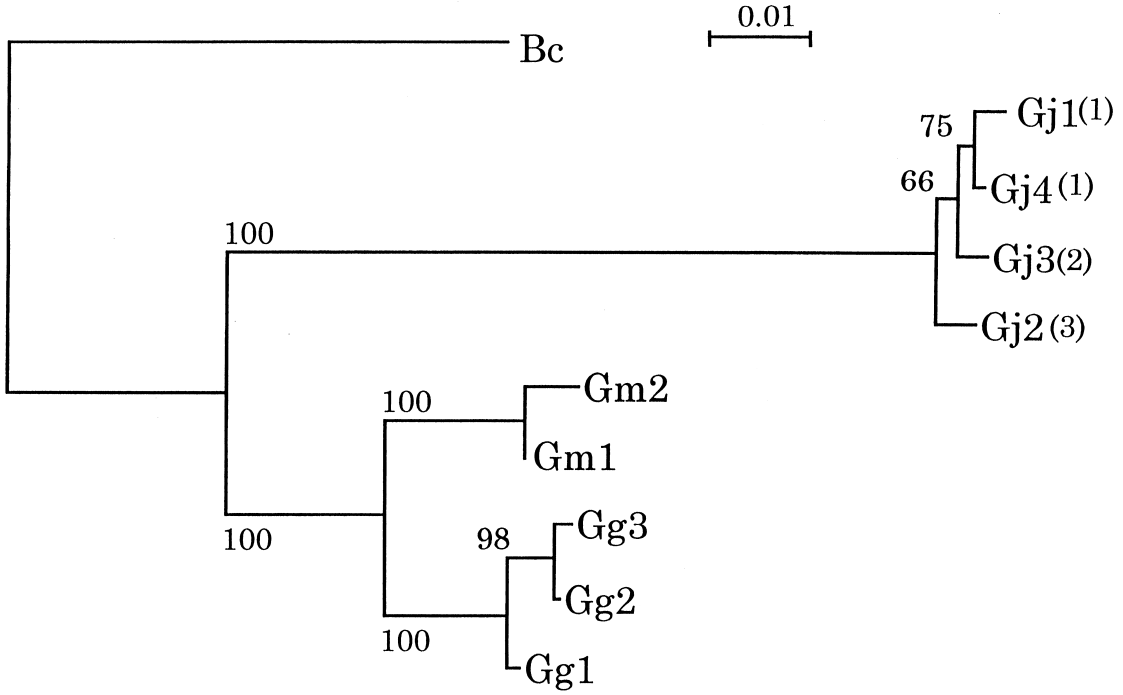

Fig. 3. Neighbor-joining phylogenetic tree of the entire control region sequences of the three crane species studied, the red-crowned crane *G. japonensis* (Gj), the common crane *G. grus* (Gg) and the hooded crane *G. monacha* (Gm). The wattled crane *Bugeranus carunculatus* (Bc) was used as an outgroup. Number of individuals examined is shown in parenthesis. The scale bar indicates an evolutionary distance. Numbers above branches show bootstrap values (%).

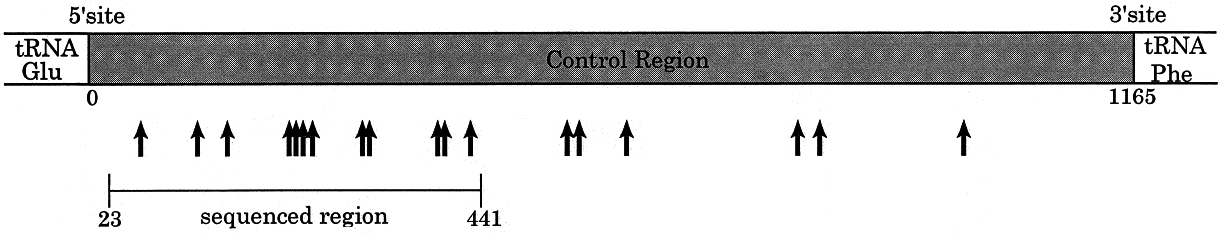

Fig. 4. Nucleotide substitution sites (arrows) identified by multiple sequence alignment of the control regions among seven individuals of *G. japonensis*. Horizontal line shows the 418 bp region used for intraspecific sequence analysis. Numbers represent the nucleotide position counted from 5' end of the control region.

Table 2. Control region haplotypes identified from the red-crowned crane *G. japonensis.*

Haplotype	Base substitution site																			No. of individuals	
	49	130	166	237	239	247	257	274	318	323	402	407	440	*572*	*584*	*644*	*830*	*858*	*1022*	Hokkaido	East Aisa mainland
Gj1	A	T	C	C	G	G	A	C	T	G	A	C	C	*T*	*C*	*C*	*C*	*G*	*A*	2 (1)	–
Gj2	·	·	T	T	A	A	·	·	·	A	G	T	T	*C*	*T*	·	*T*	*A*	*G*	13 (3)	–
Gj3	·	C	T	·	·	A	G	·	C	·	·	·	T	*C*	·	*T*	*T*	·	*G*	–	2 (2)
Gj4	G	·	T	·	·	A	·	·	·	·	·	·	T	*C*	·	·	·	·	*G*	–	2 (1)
Gj5	·	·	T	·	·	A	·	·	·	·	G	·	T							–	4
Gj6	·	·	T	·	·	A	·	·	·	·	·	·	T							–	1
Gj7	·	·	T	·	A	A	·	·	·	A	G	·	T							–	2
Gj8	·	·	T	·	·	A	·	T	·	·	G	·	T							–	2
Gj9	·	·	T	·	·	A	G	·	C	·	·	·	T							–	1

Base substitution site indicates the nucleotide position counted from 5' end of the control region. The substitution sites downstream to 418 bp region are shown in italic for parenthesized seven individuals whose entire control region was sequenced. Dots represents the same nucleotide as in the first haplotype (Gj1).

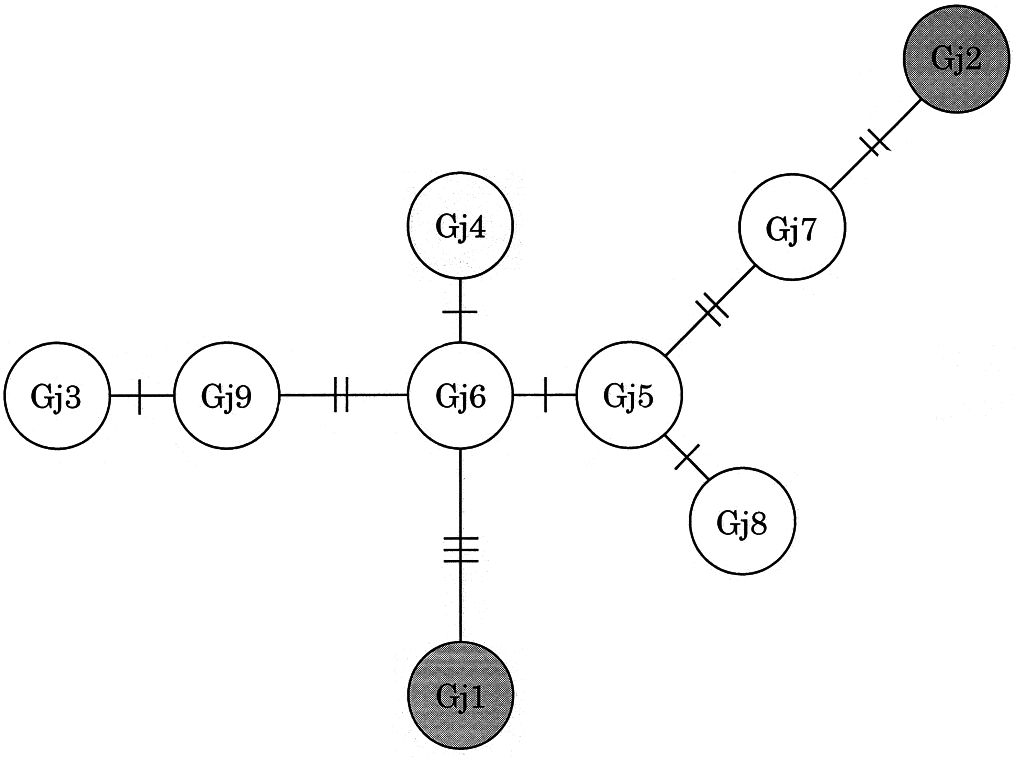

Fig. 5. Parsimony network of the control region haplotypes (418 bp sequences) of *G. japonensis* presented in Table 2. Base substitutions, all transitions, are indicated by slashes on the network. Haplotypes found in Hokkaido are shaded.

cranes including seven of *G. japonensis*, three of *G. grus* and two of *G. monacha*. The obtained topology clearly discriminated the latter two species from *G. japonensis*, suggesting a close relationship between *G. grus* and *G. monacha*, which is in agreement with previous molecular phylogenetic findings using cytochrome b sequences (Krajewski and Fetzner, 1994) and the above-mentioned overall nucleotide homology in the control regions between species studied here.

Intraspecific nucleotide sequence variation in *G. japonensis*

Multiple alignment of entire control region sequences of the aforementioned seven individuals of *G. japonensis* revealed 18 base substitution sites, mostly occurring in the variable domain I as described (Fig. 4). The observed base substitutions were transitions, giving four types of the control region sequences in these seven individuals (Table 2). Among four Hokkaido population individuals, three shared one type and one had another. Three mainland population individuals showed two other types of the nucleotide sequences, one for two individuals and another for one individual. Because of the abundant sites and higher frequency of base substitutions in the 5' half, a 418 bp region spanning the 23rd to 441st nucleotide from the 5' end, where 2/3 of the observed substitution sites are included (Fig. 4), were sequenced in the remaining 22 individuals.

As shown in Table 2, a total of 13 sites of base substitutions, all transitions, with a novel site at 274th nucleotide, were observed in the 418 bp region sequenced in 29 individuals including the above seven. This gave a total of nine haplotypes (Gj1 to Gj9) for the control region. Among these, seven (Gj3 to Gj9) were observed in 14 mainland population individuals and two (Gj1 and Gj2) in 15 Hokkaido population individuals, although no common haplotypes were found between these two local groups. Of the 15 Hokkaido population individuals, 13 shared the same haplotypes (Gj2) and two had another one (Gj1). In a parsimony network drawn on the data presented in Table 2, Gj1 and Gj2 differed by 8 substitutions connected via the Gj5 to Gj7 haplotypes, and not forming a separate branch (Fig. 5). In addition, haplotypes of the mainland and Hokkaido populations did not form a separate cluster in a bootstrapped NJ tree constructed on the 418 bp sequence and corresponding regions in *G. grus* and *G. monacha* as outgroup based on 1000 pseudoreplicates, with less than 50% of nodal values for trees of *G. japonensis* (data not shown).

DISCUSSION

Structural characteristics of crane mtDNA control regions

The length of the avian mtDNA control region so far analyzed ranges from 1072 bp in the dunlin *Calidris alpina* to 1240 bp in the greenfinch *Carduelis chloris* (Baker and Marshall, 1997). The three crane species examined herein also have similar sized control regions, i.e., 1165 bp in *G. japonensis* and 1102 bp in both *G. grus* and *G. monacha*. Size variation of avian control regions in the literature is often attributable to relatively small (1–20 bp) insertions or deletions (indels) in both the 5' domain I and 3' domain III, and to varying numbers of tandem repeats in the domain III (Baker and Marshall, 1997). Although no apparent tandem repeats were found in the domain III in the examined crane species, a 51 bp deletion observed in the domain I in *G. grus* and *G. monacha* compared to *G. japonensis* is noteworthy. Large such indels (up to about 65 bp) have so far been found only between phylogenetically distant bird species, after interordinal, interfamily, or intergeneric comparison of control region sequences (Desjardins and Morais, 1991; Quinn and Wilson, 1993; Ramirez *et al.*, 1993; Wenink *et al.*, 1994). Thus, the intrageneric large deletion observed in the present crane species may represent a useful marker for investigating phylogenetic relationships in the Gruidae, although it remains to be examined whether such a large deletion also occurs in other crane species.

In the present crane species, the TAS motifs in the domain I, the C, D and F boxes in the domain II and the CSB-1 in the domain III are apparent in the control region. These conserved motifs are thought to be putative regulatory signals implicated in transcription of mt structural genes and replication of the heavy strand (Southern *et al.*, 1988; Desjardins and Morais, 1991). Structural organization of the crane control region examined is therefore well conserved, showing a close similarity to that reported in the domestic chicken *Gallus gallus* and other bird species (Baker and Marshall, 1997). In addition, the gene order flanking the control region, i.e., $tRNA^{glu}$ at the 5' side and $tRNA^{phe}$ at the 3' side, is also conserved among the present crane species, in keeping with the order found in *G. gallus* (Desjardin and Morais, 1990), the lesser snow goose *Anser caerulescens* (Quinn and Wilson, 1993), and the greenfinch *Carduelis chloris* and the common chaffinch *Fringilla coelebs* (Baker and Marshall, 1997), although a novel gene order ($tRNA^{thr}$-5' control region 3'-$tRNA^{pro}$) has been found recently in the peregrine falcon *Falco peregrinus* and the gray-headed broadbill *Smithornis sharpei* (Mindell *et al.*, 1998). Therefore, the mt gene order alone may not represent a marker for investigating avian phylogeny at higher taxonomic levels.

Genetic variation in *G. japonensis*

Higher nucleotide sequence variation in domains I and III than domain II in the studied crane species meets well with another common structural feature of avian mtDNA control regions (Baker and Marshall, 1997). The occurrence of base substitution in *G. japonensis* control region was more frequent in domain I than in domains II and III among the seven individuals examined (Fig. 4), although the number of base substitution sites is small compared with those found in the control region of other bird species such as *C. alpina* (Wenink *et al.*, 1993) and *F. coelebs* (Marshall and Baker, 1997). Thus, intraspecific genetic variation in *G. japonensis* was examined with the 5' control region sequence spanning the domain I and the beginning of domain II. By determining this 418 bp sequence, only one novel substitution site was found by sequencing of this portion among 22 individuals, in addition to

12 sites found in seven individuals, of two local populations. This strongly suggests that no additional substitution sites could occur in the less variable domains II and III in these 22 individuals, verifying the 5' control region as the best target for genetic variation analysis in *G. japonensis*.

Seven and two control region haplotypes were found in 14 individuals of the East Asia mainland population and 15 individuals of Hokkaido population, respectively. The lower number of haplotypes and prevalence of the Gj2 haplotype in Hokkaido population may reflect a possible "bottleneck" effect, since the number of individuals in Hokkaido once decreased to about 30 in the years following World War II (Masatomi, 1981). Another possibility is that most of the Hokkaido individuals examined are kin, because they were captured accidentally, without the information of their habitat. The Gj2 haplotype was found in individuals captured from Tokachi, Kushiro and Nemuro areas covering nearly whole the eastern part of Hokkaido, whereas the Gj1 haplotype was found in those captured in Kushiro area (data not shown). Such a haplotype distribution, however, should be substantiated by an extensive phylogeographic study on a larger number of individuals with a known habitat. The Hokkaido population has long been debatable whether it is a local population or diverged from the mainland population at the level of subspecies as has been suggested by differences in the note structure of the female unison call (Archibald, 1976). However, the intraspecific genetic distance in *G. japonensis is* similar to that found in the other two *Grus* species (Fig. 3). In addition, the parsimony network (Fig. 5) and a NJ tree (not shown) strongly suggest that the Gj1 and Gj2 haplotypes of the Hokkaido population and the Gj3 to Gj9 haplotypes of the mainland population do not belong to a separate lineage. These findings indicate that the genetic difference between the two local groups of *G. japonensis* is small compared with the difference among subspecies of other birds such as *C. alpina* (Wenink *et al*., 1996), *F. coelebs* (Marshall and Baker, 1997) and the Canada goose *Branta canadensis* (Baker and Marshall, 1997), inferring little genealogical separation of the mainland and Hokkaido populations.

Further studies using individuals with a known birth place or breeding site will be needed to evaluate more precisely intrapopulational genetic structure in *G. japonensis*. A battery of other competent molecular markers such as microsatellite DNA and polymorphic nuclear genes will become useful for such studies, and to extend the present results.

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