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Studies on genetic diversity of the endangered red-crowned cranes (*Grus japonensis*) on Hokkaido Island, Japan

(北海道に生息するタンチョウ集団の遺伝的多様性に関する研究)

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Abstract

Populations that have drastically decreased in the past often have low genetic variation, which may increase the risk of extinction. Therefore, in conservation actions for endangered species, not only an increase in population but also maintenance of genetic diversity in populations is required for ensuring stable procreation. The molecular-based approaches were highly informative and could allow establishing the effective conservation decisions by highlighting fundamental information about genetic diversity.

The red-crowned crane (*Grus japonensis*) is recognized internationally as an endangered species. There are two geographically separated populations of this species; a continental population distributed in the eastern Eurasia Continent, and an island population distributed in limited regions on Hokkaido Island, Japan. Although the island population had experienced a severe bottleneck by the end of the 19th century, the population size has recovered to about 1500 and continues to increase now thanks to conservation efforts. Most of the previous studies used neutral mitochondrial DNA and microsatellite markers on limited samples. For the conservation action, it is needed to understand the precise genetic structure in the past and present, and to estimate the genetic diversity focusing on neutral and adaptive genes.

The gene duplication in mitochondrial DNA (mtDNA) has been reported in diverse bird taxa so far. Although many phylogenetic and population genetic analyses of cranes were carried out based on mtDNA diversity, whether mtDNA contains duplicated regions is unknown. In Chapter I, to address the presence or absence of gene duplication and investigate the molecular evolutionary features in mtDNA, spanning from ND5 to $tRNA^{Phe}$ genes in mtDNA of 13 crane species belonging to family Gruidae were analyzed. Based on these sequences, the gene order and the molecular phylogeny were reconstructed. I found that these crane species shared a tandem duplicated region. In addition, phylogenetic trees based on homologous sequences of control region (CR) and cytochrome *b* (Cytb) indicated the possibility of concerted evolution among the duplicated genes. The present study demonstrated the existence of a duplicated region in mtDNA. Thus, in a study about cranes using mtDNA markers, it is important not only to use homologous sequences, but also to distinguish paralogs from orthologs.

In Chapter II, to better understand the spatiotemporal changes in the genetic structure and genetic diversity of the red-crowned cranes on Hokkaido Island, CR in mtDNA of stuffed specimens (years 1878–2001) and tissue or blood samples (years 1970–2014) were analyzed. Three haplotypes were found, and one of them was a novel mtDNA

haplotype shared by 1997 and 2007 samples. The previous studies found ten haplotypes from a small number of samples derived from the continental populations. In addition, there were no clear differences in the haplotype composition through the time span. These results suggest that genetic diversity of the island population was lower than that of the continental population, and the low genetic variation of the island population persisted for the last hundred years.

On the other hand, the genes of major histocompatibility complex (MHC) is known to be functional genes playing an important role in the adaptive immune response of jawed vertebrates, and whose diversity related to disease resistance. In Chapter III, to evaluate genetic variation of the red-crowned cranes on Hokkaido Island, genotypes of exon 3 of MHC class IA genes (MHCIA) and exon 2 of MHC class IIB genes (MHCIIB) were determined. From 152 individuals, 16 and six alleles of MHCIA and MHCIIB were identified, respectively, and evidence of a positive selection at the antigen-binding sites in these genes was suggested. The phylogenetic analyses indicated evidence of trans-species polymorphism among the crane MHC genes, as well as those for other vertebrates. In addition, the genetic diversity in both classes of MHC genes at the population level was low. No geographic structure was found based on the genetic diversity of MHC genes. These results were

supported by microsatellite analysis.

General Introduction

In conservation actions for endangered species, not only an increase in population but also maintenance of genetic diversity in populations is required for ensuring stable procreation (Lande 1988; Amos and Balmford 2001). Deficiency of genetic diversity in small populations would cause inbreeding depression, increasing disease susceptibility and reduction of long-term survival of wild populations in changing environments (Keller and Waller 2002; Brook et al. 2008). These negative effects of low genetic diversity may further increase the rate of population decline. Such negative feedback loop is called 'extinction vortex' (Gilpin and Soulé 1986; Blomqvist et al. 2010), and its genetic influences have a more severe effect on smaller populations (Frankham 2005). The maintenance of genetic diversity can prevent these negative feedback, and promote a continuing ability to resist pathogens and adapting to changes in habitat environments. Molecular-based approaches were highly informative and could allow establishing the effective conservation decisions by highlighting fundamental information about genetic diversity.

The crane family Gruidae consists of 15 species and one of the most threatened families (Meine and Archibald 1996), and species of which distributed across five continents. Unfortunately, 11 species of them are categorized as being at risk of extinction by the International Union for Conservation of Nature (IUCN) Red List of the Threatened Species

(BirdLife International 2016). Previous studies (e.g. Glenn et al. 1999; Peterson et al. 2003; Jones et al. 2005) have investigated genetic information about these species using molecular markers such as mitochondrial DNA (mtDNA) and microsatellite for the species conservation.

The red-crowned (or Japanese) crane *Grus japonensis* has also been listed as 'Endangered C1' on the IUCN Red List and as a national endangered species under the Japanese law for conservation. There are two geographically separated populations of this species: a continental population distributed in the eastern Eurasia Continent, and an island population distributed on Hokkaido, a northernmost island of Japan (Meine and Archibald 1996). The continental population is migratory, and they move long-distance from breeding grounds in east-central China and the Korean peninsula, whereas the island population is non-migratory (Johnsgard 1983; Higuchi et al. 1998; Sun et al. 2015).

In Japan, nonetheless, the past population of red-crowned cranes was also migratory and had been observed in central Honshu in winter until the middle of the 18th century (Masatomi 2000; Hisai 2009). The size of the island population reduced to near extinction at the end of the 19th century owing to extensive industrial and agricultural developments (Masatomi 2000), and this population had been thought to be extinct. It is 1924 when about 20 individuals were rediscovered in the Kushiro Wetland, eastern Hokkaido. Thanks to conservation efforts

such as artificial feeding in winter and the wetland protection, the island population has recovered to over 1400 until 2012 (Masatomi et al. 2014). Additionally, Masatomi et al. (2007) estimated the immediate extinction risk from accidental death rate and the carrying capacity of the island population, and reported its risk was low. Although the number of individuals continues to grow, the habitat range is currently limited to a narrow area (eastern, northern and central Hokkaido). Besides, most of the island population concentrates for wintering in artificial feeding areas of the Eastern Hokkaido (Masatomi 2000). There was a concern that some kinds of infectious disease cause easily among wild individuals inhabiting at high density. The red-crowned crane was considered as an umbrella species in habitat environments (wetland), because the breeding sites of this species had higher bird species richness (Higa et al. 2016). Therefore, the conservation of this species' habitat accompanies protection of the other species habitat. Generally, populations that have experienced a severe population decline show manifestations of genetic effects, such as decreased effective population size, fitness, genetic variation, disease resistance, and long-term survival of wild populations in changing environments (Keller and Waller 2002). Although the population size of the red-crowned crane inhibiting Hokkaido was tending to be recovered, their genetic diversity was unknown.

Understanding the genetic characteristics of such bottlenecked population is essential for planning the wildlife conservation actions.

The contents of this thesis are as follows:

The Chapter I presents the study on determining the characteristics of crane mtDNA, such as the gene order and the existence of duplicated regions, and clarifying the phylogenetic relationships based on duplicated cytochrome *b* and control region sequences.

The Chapter II presents the study on assessing the genetic diversity of the red-crowned cranes on Hokkaido Island with mtDNA sequences and investigates its spatiotemporal changes with stuffed samples.

The Chapter III presents the study on the estimate the genetic diversity, adaptive variation and genetic structure of the red-crowned cranes in Hokkaido by analysis of microsatellites and functional major histocompatibility complex genes.

Chapter I

Gene duplication and concerted evolution of mitochondrial DNA in crane species

Introduction

The contents of this chapter are mainly based on Akiyama et al. (2017c). MtDNA is a useful tool for phylogenetic, phylogeographic, and population genetic studies because of the rapid rate of evolution, haploidy, and maternal inheritance (Sorenson and Quinn 1998). Although classification of species based on mtDNA sequence alone may result in misunderstanding (Edwards et al. 2005), analyzing mtDNA features is still a profitable approach for such phylogenetic studies. With a few exceptions, most animals' mitochondrial genomes are highly conserved and consist of 37 genes (13 protein-coding, two rRNA, and 22 tRNA genes) and a non-coding CR involved in initiating transcription and replication (Boore 1999; Clayton 1991; Moritz et al. 1987). Therefore, the gene order of animal mtDNA is also a useful index for phylogenetic studies of many taxa (Boore 1999; Quinn and Wilson 1993). Whereas the nucleotide sequences of each gene in mtDNA are homologous among vertebrates, the gene orders of mtDNA in avian species are especially different from other vertebrates.

Desjardins and Morais (1990) reported the gene order within chicken mtDNA is different from that of other vertebrates according to one-time duplication of mitochondrial genes followed by losing some of them. The gene order of chicken mtDNA is widespread in many avian taxa as an avian standard order. To date, the variation of gene order in avian mtDNA has been reported in many species such as parrots (Eberhard et al. 2001), albatrosses

(Abbott et al. 2005), ruff (Verkuil et al. 2010) and falcons (Mindell et al. 1998). Schirtzinger et al. (2012) reported that the gene duplications have occurred many times in mitochondrial genome in parrots. It is commonly described that the derived gene order in avian mtDNA has evolved independently multiple times (Mindell et al. 1998; Singh et al. 2008). On the other hand, Gibb et al. (2007) reported that the completely conserved tandem duplication as found in albatross could be an initial reorganization of the avian gene order, and that gene loss or reduction makes the change of gene orders. The derived avian mitochondrial gene orders appear to have the feature of an initial tandem duplication of the Cytb, $tRNA^{Thr}$, $tRNA^{Pro}$, *NADH6 (ND6)*, $tRNA^{Glu}$ genes followed by CR. The completely conserved tandem duplicates of $tRNA^{Thr}$, $tRNA^{Pro}$, *ND6*, $tRNA^{Glu}$ and CR have been reported in some avian species including albatrosses (*Thalassarche* (Abbott et al. 2005); *Phoebastria* (Eda et al. 2010)), black-faced spoonbill (Cho et al. 2009), boobies (Morris-Pocock et al. 2010) and hornbills (Sammler et al. 2011). In addition to the protein-coding regions, nucleotide sequences of the second CR of these species are highly homologous to those of the first (original) CR, and seem to be able to function. Among paralogous CRs within individuals, the concerted evolution maintained the sequence similarity to avoid transcription error (Tatarenkov and Avise 2007). In addition, Abbot et al. (2005) reported that the concerted evolution could have

occurred among each duplicated tRNAs and each *ND6*s.

The gene conversion caused by recombination has been reported in taxa with tandem duplications of large mtDNA fragments, such as the albatrosses (Abbott et al. 2005) and boobies (Morris-Pocock et al. 2010). For comparative analyses among species, it is important to consider the copy number and to identify orthologous regions of the target region. The family Gruidae in order Gruiformes consists of 15 crane species: 11 of which are categorized as being at risk of extinction by the IUCN Red List of the Threatened Species (BirdLife International 2016). Although mtDNA of the crane species had been studied to evaluate the genetic variations and phylogenetic relationships within and among the species (Krajewski and Fetzner 1994; Rhymer et al. 2001; Wood 1996), it is still unclear whether or not any duplicated region in the mtDNA exists. The part of mtDNA was duplicated in a similar way of gene order (e.g., Pelecaniformes, Sula boobies; Procellariiformes, Thalassarche/*Phoebastria* albatrosses). Although it is considered that the duplication event has occurred independently in different avian lineages, it is not clear how species have shared such gene duplications.

In this study, to clarify whether or not the genomic duplication occurs in mtDNA of crane species, the mtDNA sequences spanning a region from Cytb to CR reported as the

crane gene order (Krajewski et al. 2010) was investigated. In addition, to reveal the molecular evolutionary features, I examined the phylogenetic relationships based on corresponding sequences (two Cytbs and two CRs) obtained from 13 crane species. The results showed the duplication event shared by the crane species, and also evidence of concerted evolution among paralogous genes in mtDNA.

Materials and Methods

Samples and DNA extraction

To investigate the existence of the tandem duplication and the patterns of evolution in crane mtDNA, 16 samples from 13 crane species were analyzed: four individuals of the red-crowned crane and one individual from each of 12 other species shown in Table I-1. Blood, lung or liver tissues were preserved in 99% ethanol at -20˚C until use. Fibroblasts were cultured from small pieces of skins according to Nishida et al. (2013), and stored in liquid nitrogen until use. Feathers were non-invasively obtained from individuals in zoos (Table I-1), and stored at -20˚C until use. Total DNA was extracted from the tissues and fibroblasts with the DNeasy Blood & Tissue Kit (Qiagen), and from feather shafts with the QiaAmp DNA Micro Kit (Qiagen). All the kits were used according to the manufacturer's protocols.

DNA amplification and sequencing

First, to reveal the gene order and the possibility of the existence of duplicated regions in crane mtDNA, I attempted to perform polymerase chain reaction (PCR) amplification and determine nucleotide sequences of a region encompassing from the 3' end of ND5 to 5' end of $tRNA^{Phe}$ genes in mtDNA of two red-crowned cranes. The PCR amplification was performed in a total 20 μl of the reaction mixture containing 70-100 ng of the DNA template, 0.4 μl of PrimeSTAR GXL Polymerase (TaKaRa), 4.0 μl of 5× buffer, 1.6 μl of 2.5 mM dNTPs, and 0.3 μl of each primer (20 pmol/μl). The PCR conditions were 15 × [98 °C/10 s, 58–62 °C/5 s, 68 °C/15 min]. Primers used for PCR and sequencing were listed in Table I-2. The almost whole mtDNA was PCR-amplified with primer set, LCO3g/HCO3g, designed in the COIII gene region to minimize the possibility of amplification of nuclear copies of mtDNA (*numts*). Using 1.0 μl of 1/20 dilution of the first PCR product, nested PCR was performed to amplify Fragment A (Fig. I-1). Fragment A between two presumed CRs was amplified with the primer set, Ldbox/Hbirdsb. These primers can anneal the 3' or 5' ends of the target region only when the CR regions are duplicated. Because this amplification yielded PCR products, I judged that the mtDNA in the red-crowned crane has the duplicated region. The duplicated

region between the Cytb and $tRNA^{Phe}$ genes was amplified in three overlapping fragments using primer sets, Lcfb58/H463cr (Fragment B); LtrnaP/H15655 (Fragment C); and LtrnaG/H01251 (Fragment D).

Second, the duplicated regions of 12 other cranes species were analyzed for comparison with that of the red-crowned crane. Because Pereira and Baker (2004) indicated that the PCR products of about 1500 base-pairs (bp) were likely to represent true mtDNA fragments from the chicken, those having over 1500 bp were selected and also amplified in the crane samples to avoid amplification of *numts* having mtDNA-like nuclear sequences. The presence of the duplicated regions in mtDNA of other crane species was examined by using primers, Ldbox and H463cr (Fragment A' in Fig. I-1); these primers were oriented so as to obtain the PCR product only when the CRs were duplicated. Fragments B, C, and D in the duplicated region between Cytb1 and 12SrRNA were then amplified by using the primer pairs shown in Figure I-1.

The PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen) or the QIAquick PCR Purification Kit (Qiagen), and sequenced with the BigDye v3.1 Cycle Sequencing Kit (Life Technologies) and an ABI 3730 DNA automated sequencer. The PCR products were sequenced using the same primers as used for amplification of each fragment

or the internal primers (Table I-2).

Phylogenetic analyses

Nucleotide sequences were aligned using software MEGA v5.0 (Tamura et al. 2011). For further investigation of the evolution of the duplicated regions, I focused on Cytbs and CRs in each duplicated sequence (Fig. I-1). I used sequences of Cytbs (Cytb1 and Cytb2) and CRs (CR1 and CR2) obtained from all samples, and made phylogenetic analyses with both the maximum likelihood (ML) and Bayesian inference (BI) methods. The best-fit nucleotide substitution models under the Bayesian information criterion (BIC) for ML and BI were estimated using Kakusan4 (Tanabe 2007, 2011). The ML analysis was executed in RAxML v8.0.20 (Stamatakis 2014). Nodal supports were evaluated through 10^3 bootstrap replicates. The BI analysis was executed in MrBayes v3.2 (Ronquist and Huelsenbeck 2003). Parameter spaces were explored in MrBayes using one cold chain and three incrementally heated chains; each analysis was run for 2×10^6 generations, with sampling every 100 generations, and first 25% of saved trees were discarded as burn-in. To verify convergence of the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) process, two simultaneous runs were continued until the standard deviation of split frequencies between the chains was lower

than 0.01. Convergence of MCMCMC parameter estimates was confirmed by using the Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/). Effective sample size values of all parameters were at least 200. The ML and BI trees were edited with the FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Results

Order of mtDNA genes in family Gruidae

About 5.3-kbp nucleotide sequences including the duplicated region spanning from the 3' end of ND5 to the 5' end of $tRNA^{Phe}$ genes (Fig. I-1) were obtained from the 13 crane species examined. The duplicated sequence I and II included the $tRNA^{Thr}$, $tRNA^{Pro}$, ND6, and $tRNA^{Glu}$, CR and Cytb sequences. Fragments of 20 bp in genus *Balearica* and 90 bp in genus *Grus* were also found between the duplicated sequence I and II. The obtained sequence data of 13 crane species were deposited to the DDBJ/EMBL/GenBank databases under accession nos. LC114470-LC114485. The gene order within the duplicated region (Fig. I-1) was identical not only in all the 13 crane species but also diverse species such as black-faced spoonbill (Cho et al. 2009), hornbills (Sammler et al. 2011), and albatross (Abbott et al. 2005). The length of each of tRNA genes and ND6s were identical among the species analyzed. Nucleotide sequences of the tRNA genes and *ND6*s are highly homologous, except for *ND6*-2

of the sandhill crane (*Grus canadensis*) that had a stop codon within it. The Cytb2 composed of 507 bp was highly homologous to the 3' end of Cytb1. Since the Cytb2 lacked the 5' region, compared with Cytb1 with full-length, it could be malfunctional. The 5' ends of CRs containing a C-rich site (Quinn and Wilson 1993) were highly variable and showed low sequence similarity among all the crane species. CRs found in species shared highly homologous sequences, except the 5' and 3' ends, and all of them seemed to be functional. The CR1 and CR2 found in all 13 crane species examined had some conserved regions, such as the C, D and F boxes and a short conserved sequence box (CSB-1) (Table I-3), as reported in other avian species (Marshall and Baker 1997). The 3' end of CR2 in the black-crowned crane (*Balearica pavonina*) and gray-crowned crane (*B. regulorum*) shared short repetitive sequences, of which the number of the repeats could not be technically determined, that were not found in CR1.

Molecular evolution of each duplicated gene in mtDNA

Phylogenetic analyses were performed based on a 700 bp region of CRs and a 507 bp region of Cytbs, excluding the 5' and 3' ends containing the variable regions and the repetitive sequences in the black-crowned and gray-crowned cranes (Fig. I-1). The ML analyses were

carried out under the general time reversible model with a proportion of invariant sites and gamma-distributed rate variation (GTR+I+G). The BI analysis using Cytbs sequences were also performed with the optimal nucleotide substitution models determined by BIC using Kakusan4 as SYM+G for codon position 1, HKY85+I+G for codon position 2 and HKY85+G for codon position 3. Because the ML tree showed the same clade and divergence pattern as the BI tree, only the BI tree with ML bootstrap values was shown in Figure I-1. The paralogous CRs (CR1 and CR2) in all the 13 species were more closely related to each other than to the orthologous CRs, mostly with >70% bootstrap supports and >90% Bayesian posterior probabilities. The paralogous Cytbs (Cytb1 and Cytb2) of most crane species except *G. monacha* and *G. nigricollis* (shown by a square in Fig. 2B) were more closely related to each other than to the orthologous Cytbs. The CR1 and CR2 haplotypes from the four red-crowned cranes (*G. japonensis*) formed a well-supported clade (Fig. 2A). Focusing on the red-crowned crane (sequence data obtained from multiple individuals), the orthologous sequences (CR1 and CR2; Cytb1 and Cytb2) were more closely related to each other within each individual than the paralogous sequences among individuals.

Discussion

Gene order of Gruidae species and timing of duplicated event

A previous phylogenetic study of cranes based on mtDNA sequences (Krajewski et al. 2010) did not consider the existence of duplicated region. The present study revealed the existence of gene duplication, and showed the characteristic features of mtDNA sequence of 13 crane species. Although the nucleotide sequences of the entire duplicated region of all examined samples were determined with only one individual per each species (except for four individuals of the red-crowned crane), it was shown that all the duplicated regions shared the duplicated unit consisting of Cytb, $tRNA^{Thr}$, $tRNA^{Pro}$, *ND6*, $tRNA^{Glu}$, and CR. The gene order in the tandem duplicated region was also conserved among the 13 crane species. The results indicate that the duplication event occurred in the common ancestral lineage of the crane species. Krajewski et al. (2010) showed that the *Grus* lineage diverged from the *Balearica* lineage approximately 31-37 million years ago, suggesting that the duplication event occurred at the time or earlier; however, more data are needed to determine the precise timing of the gene duplication. In mitochondrial genome of many avian species, the gene organization has been driven by the initial tandem duplication followed by several gene losses or reductions (Gibb et al. 2007). In Gruidae, however, no evidence of the gene losses or degenerations has been found in the duplicated region except in Cytb2 and the sandhill crane ND6-2. Furthermore, the gene order within the duplicated region in the crane species was similar to

that in diverse birds species having the completely conserved tandem duplicated region (albatrosses: [Abbott et al. 2005; Eda et al. 2010], spoonbills: [Cho et al. 2009], boobies: [Morris-Pocock et al. 2010]). These facts suggest that the duplication event occurred more than 34 million years ago. Alternatively, it is possible that the duplication event has occurred multiple times within Gruidae. However, the frequency of occurrence of gene duplication in avian mtDNA is under discussion (Bensch and Härlid 2000; Gibb et al. 2007), and the similar structure within the duplicated region seems to be precluded by repeated convergent duplications (Morris-Pocock et al. 2010).

Molecular evolution of duplicated genes in mtDNA

In this study, the CRs were phylogenetically grouped together within each species as paralogous, rather than across species as orthologous. The phylogenetic trees based on Cytbs and CRs showed high similarity except for the hooded crane and black-necked crane. These results indicate that the duplicated sequences have evolved concertedly within crane species, which was also reported by Abott et al. (2005) and Morris-Pocock et al. (2010) on the duplicated CRs in other avian taxa. Because the topology of the phylogenetic tree based on Cytbs was not completely identical with that on CRs among species (shown by a square in

Fig. I-2B), concerted evolution of short regions in the duplicated region might have occurred independently. All the other duplicated genes in each individual could be functional and showed high sequence similarities among orthologous sites except the *ND6*-2 in the sandhill crane containing a stop codon. The functional advantage of having duplicated control regions was shown to be increasing efficiency in the initiation of replication or transcription (Kumazawa et al. 1995; Shao 2003). However, the advantage of having extra copies of functional genes remains unclear, because one of the two *ND6* (*ND6*-2) in the sandhill crane has a stop codon and may be malfunctional. Among the red-crowned crane individuals, the orthologous CRs and Cytbs formed a phylogenetic group, indicating that the paces of concerted evolution between CR1 and CR2 or between Cytb1 and Cytb2 are faster than the rates of interspecific divergence, but slower than the rate of intraspecific divergence.

Chapter II

Mitochondrial DNA analyses revealed the low level of genetic diversity of red-crowned cranes on Hokkaido Island over the hundred years

Introduction

The contents of this chapter are mainly based on Akiyama et al. (2017b). MtDNA could provide an information about phylogenetic and population genetic relationships because of the rapid rate of sequence divergence, absence of recombination and maternal inheritance (Sorenson and Quinn 1998; Harrison 1989). The variable regions (e.g. the CR) in mtDNA is relatively easy to use in examination because mtDNA appears in multiple copies in a cell and it typically flanked by highly conserved regions in which PCR primers could be designed (Galtier et al. 2009). Although species classification based on mtDNA sequences alone may sometimes result in misunderstanding (Edwards et al. 2005), analyzing mtDNA features is a useful approach for population genetics.

Hasegawa et al. (1999) reported 18 polymorphic sites in 418 bp of the CR2 in mtDNA to determine haplotypes. Previous mtDNA analyses identified only two haplotypes in the island population, whereas another 10 haplotypes were found in the continental population (Hasegawa et al. 1999; Miura et al. 2013; Sugimoto et al. 2015). However, as shown as in Chapter 1, I confirmed that the mtDNA of the red-crowned crane contains a duplicated region, including the control region; the existence of orthologs (CR1) was not addressed in previous studies. I therefore focused on the overlapping region to analyze mtDNA haplotypes of red-crowned cranes. In addition, Sugimoto et al. (2015) analyzed microsatellite variation and

found no regional genetic differences within the island populations. This also suggested that the genetic structure of the island population has changed as an effect of the increased number of cranes on Hokkaido (Masatomi et al. 2014). However, no direct comparison between the historical and recent genetic structures on the island population of the red-crowned crane has been performed to date.

In this study, the mtDNA analyses were conducted using samples collected between 1878 and 2014. The lower genetic variation among island populations and spatiotemporal changes in the island population were discussed.

Materials and Methods

Samples and DNA extraction

Information on stuffed specimens was collected with the cooperation of the Ministry of the Environment. Specimens with clear information on sampling localities in Hokkaido were selected. From these, feather roots were obtained from 17 stuffed specimens preserved in museums or research institutes in Japan (with the oldest samples from the 1870s: Table II-1). In addition, 213 samples of blood or tissues obtained from chicks or young birds were collected: 29 samples from carcasses stored in the Kushiro City Zoo (Yoshino 2017); 184 samples captured for the bird banding by the Red-Crowned Crane Conservancy or wounded

in accidents and rescued by the Kushiro City Zoo. As all the crane individuals mentioned above were captured in Japan, information on capture points or birth locations was available. Total DNA was extracted using the QIAamp DNA Micro Kit (Qiagen) or the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's protocols. To avoid contamination during the analysis of feather samples, DNA extractions were performed using disposable plastic gloves, tubes, and other equipment sterilized by using UV radiation.

Amplification and sequencing of mtDNA

Because two control region loci (CR1 and CR2) originated from the gene duplication were found in mtDNA of the red-crowned crane (as shown as Chapter I), new primers for PCR were designed to specifically amplify the CR2 region. Fragment 1 (Fig. II-1), containing the complete CR2, was amplified from 210 samples consisting of the stuffed specimens and the contemporary individuals, using primers (primer *1:* Lcr1cyt2, 5′-GCC CAT ATT CCA CCC CTA TT-3′; primer *8*: H15030, Omote et al. 2013: the primers' positions are shown in Fig. II-1), and sequenced by using internal primers (*2:* L90nd6, 5′-CCA ACC CTC CCA ATA CAA AA-3′; *6:* H680cr, 5′-TGT AAG CAC CCG CTG CAC-3′; *7:* H01251, Omote et al. 2013). PCR amplifications were carried out using the PrimeSTAR GXL DNA Polymerase

(Takara) in a volume of 20 μl containing 70–100 ng of DNA template, 0.4 μl of PrimeSTAR GXL Polymerase, 4.0 μl of 5× buffer, 1.6 μl of 2.5 mM dNTPs, and 0.3 μl of each primer (20 pmol/μl). The reaction conditions were 25 × [98 °C/10 s, 60 °C/5 s, 68 °C/2 min]. The amplicons were purified using the QIAquick PCR Purification Kit (Qiagen), and sequenced with the BigDye v3.1 Cycle Sequencing Kit (Life Technologies) and an ABI 3730 DNA automated sequencer (Life Technologies).

Amplification and sequencing of mtDNA with old feather samples

Because the stuffed specimens included feather samples more than 100 years old, the genomic DNAs in the old specimens were often highly fragmented, resulting in difficulties in the PCR amplification of long sequences; other primers therefore were designed to amplify the shorter fragments of the CR2 region. Because the 418 bp region was located at the 5′ end of the CR2 including 18 polymorphic sites, it was thought to be of sufficient length to distinguish haplotypes. This region was divided into two fragments, and each fragment was amplified by an independent PCR using primers (*2:* L90nd6; *4:* H196cr2, 5′-ATT GGG TTG GAC AGT GGT TG-3′) for the fragment 2 (286 bp), and the primers (*3:* L128cr2, 5′-CAT TCA ATG TAA AAG ACA CAT CAC A-3′; *5:* H463cr, Akiyama et al. 2017c) for fragment 3 (381 bp).

The PCR amplifications were performed in a volume of 20 µl containing 20–60 ng of DNA template, 0.4µl of PrimeSTAR GXL DNA Polymerase, 4.0 µl of 5× buffer, 1.6 µl of 2.5 mM dNTPs, 0.2 µl of 20mg/ml bovine serum albumin (BSA, Roche Diagnostics), and 0.3µl of each primer (20 pmol/µl). The BSA binds to various PCR inhibitors possibly included in old samples. The PCR conditions for the old samples were 35 × [98 °C/10 s, 60 °C/5 s, 68 °C/20 s]. The PCR products were purified and sequenced as described above.

Analyses of mtDNA sequences

The mtDNA sequences were aligned with MEGA v5.0 (Tamura et al. 2011). To investigate the relationships among mtDNA haplotypes, a parsimony network of haplotypes was generated using TCS v1.21 (Clement et al. 2000). Ten additional haplotypes reported in previous studies (Hasegawa et al. 1999; Miura et al. 2013b) were obtained from the DNA Data Bank of Japan (DDBJ) (accession numbers AB714138–AB714140 for Gj3–5; and AB669093–AB669095 for Gj10–12; Table II-2). For further study on the evolutional relationships among mtDNA haplotypes, a phylogenetic tree was reconstructed with BI. This analysis used the complete CR2 sequences (haplotypes Gj1–5, Gj9–13 and that of *Grus leucogeranus* as an outgroup; LC114478) aligned with the MUSCLE algorithm (Edgar 2004).

The best-fit nucleotide substitution models under the BIC for BI analysis were estimated using Kakusan4 (Tanabe 2007, 2011). The BI analysis was executed with MrBayes v3.2 (Ronquist and Huelsenbeck 2003). Parameter spaces were explored in the analysis using one cold chain and three incrementally heated chains; each analysis was run for one million generations, with sampling every 10^3 generations, and first 25% of saved trees were discarded as burn-in. To verify convergence of the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) process, two simultaneous runs were continued until the standard deviation of split frequencies between the chains was lower than 0.01. Convergence of MCMCMC parameter estimates was confirmed using Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer). Effective sample size values of all parameters were at least 200. The BI trees were edited using FigTree v1.4.2 (http://tree.bio.ed.ac.uk.software/figtree/). To visualize the spatiotemporal changes of distribution and frequencies of mtDNA haplotypes, its haplotypes were plotted on a map of Hokkaido over the last several decades.

Results

Relationships of mtDNA haplotypes

Partial (440 bp containing the 418 bp region) or complete (1162 bp) sequences of the mtDNA CR2 were determined on the samples, including 17 stuffed specimens (collected in 1878–

2001) and 213 tissue or blood samples (collected in 1970–2014). In the present study, three haplotypes (Gj1, Gj2 and Gj13) were identified from the island population. Haplotypes Gj1 and Gj2 were frequently found among individuals of the island population (Hasegawa et al. 1999; Miura et al. 2013a; Sugimoto et al. 2015). In contrast, in the present study, haplotype Gj13 was identified for the first time as a novel haplotype from a single stuffed specimen and one contemporary sample. The obtained sequence data were deposited to the DDBJ/EMBL/GenBank databases under accession no. LC129041. This new haplotype Gj13 had only two substitutions from haplotype Gj1 at nucleotide positions 440 and 550, both of which were highly polymorphic sites within the CR2 (Table II-2).

Figure II-2 shows a parsimony network of the mtDNA haplotypes based on the 5' end of 440 bp in the CR2. Haplotype Gj13 was a missing link between Gj1 and Gj6. Haplotypes Gj3–9 were found in the continental population (Hasegawa et al. 1999), and haplotypes Gj10–12 were found in the feather samples of field-collected cranes in the Honshu main island of Japan, which may have been derived from continental individuals flying to Japan; no information was available on whether the individuals were wild or bred (Miura et al. 2013b). As the previous study (Hasegawa et al. 1999), the three haplotypes identified from only the island population (Gj1, Gj2 and Gj13) and ten haplotypes identified from only

continental individuals (Gj3–12) formed a common cluster. In addition, continental haplotypes (Gj5, Gj6 and Gj7) were positioned between the Hokkaido haplotypes Gj2 and Gj13 and other continental haplotypes connected to the Gj5, Gj6 or Gj7 (Fig. II-2). Figure II-3 shows the phylogenetic relationships among haplotypes, reconstructed by the 1141 bp of CR2. The optimal nucleotide substitution model determined by BIC4 (sample size = the number of sites) using Kakusan4 was HKY85+Gamma. Haplotypes from the island population were polyphyletic, whereas Gj1 and Gj13 formed a well-supported clade, and Gj2 formed a clade with Gj12, which was thought to be a continental haplotype.

Frequencies of mtDNA haplotypes in the island population

The mtDNA haplotype frequencies in the island populations were as follows: 8.7% (20/230 individuals) for Gj1; 90.4% (208/230 individuals) for Gj2; 0.9% (2/230 individuals) for Gj13. Figure II-4 shows the transition of the mtDNA haplotype distribution in the island population over the past several decades, although information on the old feather samples such as capture areas and birth years was incomplete. Haplotype Gj2 occurred more frequently in all time-spans, and this haplotype was widespread in eastern Hokkaido. In addition, Gj1 was also found in most regions in Hokkaido through 1878–2014, except in few occasions such as

Nemuro in the 2010s (Fig. II-4E) and Abashiri across all time-spans. The novel haplotype Gj13 was identified from one stuffed specimen sample obtained in Kushiro in 1997 and one contemporary sample, also obtained in Kushiro in 2007. In most contemporary samples in the 2010s (Fig. II-4E), no additional novel haplotypes were found.

Discussion

Frequencies of mtDNA haplotypes in the island population

In the present study, three CR2 haplotypes were identified from samples of the island population over the past several decades. No common haplotypes were found between the continental and island populations. Haplotype Gj13, which found in only two samples in Hokkaido, was positioned at a missing haplotype near haplotype Gj1 in the haplotype network, indicating that Gj13 is specific but infrequent in the island population. Nevertheless, it is uncertain whether Gj13 remains in the modern population, as it has not been identified from any samples collected after 2007. This haplotype could have disappeared due to inbreeding or genetic drift after the bottleneck. Haplotype Gj2 was found most frequently among samples, irrespective of region and period (Fig. II-4). The higher frequencies of haplotypes Gj1 and Gj2 were in agreement with those in previous reports (Miura et al. 2013a; Sugimoto et al. 2015). Sugimoto et al. (2015) reported no apparent spatial genetic structures in the current island

population from microsatellite and mtDNA analyses. The present study also showed a low genetic variation of the island population, and that this state has continued for about 100 years. The lower levels of genetic variation in the red-crowned crane of Hokkaido may have resulted from the population recovery from a very small number of individuals over the past few decades, as reported by Masatomi (2000). Unfortunately, the number of historical stuffed specimens samples examined in the present study was too low to assess the effects of the past population decrease in Hokkaido. It is necessary to analyze older samples, such as archaeological remains or greater numbers of historical samples of the red-crowned crane for the investigation of the following points: (1) whether there were any common haplotypes in mtDNA between the continental and island populations in the past; (2) detailed genetic structure changes during and before the population decline.

Evolutionary relationships among mtDNA haplotypes of the red-crowned crane

The haplotypes of the red-crowned crane mtDNA found in the island population did not form a single clade. This suggests that the population was divided after the divergence of the haplotypes, and it is possible that the island population had other haplotypes and then lost some of them through a severe population decline. To investigate the process of the genetic

structure formation, the migration rate and the diverged period between the continental and island population, it is necessary to precisely compare the genetic variation of the island population with those of the continental population.

In a previous study on the structure of the female unison calls (Archibald 1976), the island population has diverged from the continental population at a subspecies level. However, the phylogenetic analysis of the present study shows that the mtDNA haplotypes found from the two populations were not largely differentiated. Although it was reported that some individuals flew from the continent to Japan in 2008–2011 (Miura et al. 2013b), this study suggests no gene flow (Fig. II-4D, E). Genetic exchange between two populations may improve the low genetic variation in Hokkaido. It seems difficult for the island population, in which genetic structure was equalized and genetic variation was low, would be able to significantly recover the genetic variation on its own. Accordingly, if mating between the continental and island individuals is possible; conservation plans might include the artificial introduction of continental individuals to Hokkaido for the recovery of genetic variation in the island population. If that is pursued, however, it will be important to consider that encounters between individuals from different habitats carry the risk of transmitting pathogens derived from each habitat. Because red-crowned cranes live in narrow regions in Hokkaido at a high density, there is a serious

concern about outbreaks of infectious disease. In future conservation efforts, it is important to promote an expansion of its distribution by artificial movement and dispersal in order to reduce the risk of morbidity and population extinction. In addition, further studies on functionally important genes, such as major histocompatibility complex genes, could be useful for evaluating adaptive genetic variation in the island population and monitoring the genotypes of the continental individual to support potential introduction strategies.

Chapter III

Genetic diversity at microsatellite loci and MHC of red-crowned cranes on Hokkaido Island

Introduction

The contents of this chapter are mainly based on Akiyama et al. (2017a). The genetic variation of the island population of red-crowned cranes has been examined using neutral genetic markers such as mtDNA sequences and microsatellites, and it was reported that the island population has lower genetic diversity than the continental population (Hasegawa et al. 2000; Miura et al. 2013b; Sugimoto et al. 2015; Akiyama et al. 2017b). The neutral markers can provide information about the population history (past demographic expansions/contractions), dispersal patterns, gene flow and genetic drift (Zhang and Hewitt 2003), but information on adaptive potential is not provided. Information on adaptive genetic variation is essential for making optimal management decisions in consideration of adaptive process (Funk et al. 2012).

Genes of the MHC encode the proteins that are associated with a primary factor in initiating the vertebrate immune system. The MHC multigene family contains two main classes (class I and II genes). Genes in the two classes encode cell-surface glycoproteins that bind antigen peptides derived from intracellular or extracellular pathogens and present them to T-cells, initiating an immune response (Klein 1986; Piertney and Oliver 2005). The MHC class I proteins consist of an alpha chain and a β2-macroglobulin. In particular, the alpha chain has antigen-binding site (ABS) encoded by exons 2 and 3 in MHC class IA gene. On

the other hand, the MHC class II proteins consist of alpha and beta chains, which have ABS, encoded by exons 2 of MHC class IIA and class IIB gene. The MHC proteins display high polymorphisms especially at amino acid sequences lying in ABS. The high levels of sequence polymorphisms in ABS were likely maintained for recognizing diverse pathogens (Piertney and Oliver 2005). Kohyama et al. (2015a) isolated and sequenced spanning exons 1 to 4 of MHC class IIB gene from 13 crane species and three other species closely related to cranes, and reported that the most polymorphisms were located in exon 2. Alcaide et al. (2009) developed primers for the amplification of exon 3 of MHC class IA gene based on the sequences of some bird species including the crane species.

High polymorphisms of MHC could be maintained due to balancing selection mediated in pathogen pressure with gene duplication (Hughes and Nei 1988; Hughes and Nei 1989; Garrigan and Hedrick 2003), and likely affect on sexual selection in mate choice (Ziegler et al. 2005). In addition, balancing selection on MHC genes gives rise to the maintenance of MHC allelic lineages among related species for longer terms, and has resulted in the pattern of trans-species polymorphism (Klein 1987). Low MHC variation in wild populations, which experienced population reduction in their history, was reported in some vertebrate species (Bollmer et al. 2007; Siddle et al. 2007; Niskanen et al. 2014; Kohyama et

al. 2015b), and such populations are potentially susceptible to pathogens and increase the risk of extinction. In addition, inbred populations tend to have low MHC variation, followed by lower resistance to parasites (Radwan et al. 2010). In conservation genetics for wild animal populations, measuring polymorphisms in MHC genes provides useful information of the immunological adaptation and fitness (Ujvari and Belov 2011).

In this study, the allelic variation of MHC class IA (MHCIA) and IIB (MHCIIB) genes were investigated in 152 red-crowned cranes of the island population by the next generation sequencing approach. In addition, neutral genetic diversity using microsatellite markers was examined for the comparison with it of functional MHC genes. The sequence polymorphisms were evaluated and the evidence of selection was provided at IA exon 3 and IIB exon 2

Material and Methods

Samples, DNA extraction, RNA extraction and cDNA synthesis

Blood or liver tissue samples of 152 red-crowned cranes were collected from Abashiri (n = 3), Kushiro (n = 61), Nemuro (n = 41) and Tokachi (n = 47) districts on Hokkaido Island, Japan, between 2006 and 2014 (Fig. III-1). All samples were obtained from chicks or first-year juveniles captured for leg banding as the conservation activities by the Red-crowned Crane

Conservancy or rescued from accidents by the Kushiro Zoo. Information on the birth years and locations were available for all samples. In addition, nine blood or fibroblast samples (each one individual per species) in family Gruidae were collected (Table III-1). Tissue samples were preserved at −20 °C in 99% ethanol, and blood samples were dried on filter papers until DNA extraction. Total genomic DNA (gDNA) was extracted with the DNeasy Blood and Tissue Kit (Qiagen). In addition, total RNA was extracted from fibroblasts from two individuals with the RNeasy Plus Mini Kit (Qiagen), and then cDNA was synthesized with SuperScript III First-Strand Synthesis System (Invitrogen). Fibroblasts were cultured from small pieces of skins of the two individuals in island population according to the method of Nishida et al. (2013).

Design of primers for 454 sequencing of the rad-crowned crane MHC genes

New primer sets (GrjaI3F, 5'-TCT CCC TGG TCR TGT TTC AGG GGC-3'/GrjaI3R, 5'-TAG CTC ACG TAT TTC CTC AGC CAC-3'; GrjaII2F, 5'-CTG ACC TGC CTC CCT GCA CAC ACA G-3'/GrjaII2R, 5'-GTG AGT GCG TGG CAG AAC ATT TCC C-3': Fig. III-2) were designed to amplify partial sequences of MHCIA exon 3 and complete sequences MHCIIB exon 2. In MHCIIB, primers were developed based on the consensus from published

sequences of 13 crane species (LC062901–LC062955). In MHCIA, PCR was firstly performed for amplifying the partial sequences spanning from 3'-end of exon 2 to 5'-end of exon 4 with gDNAs from eight and cDNA from two individuals for identifying exon/intron using three primers: MHCI-ex2F or MHCI-int2F/MHCI-ex4R, which were developed to bind within the conserved regions of avian MHCIA (Alcaide et al. 2009). These products were purified with the QIAEX II Gel Extraction Kit (Qiagen), and were cloned into the plasmid vector pBluescript II SK+ (Agilent Technologies) and transformed into *Escherichia coli* strain DH5α. Plasmids were isolated with the QIAprep Spin Miniprep Kit (Qiagen), and eight clones/samples were sequenced with the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies) and an ABI 3730 DNA automated sequencer (Applied Biosystems). Sequences obtained were aligned using MEGA v6 (Tamura et al. 2013), and primers were developed based on these sequences and the coding region sequence of the sandhill crane (*Grus canadensis*) (AF003106: Jarvi et al. 1999). The PCR amplifications were performed using the high fidelity DNA polymerase PrimeSTAR GXL (Takara) in a total volume of 20 μl containing 0.4 μl of PrimSTAR GXL Polymerase, 4.0 μl of 5× buffer, 1.6 μl of dNTPs (2.5 mM each), 0.3 μl of each primer (20 pmol/μl), and gDNA or cDNA (approximately 50 ng). The PCR conditions were 30 × [98 °C/10 s, 60 °C /5 s, 68 °C/90 s].

454 sequencing

454 sequencing was performed with the amplicon library pooling 338 amplicons (169 amplicons for each MHC class) constructed from 152 the red-crowned crane individuals. Seventeen individuals were independently amplified and sequenced twice to estimate the genotyping error.

For the amplicon library preparation, I used fusion primers consisting of the GS FLX Titanium Primer sequence (forward sequence, 5'-CGT ATC GCC TCC CTC GCG CCA TCA G-3' and reverse sequence, 5'-CTA TGC GCC TTG CCA GCC CGC TCA G-3'), followed by a 10 bp multiplex identifier (MID), and sequences of the specific primer (GrjaI3F/GrjaI3R for MHCIA; GrjaII2F/GrjaII2R for MHCIIB). Twenty-six MIDs (Nos.48–73) were chosen from the 10-base extended MID set from Roche Diagnostics (454 Life Sciences Corp 2009). Amplicons from different PCR reactions within the library were distinguished based on these MIDs in the forward and reverse primers. The PCR amplifications were performed in a total volume of 25 μl containing 0.5 μl of the PrimeSTAR GXL polymerase, 5.0 μl of 5× GXL buffer, 2.0 μl of dNTPs (2.5 mM each), 0.5 μl of each primer (10 pmol/μl), and gDNA (approximately 50 ng). The PCR conditions were 25 ×

[98 °C/10 s, 68 °C/90 s] and PCR products were purified with the MinElute PCR Purification Kit (Qiagen). To decrease the effect of chimera formation, the number of PCR cycles was minimized (Lenz and Becker 2008). After quantification by 1.5 % agarose-gel electrophoresis, the purified amplicons were mixed in approximately equimolar quantities (including approximately 30 ng of each product). The library was commercially sequenced on a 1/4 Titanium Pico Tire Plate with the GS FLX Titanium Sequencing Kit XLR70 (Roche) at Hokkaido System Science Co. (Sapporo, Japan).

MHC genotyping of the red-crowned cranes

I used commercially available CLC Genomics Workbench v8.5.1 to trim GS FLX Titanium primer sequences, and removed unapt sequence reads based on length and quality from the 454 output data. Allele detection and genotyping were performed with the pipeline ngs_genotyping (Pavey et al. 2013; https://github.com/enormandeau/ngs_genotyping). The parameter settings for steps in this pipeline are the same as that in Kohyama et al. (2015b), and these settings of the threshold value in this pipeline steps fulfill the two-PCR criterion that is the standard condition in MHC genotyping (Babik 2010). Although this is not strictly correct, the term 'alleles' was used to refer to unique sequence variants detected from multiple

loci for convenience.

Detecting signatures of historical selection and recombination in MHC

The number of segregating sites (S) and summary statics of sequence diversity including nucleotide diversity (π), Watterson's θ (Watterson 1975), and Tajima's *D* (Tajima 1989) among allele sequences were calculated with DnaSP v5 (Librado and Rozas 2009). To look for signs of positive selection on the ABSs in MHCIA and MHCIIB, the ω value was calculated. The ω value [non-synonymous substitution rate (d_N)/ synonymous substitution rate (d_S)] provides a measure of selection pressure (Nei and Kumar 2000; Yang 2001). The d_N values involve changes in amino acid sequences and are more likely to undergo selection than are d_S. The $\omega = 1$ supposes neutral genes, whereas $\omega > 1$ means that d_N had been maintained with processes such as positive selection (Spurgin and Richardson 2010). The putative ABSs were estimated from the chicken MHCIA (Wallny et al. 2006) and from human MHCIIB (Brown et al. 1993). The overall ω estimated by calculating the average values of d_N and d_S by the Nei-Gojobori method with the Jukes-Canter correction (Nei and Gojobori 1986), standard error estimates were based on 1000 replicate bootstrap procedure, and the *Z*-tests was performed for detecting the signature of selection using MEGA v6. These calculations were

performed separately for total sequences, the putative ABS and the non-ABS of each MHC class.

To identify a codon-specific signature of positive selection for alleles, the program omegaMap (Wilson and McVean 2006) was performed. This program uses a population genetics approximation of the coalescent with recombination, and a reversible-jump Markov chain Monte Carlo to perform Bayesian inference on both ω and the recombination rate (ρ). To calculate a unique ω and ρ for each codon, the prior distributions of the omegaMap were set with the following parameters ω and ρ: codon frequency = all codons had equal equilibrium frequencies, omega and rho prior distributions = inverse, omega model = independent, rho model = variable, rho block = 3. From gene pool of each MHC allele, 200 sequences for each MHC classes were randomly sampled. Twice independent MCMC chains were run for each random-sample with 10^6 iterations and combined each run with burn-in of 10% (10^5 iterations). Codons were considered to be under positive selection if the posterior probability of $\omega > 1$ exceed 0.95.

To explore the presence of recombinant sequences in identified alleles of each MHC class, recombination analysis were performed using the RDP4 beta 4.27 (Martin et al. 2010) with the following seven methods: RDP (Martin and Rybicki 2000), GENECONV

(Padidam et al. 1999), BootScan (Martin et al. 2005), MaxChi (Smith 1992), Chimaera (Posada and Crandall 2001), SiScan (Gibbs et al. 2000), and 3seq (Boni et al. 2007). Default settings as follow were used: the maximal acceptable *P*-value was 0.05; 100 permutations were performed for seven methods. Evidence of recombination events was detected when it was found to be significant in at least three or more methods of examined seven methods.

To estimate genetic differentiations among subpopulations on Hokkaido, population pairwise values of Nei's G_{ST} was calculated based on genotypes of MHC genes using the package mmod v1.2.1 (Winter 2012) in R, and the significance of the G_{ST} values was evaluated by 1000 replicate permutations.

Design of primers, cloning and sequencing for other Gruidae species

In MHCIA, only one sequence of Gruidae species was registered in NCBI nucleotide database (AF003106: Jarvi et al. 1999). To estimate the phylogenic relationship of MHC allele on Gruidae species, the partial sequence in MHCIA of nine species were detected. Genomic MHCIA sequences spanning 3'-end of exon 2 to 5'-end of exon 4 were amplified by PCR with MHCI-ex2F or MHCI-int2F/MHCI-ex4R. After sequence detected, new inner primer set was designed based on the alignment of these sequences (GrIint2F, 5'-TCT CCC

TGG TCR YTT TCA-3'/ GrI3R, 5'-GCC CGT AGC TCA CGT ATT TC-3': Fig. III-2). The PCR, purifying, cloning and sequencing were performed as seen above. PCR amplification for each sample was performed twice time, and at least 32 clones per PCR product were sequenced. The sequence was considered as valid alleles only if the identical sequences were obtained at least two times in independent plasmid clone.

Phylogenic reconstruction

To infer the evolutionary relationships among MHC alleles of red-crowned cranes and other birds, Bayesian phylogenetic analyses were executed with nucleotide sequences of each MHC class from following species: for MHCIA, Gruiformes, Pelecaniformes, Charadriiformes, and Procellariiformes; for MHCIIB, Gruiformes, Pelecaniformes, Procellariiformes, and Otidiformes (GenBank accession numbers were showed in Fig. III-9). After alignment of all sequences with the MUSCLE algorism (Edgar 2004), the best-fit nucleotide substitution models were estimated under the BIC4 (sample size = the number of sites) in Kakusan 4 (Tanabe 2011). Phylogenetic relationships were reconstructed by the Bayesian inference with MrBayes v3.2.4 (Ronquist et al. 2012). I run the program for 3×10^7 generations, with a sampling of every 1000 generations, and first 25% of sampling trees were discarded as

burn-in. Convergence of parameters was confirmed by the standard deviation of split frequencies (<0.01) and by visually using the Tracer v1.6 (Rambaut et al. 2014). Effective sample size values of all parameters were at least 200. The trees were visualized with the FigTree v1.4.2 (Rambaut 2014).

Microsatellite genotyping of the red-crowned cranes

Samples were genotyped at nine microsatellite markers: Gj-M8, Gj-M11a, Gj-M13, Gj-M15 and Gj-M34 (Zou et al. 2010); Gj4066, Gj1303, Gpa33, and Gpa34 (Hasegawa et al. 2000). Forward primers were fluorescently labeled, and three multiplex primer-pair mixes were created as follows: Mix 1 with Gj-M8 (PET) and Gpa33 (VIC); Mix 2 with Gj-M11a (NED), Gj4066 (PET) and Gj1303 (FAM); Mix 3 with Gj-M13 (FAM), Gj-M15 (VIC), Gj-M34 (NED) and Gpa34 (PET). The PCR amplifications using the Multiplex PCR Kit (Qiagen) were performed in a total volume of 5.0 μl including 1.0 μl of DNA template, 2.5 μl of 2× Qiagen Multiplex PCR Master Mix, 1.0 μl of distilled water, and 0.5 μl of primer mix (2 pmol/ml each primer). The PCR conditions were as follows: 95 °C/15 min, 30 × [94 °C/30 s, 60 °C/90 s (primer Mix 2) or 56 °C/90 s (primer Mix 1), 72 °C/90 s], 72 °C/10 min. The

fragment analyses were performed with an ABI 3730 DNA automated sequencer using the GeneScan 600 LIZ Size Standard (Life Technologies) as a size marker.

Microsatellite analysis and genetic diversity

Microsatellite allele size was analyzed by GENEMAPPER v4.0 (Life Technologies). Input files for population genetics programs were generated using PGDSpider (Lischer and Excoffier 2012). Allele richness (A_R) and the inbreeding coefficient (F_{IS}) were calculated using FSTAT v2.9.3.2 (Goudet 1995). Tests for deviations from Hardy-Weinberg equilibrium (HWE), and calculations of observed (H_O) and expected heterozygosities (H_E) were performed with ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010). The population pairwise values of Nei's G_{ST} based on genotypes of microsatellite was calculated by the same method as seen above. Additionally, to estimate the number of genetic clusters (K), the Bayesian clustering software STRUCTURE v2.3.4 (Pritchard et al. 2000) was run based on genotypes of microsatellites with default settings of an admixture model (sampling locations were set to prior) and correlated allele frequencies among subpopulations. The number of K ranged 1–5 was tested with ten independent replicates for each K. The running length was set 10^6 steps after an initial burn-in of 5×10^4 steps. Ten independent runs were conducted to quantify the

amount of variation in likelihood values. The support for different values of *K* was assessed by comparing the average log probability of *K* (LnP(*K*)), and the delta-*K* (Evanno et al. 2005) associated with each model was evaluated by using the program STRUCTURE HARVESTER (Earl and vonHoldt 2012).

Results

Identification of MHC alleles by 454 sequencing

A total of 219,672 reads with complete forward and reverse MIDs sequences were obtained: 109,201 reads ranging 162 to 2,438 reads per amplicon corresponding to MHCIA exon 3, and 110,471 reads ranging 613 to 1,932 reads per amplicon corresponding to MHCIIB exon 2. In MHCIA, because the reads from id034 sample (Table III-2) were not obtained, this data was excluded from subsequent analyses. Finally, 107,919 reads (the mean coverage [number of reads per amplicon] ± *SD* = 639 ± 355, ranging 157 to 2,411) were used for genotyping for MHCIA, and 108,622 reads (the mean coverage ± *SD* = 643 ± 327, ranging 186 to 1,941) were used for MHCIIB. Because the small library size (number of reads) could cause to underestimate allelic diversity for missing allele, the correlation between library size and allele number within an individual were estimated after genotyping. No significant correlation was identified between the library size (each MHC classes with >150 reads) and the allele

number per individual (Fig. III-3). Except for one of the 17 pairs sequenced twice to estimate the genotyping error in MHCIA, the same MHCIA/MHCIIB alleles were identified from the same sample pair. As a result, total 151 individuals in MHCIA and 152 individuals in MHCIIB were genotyped (Table III-2).

Sixteen alleles were identified from partial sequences of MHCIA exon 3 (222 bp: 82% of the complete exon 3 sequence) and the six alleles were identified from complete sequence of MHCIIB exon 2 (270 bp). Neither frameshift mutations nor stop codons were found in any obtained nucleotide sequences. The four MHCIIB alleles (Grja-DAB*01-04) had been previously identified from a red-crowned crane inhabiting Hokkaido (LC062928–LC062931; Kohyama et al. 2015a). Therefore, the remaining 16 MHCIA (Grja-UA*01-16) and two MHCIIB alleles (Grja-DAB*05-06) were named following the nomenclature proposed by Klein et al. (1990). Sequences of novel MHC alleles identified in this study were deposited in the DDBJ/EMBL/GenBank databases under accession nos. LC132723-LC132740.

The nucleotide sequences of alleles contained 26 variable sites for MHCIA, and 39 variable sites for MHCIIB (Fig. III-4). Fifteen distinct amino acid sequences with 12 variable sites were translated from the 16 MHCIA alleles, and all of six MHCIIB alleles have distinct

amino acid sequences with 19 variable sites (Fig. III-5). Allele frequencies ranged from 0.21 (Grja-UA*14–16) to 0.95 (Grja-UA*01) in MHCIA, and 0.21 (Grja-DAB*03) to 0.89 (Grja-DAB*01) in MHCIIB (Fig. III-6). The number of alleles per individual of each MHC class ranged as follows: 4–11 (mean = 8.636) for MHCIA; 2–4 (mean = 3.046) for MHCIIB (Fig. III-7). This result suggested the existence of two to six MHCIA loci and one to two MHCIIB loci. Always coexisting alleles in the same individuals were as follows: Grja-UA*04–07; Grja-UA*09, 10; Grja-UA*12, 13; Grja-UA*14–16; Grja-DAB*02, 06 (Table III-2). This shows the possibility that these allele sets were genetically linked.

Selection analysis on each MHC class

Both of overall values of Tajima's *D* for MHCIA exon 3 and MHCIIB exon2 were positive, whereas they were not significantly different from zero (Tajima's *D* =1.486 for MHCIA and 0.219 for MHCIIB: Table III-3). Fifteen codon positions for MHCIA and 24 codon positions for MHCIIB were inferred as ABSs, compared with chicken and human MHC sequences (Fig. III-5). The ω values of the putative ABSs were 1.143 for MHCIA and 1.063 for MHCIIB; therefore, these positions are thought to be under positive selection. However, these values were not significantly different from 1.0 (*Z*-values = 0.40 for MHCIA and 0.55 for MHCIIB;

P-values = 0.25 for MHCIA and 0.29 for MHCIIB: Table III-3). The ω values of non-ABS and all sites on each MHC class were less than 1.0. These values were also not statistically significant by *Z*-test. In addition, the π values of putative ABSs for each class were higher than those of the other sites.

The codon-based omegaMap analysis (Fig. III-8) indicated that nine of 73 codons within the MHCIA and 12 of 89 codons within the MHCIIB provided evidence for positive selection (minimum 95% highest posterior density of codon positions with $\omega > 1$), of which six (67%) for MHCIA, and eight (67%) for MHCIIB are located at in the putative ABS. Codon position 46 in MHCIA (Fig. III-8A) showed a posterior probability > 0.95, but ω value was not higher than 1.0. Some codon positions (3, 10, 16, 39 and 46 in MHCIA; 11, 21, 24, 67, and 68 in MHCIIB) were different from putative ABS sites, but the $\omega > 1.0$ with a posterior probability > 0.95. The averages of the ω of the entire sequences examined were 4.287 for MHCIA and 4.389 for MHCIIB. No codons within each MHC class showed significant evidence of recombination (minimum of 95% highest posterior density of ρ and mean ρ was not higher than 1.0) with omegaMap and RDP4. The averages of mean ρ were 0.161 for MHCIA and 0.080 for MHCIIB.

Phylogenetic analysis of MHC allelic sequences

Figure III-9A shows the Bayesian phylogenetic tree based on partial sequence of MHCIA exon 3 alleles. A division between families was not supported with high Bayesian posterior probability (BPP) and alleles of the same family were not formed monophyletic groups. Alleles of each Gruidae species did not form monophyletic groups. However, the alleles of the Gruidae were closer than other families. Figure III-9B shows the phylogenetic relationship based on MHCIIB exon 2 including two novel alleles found in this study. In Gruiformes, although the alleles of Rallidae (Eurasian coot) were separated, its of Gruidae and Psophiidae (Grey-winged trumpeter) formed monophyletic groups. In addition, one allele from the sandhill crane formed a group with that of genus Balearica. The alleles from the red-crowned crane in MHCIA and MHCIIB did not form a clade by themselves, and some of the alleles were genetically closely related to that of another crane. These trees also showed evidence of trans-species polymorphism in the allelic lineage of crane species.

Genetic diversity of microsatellites and population structure

All samples were successfully genotyped at nine microsatellite loci. The number of alleles per locus ranged from two to eight. Eight private alleles (A_P) were found when comparing an area

population with other areas population (Table III-4), and the six per eight alleles belonging to Kushiro area and two alleles belonging to Tokachi area, with a low frequency ranging 0.003–0.007. The H_O ranged from 0.55 to 0.59 (Table III-4). When all samples were analyzed as a single population, three loci (Gj-M34, Gpa34 and Gj-M15) significantly departed from HWE. One locus (Gj-M34) significantly departed from HWE in all pre-defined four areas, and one locus (Gj-M15) significantly departed in Abashiri and Kushiro area. F_{IS} ranges from -1 to 1, whose positive numbers indicate heterozygosity deficiencies, and F_{IS} values of each area were ranged from 0.02 to 0.06 (Table III-4), but there were not a significant deficit of heterozygotes. The pairwise G_{ST} values between sampling locations were not significant (Table III-5), and each value is as follows: median of the pairwise G_{ST} value with microsatellite = 0.005; median of the pairwise G_{ST} value with IA = -0.002; median of the pairwise G_{ST} value with IIB = -0.005. In addition, all samples were assigned to the same cluster in the STRUCTURE analyses with $K > 1$, $K = 1$ has the highest Ln P (K) value, indicating no apparent genetic diversity in the current island population (Fig. III-9).

Discussion

MHC in the red-crowned crane

This study provided the first population genetic characteristics of MHC genes of the

red-crowned crane inhabiting Hokkaido Island, Japan. MHCIA and MHCIIB genes for 152 individuals were genotyped using a next-generation sequencing. Because the existence of PCR products were confirmed from all individuals through agarose-gel electrophoresis and concentration measurement, the lack of reads of id034 might be due to the mistake when combined in approximately equimolar quantities. The 16 different alleles from partial sequences of MHCIA exon 3 sequences and the six different alleles from complete sequences of MHCIIB exon 2 were found in the island population. Because the alleles identified in this study did not contain stop codons or frameshift mutations, these alleles could be functional. The allele frequency of each MHC gene ranged approximately 21–95% for MHCIA and 21–89% for MHCIIB, but the regional deflection of the frequency within Hokkaido Island was not seen. Alleles Grja-UA*04–07; Grja-UA*09, 10; Grja-UA*12, 13; Grja-UA*14–16, Grja-DAB*02, 06 seemed to be genetically linked. However, it was thought that all alleles do not compete with one another for selection. Such genetically linked relationships at a glance could be caused by that the island population was homogeneous with the low allelic variation. The maximum number of alleles per individual was eleven for MHCIA and four for MHCIIB, indicating the existence of at least six MHCIA loci and two MHCIIB loci resulted from duplicated events. Detecting 11 alleles in one individual of the wattled crane (Table III-1)

suggested the presence of at least six loci. The presence of at least two MHCIIB loci in cranes has been also indicated by the previous study (Kohyama et al. 2015a). In addition, the number of MHCIA allele sequences per individual ranged 4–11. These results suggest the following possibilities: (1) the number of MHCIA loci is different among species/individuals, similar to some reports in vertebrate MHC genes including some birds (Eimes et al. 2011; Promerová et al. 2012; Jaeger et al. 2016); (2) the same allele existing among plural loci and/or the homozygote existing in several loci; (3) some part of possible MHCIA alleles could not be amplified with the primer set using for 454 sequencing in this study. To examine these possibilities, it is required to estimate the number of loci using conservative region in the MHC genes and analyze the overall sequence of MHC genes. Besides, the expression analysis using cDNA synthesized from a fresh blood sample and the investigations of full-length cDNA are needed to estimate functional MHC loci and to better understand how MHC has evolved in the red-crowned cranes. Several hundreds of MHC alleles detected per loci is reported in the wild population of birds (Alcaide et al. 2014), and the number of alleles identified in this study is low as much as that of the other bird species that experienced a severe population decline (Zhang et al. 2006; Bollmer et al. 2007). Therefore, our results suggest that the MHCIA and MHCIIB genes were also genetically influenced by a bottleneck

effect of the crane population. Actually, the island population has gradually recovered from a small number (estimated at about 60) of individuals (Masatomi 2000), and the low genetic variation due to a bottleneck was also indicated by previous studies using microsatellites and mtDNA sequences (Miura et al. 2013b; Sugimoto et al. 2015).

Nevertheless, the historical selection has worked on the red-crowned crane MHC, and the MHC allelic lineages seem to be retained. In the sequence analysis with DnaSP and MEGA, I found that π of ABSs in each MHC class is higher than that of non-ABSs, and that ω of ABSs is also higher than 1.0. The analysis with omegaMap found nine and eight amino acid positions under positive selection in each MHC class examined. These results suggest that the positive selection has worked on ABSs encoded in the MHCIA exon 3 and MHCIIB exon 2. However, the selection seems to have become weak via bottleneck, and estimated codon positions under positive selection were not completely identical to putative ABS. Accordingly, I cannot reject the possibility of misidentifying ABSs as they are based on the crystallographic studies of chicken MHCIA and of human MHCIIB (Brown et al. 1993; Wallny et al. 2006). By omegaMap analysis, evidence of recombination was not clearly detected in the examined regions, whereas Kohyama et al. (2015a) indicated that three recombination breakpoints exist at a point near the end of exon 2 and two points within intron

1 of crane MHCIIB sequences. Because our data were calculated from only the partial MHCIA exon 3 and the complete MHCIIB exon 2, the ω and ρ may differ from those when the entire MHC gene is analyzed.

Phylogenetic trees based on the MHC genes showed that the allelic lineage of crane did not form monophyletic groups among species, suggesting some variable alleles had retained during the process of species-level diversification according to the balancing selection (Cutrera and Lacey 2007). The MHCIA and MHCIIB alleles of Gruiformes species represented evidence of trans-species polymorphism, which could have retained for at least 37 million years, because the crane species belonging to *Grus* and *Balearica* have radiated for 31–37 million years (Krajewski et al. 2010). It is necessary to obtain more data from closely related species to the red-crowned crane and longer sequence data such as complete exon 3 for more precise investigation of the allelic evolution of cranes.

Population structure in Hokkaido

The G_{ST} values among subpopulations on Hokkaido based on microsatellites, MHCIA and MHCIIB were very low and showed no significant differentiation. No remarkable regional differentiations were also found the Bayesian clustering analysis of

microsatellites. In addition, the rates of MHC allele frequencies in subpopulations were almost identical among all alleles except the Abashiri subpopulation. The Abashiri subpopulation appeared in 1999, and suggested to be derived from some pairs of the crane (Masatomi 2000). Although the sample size of the Abashiri subpopulation was only three in this study, the same allele set was obtained from each sample, probably due to inbreeding or samples derived from the same pair. Although there was the potential deflection of allele frequencies among subpopulations, no geographic structure was found based on the genetic diversity of microsatellite and MHC genes. These results also suggest that the island population had lost the genetic variation through a past population decline, followed by increasing from the small population, supported by the field study.

This study can become the foundation of the future research corresponding to the immunological study of the island population and allopatric continental population of the red-crowned crane. The previous study reported migratory birds have more various MHC than non-migratory birds, because migratory birds challenge more pathogens in many different environments (Møller and Erritzøe 1998). In addition, the genetic variation of the continental population based on a neutral gene is higher than that of the island population of the red-crowned crane (Hasegawa et al. 1999; Miura et al. 2013a). Therefore, the MHC

variation of the continental population may be higher than that of Hokkaido. It is needed to analyze the MHC on the continental population to elucidate the evolutionary history of MHC in the red-crowned crane. Because continental individuals flying to Japan in recent years were reported (Miura et al. 2013a), there is the possibility that the chance of the contact between the island population and the continental population occurred in the future. The gene flow between the continent population and the island population gives a good opportunity to promote recovery from low genetic variation (Lowe and Allendorf 2010). On the other hand, this might cause a risk of an encounter with a novel pathogen for the island population.

It is important to know the pathogens for the red-crowned crane, especially for the island population, and also obtain the data on the MHC variation for evaluation the susceptibility to the pathogens. Because the current island population of the red-crowned crane is distributed in limited ranges and inhabits at high density, it is thought that the extinction risk of the island population would be high if any novel pathogens are introduced. Therefore, the development of the plan to increase the variation of the MHC alleles is effective in the future conservative action of the red-crowned crane in Japan.

General Discussion

In the series of present studies, the genetic diversity and genetic population structure of the endangered red-crowned crane inhabiting Hokkaido were discussed based on both neutral genes (mtDNA and microsatellite) and functional gene (MHC).

Firstly, in mtDNA sequences which was generally used as the genetic marker, the existence of the tandem duplicated region was found in Chapter I. The gene order in its region was also conserved among crane species in family Gruidae, suggesting the duplicated event had occurred in the common ancestral lineage before species diversification. These results strongly suggest that it must be careful not only for using homologous sequences, but also for distinguishing paralogs from orthologs using mtDNA for family Gruidae.

To elucidate the spatiotemporal changes in the genetic structure and genetic diversity of the island population, 213 samples were collected between 1878–2014 and were analyzed with mtDNA in Chapter II. The direct comparison between the past and recent genetic structures based on mtDNA haplotypes revealed that the level of genetic diversity of island population has been lower for about hundred years. In Chapter III, it is confirmed that the MHC genes, which play an important role in the adaptive immune response, were maintained by molecular evolutionary systems in the red-crowned crane. However, a decreasing of genetic diversity was indicated in such an important gene. Additionally, no remarkable

regional differentiations were found by G_{ST} values based on functional MHC genes, suggesting that the extinction risk of the island population would be high if any novel pathogens are introduced. It is thought that the lower levels of genetic diversity and no geographic structures could have resulted from the population expansion from a very small number of individuals. In addition, mtDNA haplotype analysis and inbreeding coefficient values calculated by microsatellite data suggested that the inbreeding is possible to be causing the loss of genetic diversity in the small population. Protecting habitat environments and promoting an expansion of distribution are important, because it can cause that reducing the risk of morbidity and population extinction.

Unfortunately, enough samples of continental populations and pre-bottleneck historical stuffed specimens were not collected in this study. Therefore, it was considered that bottleneck decreased the genetic diversity, but genetic diversity of the founder population and pre-bottleneck population is unknown. The phylogeographical, phylogenetic and population genetic analyses (such as divergence time, genetic similarity) with both continental and island population are strongly required in the future study. Information from these analyses reveals not only the history of the evolution of the island population, but also can be useful for future conservation action plans. For instance, Blakiston's fish owl (*Bubo blakistoni*), which is an

endangered species in Hokkaido, experienced severe population decline similar to the red-crowned crane. The genetic differentiation among local populations in Hokkaido was reported and promoting movements among these populations could counter the decline in genetic diversity (Omote et al. 2015). The genetic exchange between populations could increase the genetic variation, but genetic information should be investigated before considering this event. The present study provides invaluable data to the optimal management of the red-crowned crane population in Hokkaido, and also contributes to the development of conservation biology of other endangered species.

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Figures and Tables

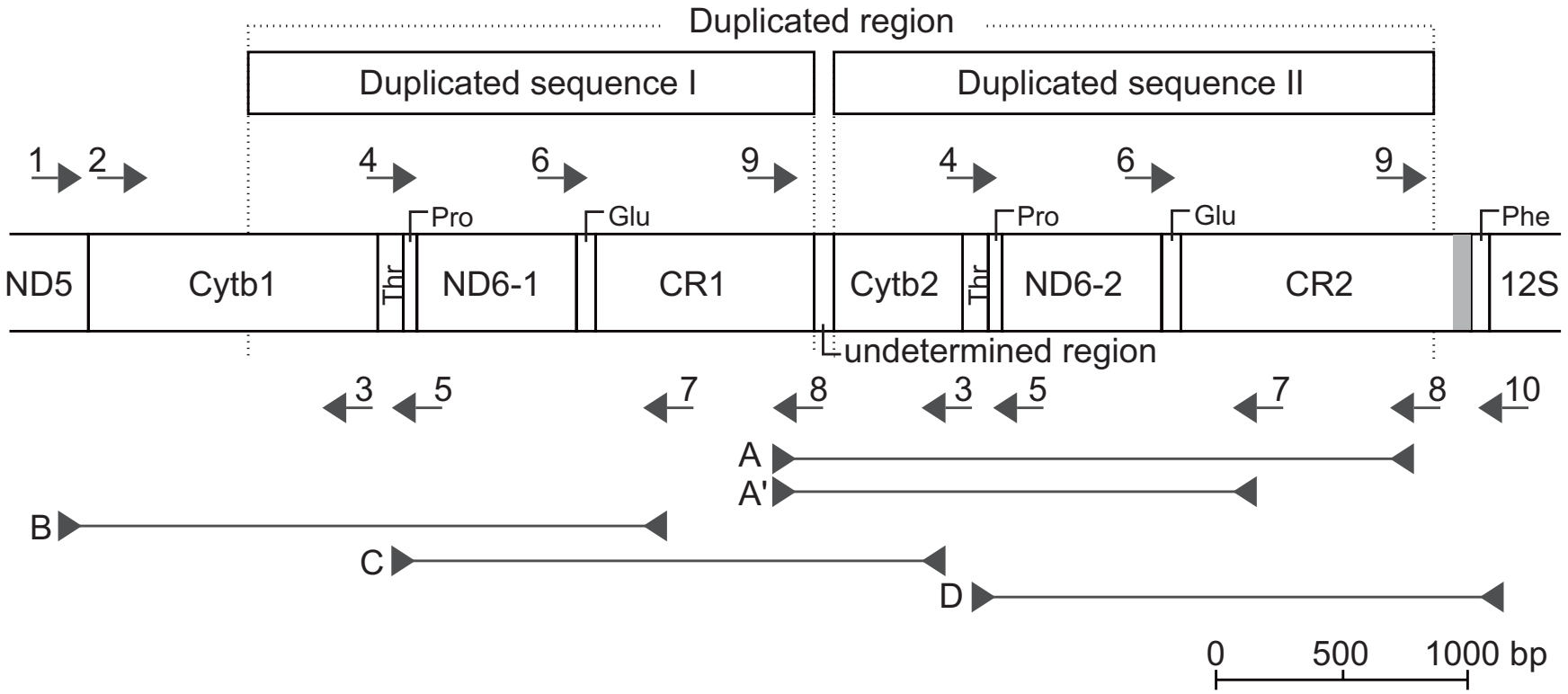

Fig. I-1 A schematic structure around the duplicated region in the mitochondrial genome of crane species from Akiyama et al. (2017c). The gray box indicates repetitive sequences in CR2 of the black-crowned crane (*B. pavonina*) and gray-crowned crane (*B. regulorum*). The PCR-amplified fragments are shown as bars with the name of Fragments A to D, and arrows indicated primer sets. Fragments A and A' were amplified only when the CR region was duplicated. The L-strand primers are shown above and the H-strand primers are shown below the gene. All primer sequences are shown in Table I-1. Abbreviations for genes or region are as follows: ND5, NADH dehydrogenase subunit 5; Cytb1 and Cytb2, cytochrome *b*1 and 2; ND6, NADH dehydrogenase subunit 6; 12S, 12SrRNA; CR1 and CR2, control regions 1 and 2; Thr, tRNA-Threonine; Pro, tRNA-Proline; Glu, tRNA-Glutamic acid; Phe, tRNA- Phenylalanine.

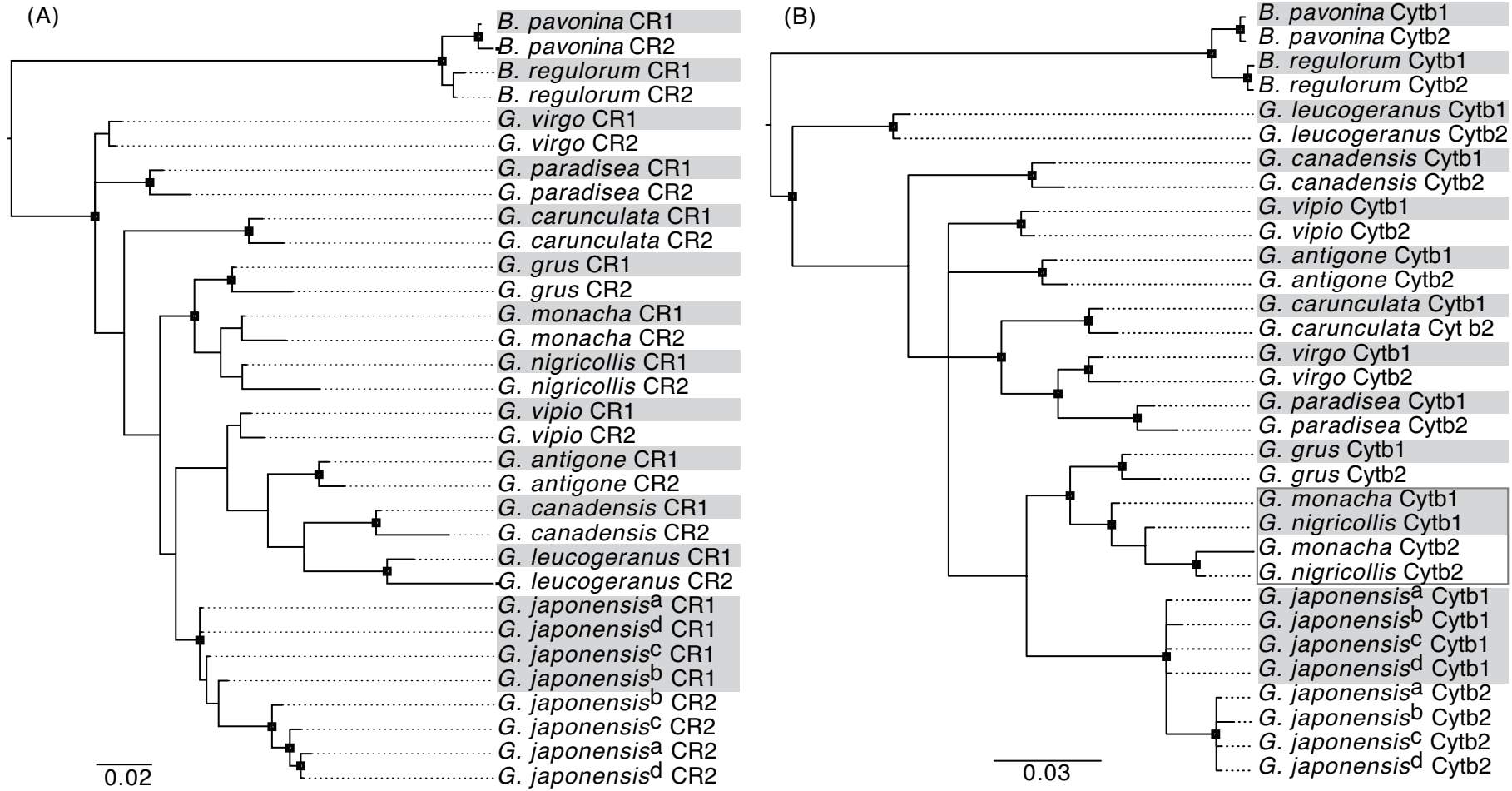

Fig. I-2 Bayesian tree based on CR or Cytb sequences from Akiyama et al. (2017c).
Bayesian trees inferred from combined sequences of CR1 and CR2 (A) and these of Cytb1 and Cytb2 (B) analyzed under the best-estimated model. Closed squares indicate nodes recovered with >0.90 posterior probabilities in the Bayesian analysis, and >70% bootstraps support values in the ML analysis of 1000 pseudoreplicate data sets. The scale bar indicates the number of substitutions per nucleotide site. The gray backgrounds show CR1 operational taxonomic units (A) and Cytb1 (B). The letters a to d of *G. japonensis* indicates different red-crowned cranes; *B, Balearica*; *G, Grus*. In the tree (B), the paralogous Cytb1 and Cytb2 of most crane species, except *G. monacha* and *G. nigricollis* (shown by a square), are more closely related to each other to the orthologous Cytbs.

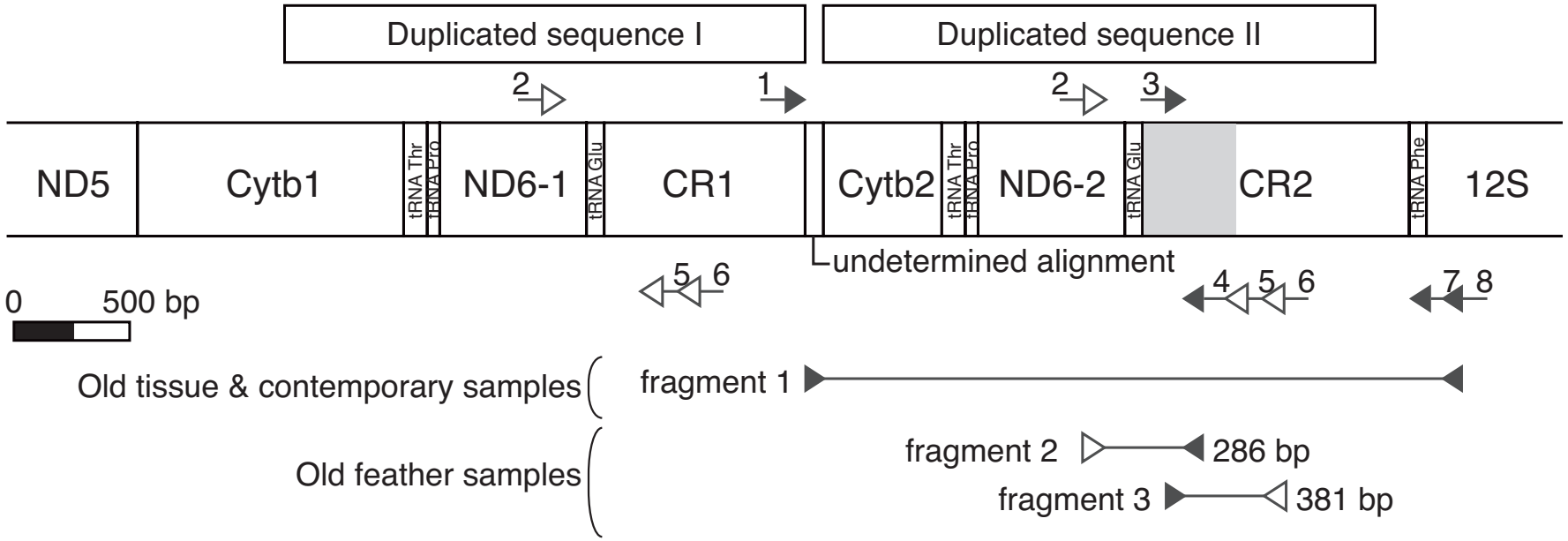

Fig. II-1 A scheme of mtDNA gene order around the duplicated region and position at which a primer was used (cited from Akiyama et al. 2017b): The gray box indicates the target region (440 bp) for haplotyping the island population of the red-crowned crane (*Grus japonensis*). For old tissues and contemporary samples, fragment 1 was PCR-amplified. For old feather samples, fragments 2 and 3 were PCR-amplified. Arrows with number show the primer positions. Closed arrows indicate primers annealing the specialized locations in the duplicated region. Abbreviations for genes or region are as follows: ND5, NADH dehydrogenase subunit 5; Cytb1 and Cytb2, cytochrome *b*1 and cytochrome *b*2; ND6, NADH dehydrogenase subunit 6; 12S, 12SrRNA; CR1 and CR2, control regions 1 and control regions 2; Thr, tRNA-Threonine; Pro, tRNA-Proline; Glu, tRNA-Glutamic acid; Phe, tRNA- Phenylalanine.

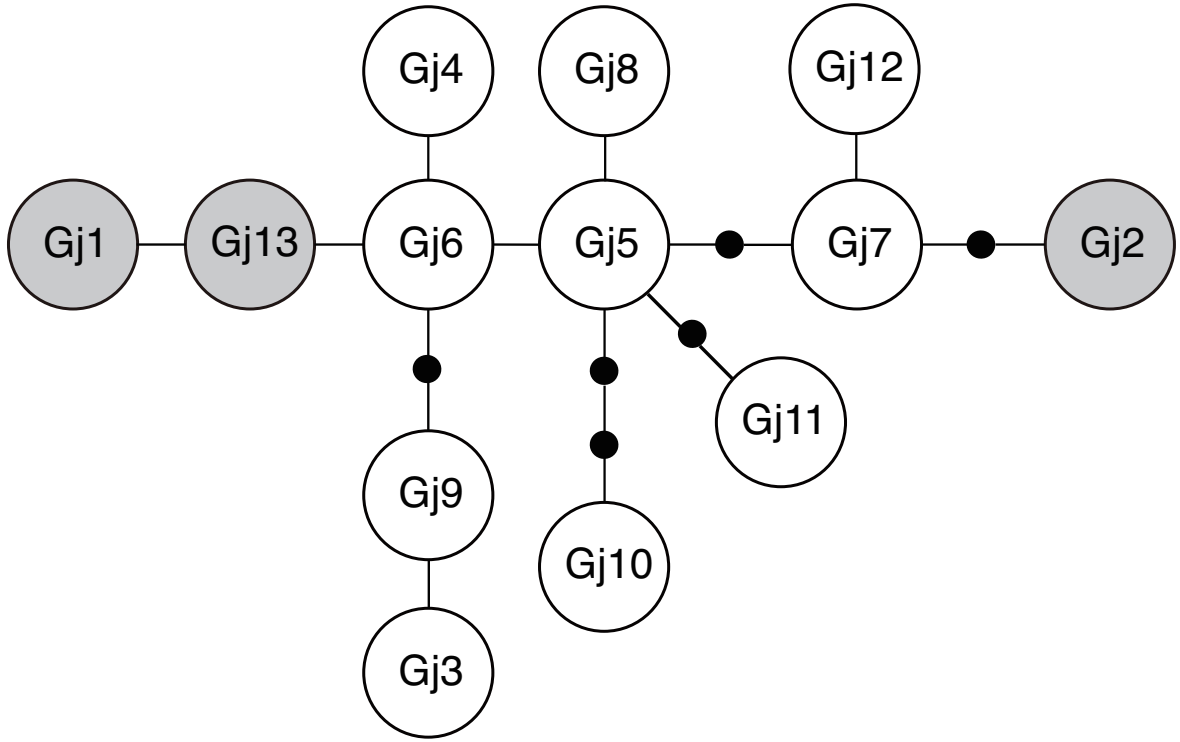

Fig. II-2 Parsimony network of CR2 haplotypes from the red-crowned crane. Gray circles (Gj1, Gj2 and Gj13) show haplotypes identified from the island population in Akiyama et al. (2017b). Open circles indicate previously reported continental haplotypes (Hasegawa et al. 1999; Miura et al. 2013a) and closed circles indicate putative haplotypes. Each line between circles indicates one base substitution.

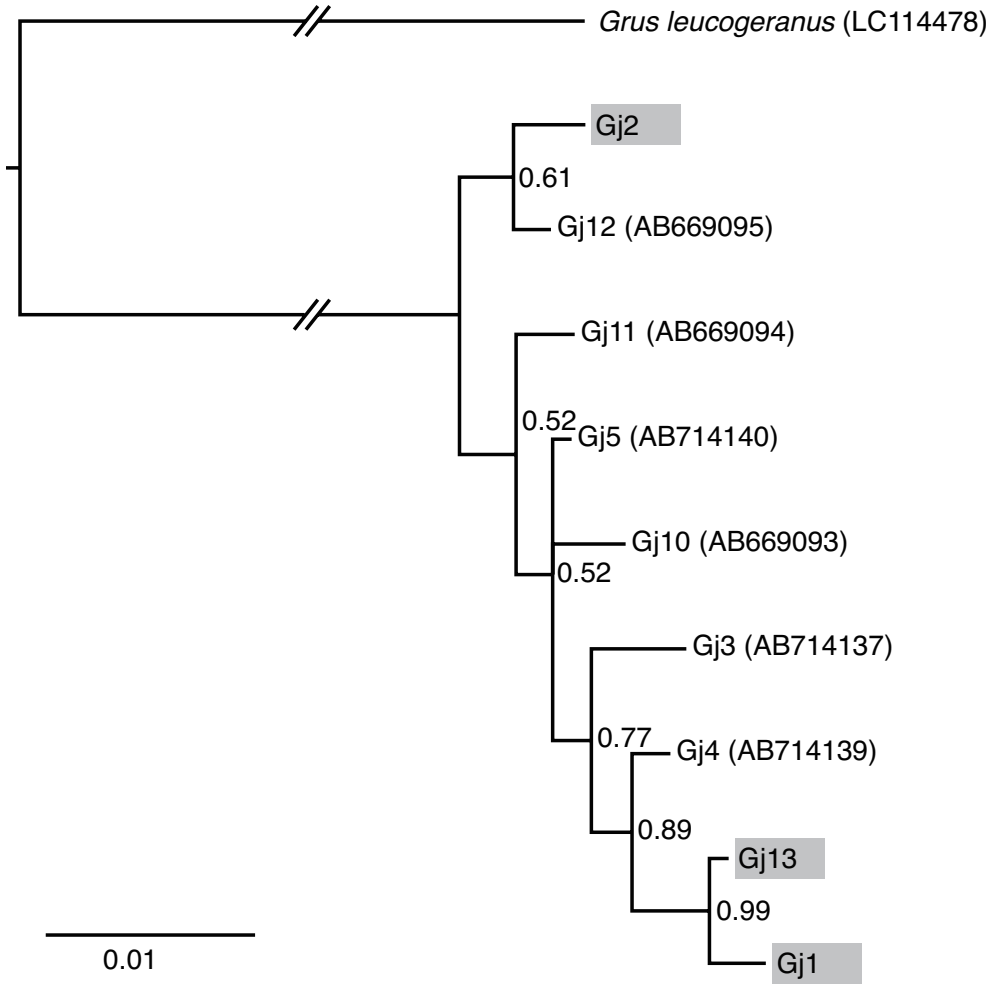

Fig. II-3 Bayesian tree based on the CR2 sequences from Akiyama et al. (2017b). Fifty percent majority-rule consensus trees inferred from CR2 sequences (1141 bp) of different haplotypes. Haplotypes with gray backgrounds were found in the island population in the present study. GenBank accession numbers of the previously reported haplotype sequences are indicated in parentheses. Numbers on node show the posterior probabilities values. The scale bar indicates the number of substitutions per nucleotide site.

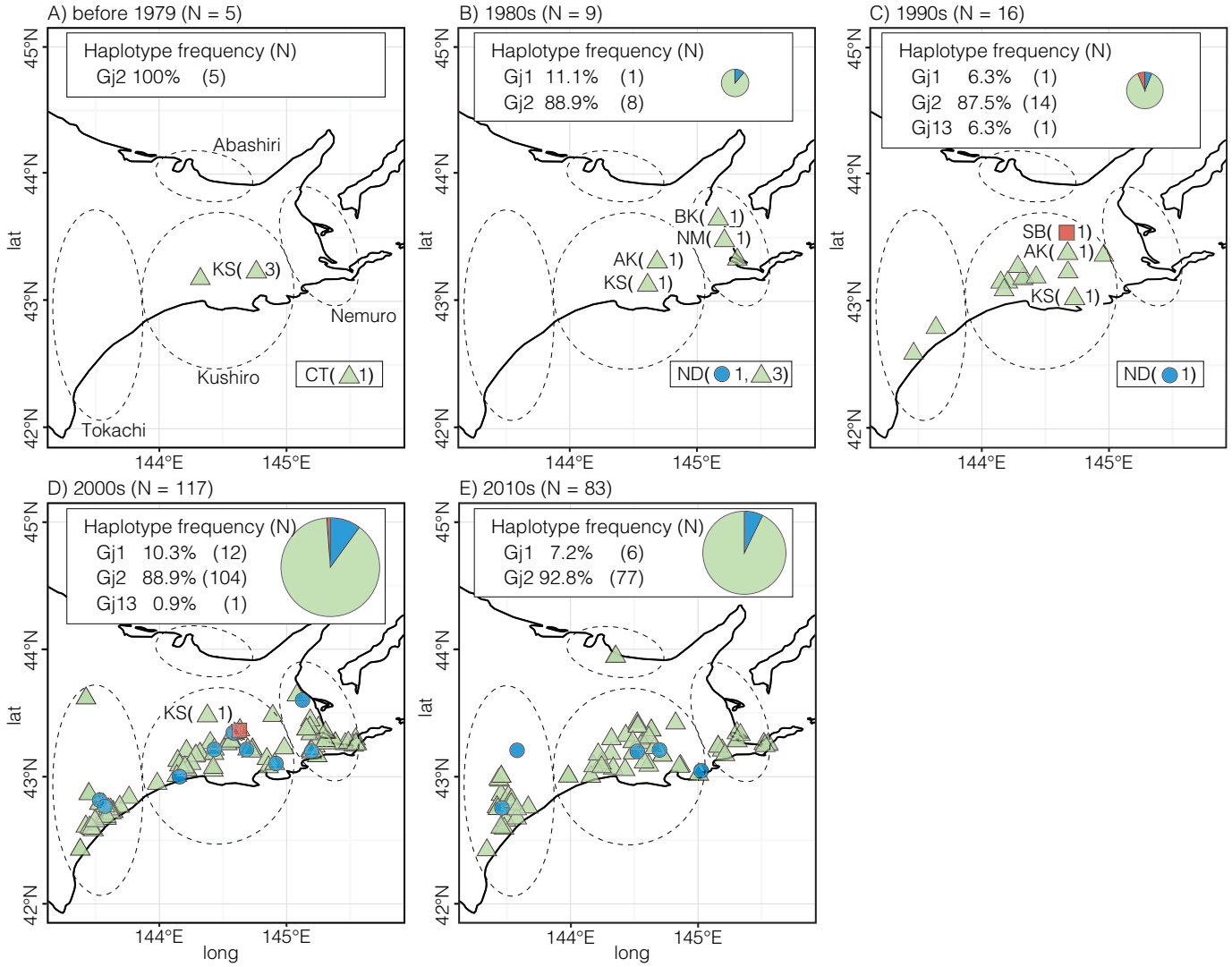

Fig. II-4 Haplotype frequencies and distribution of mtDNA haplotypes in the island population through 1878–2014 (cited from Akiyama et al. 2017b): (A) before 1979, (B) 1980s, (C) 1990s, (D) 2000s, (E) 2010s, respectively. Circles with dotted lines indicate four areas: Abashiri, Kushiro, Nemuro and Tokachi. MtDNA haplotypes were shown as follows: gray circle for Gj1; triangle for Gj2; dark gray square for Gj13. Abbreviations of sampling locations for stuffed specimens (A) are as follows: CT, Chitose; KS, Kushiro; BK, Bekkai; NM, Nemuro; AK, Akan; SB, Shibecha; ND, No data. The frequencies of haplotypes are visualized by pie charts colored by gray (Gj1), white (Gj2) and dark gray (Gj13).

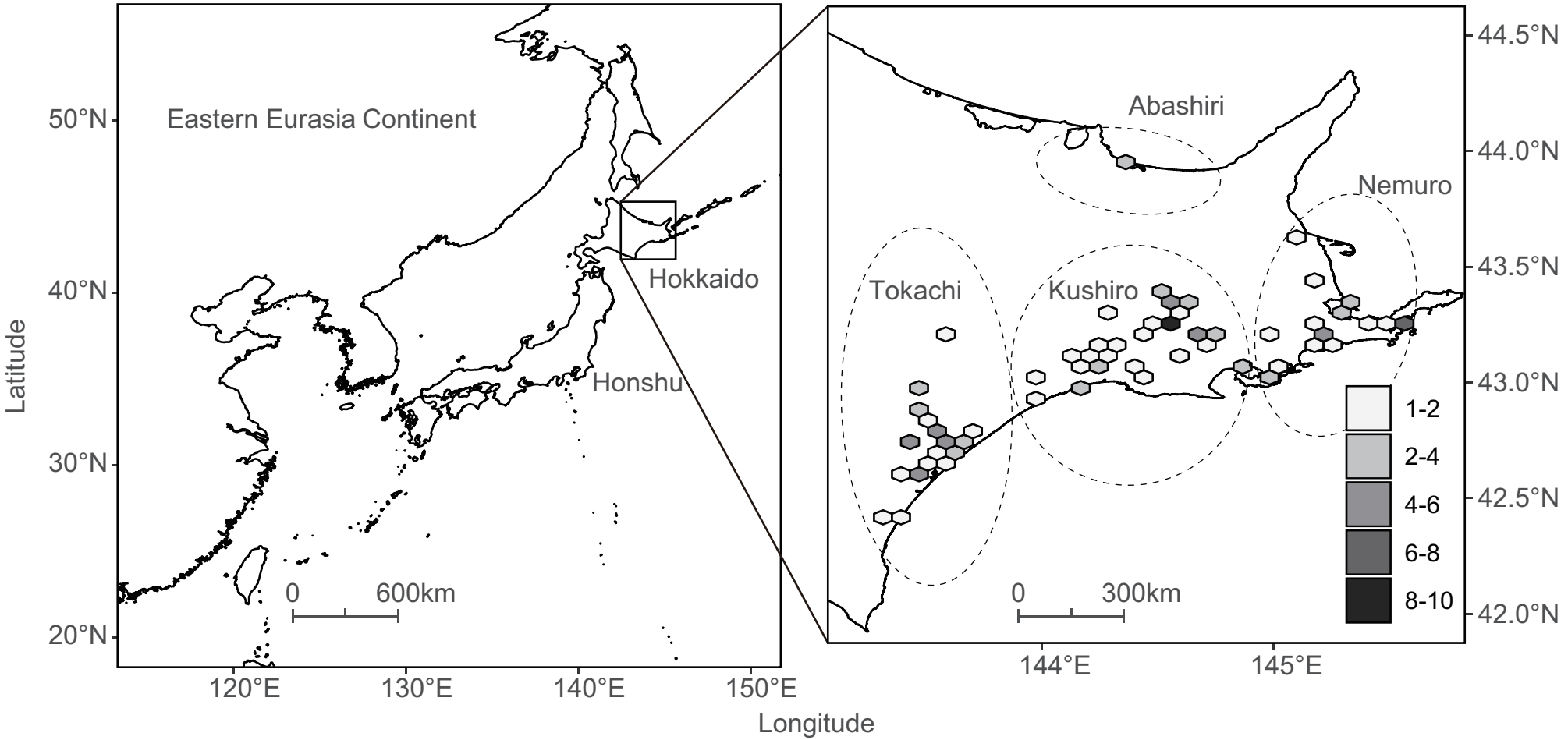

Fig. III-1 Map of the sampling locations on eastern Hokkaido (left: view of the Japanese islands; right: enlargement of Hokkaido). The circles with broken lines represent four regions in Hokkaido: Abashiri, Kushiro, Nemuro and Tokachi. The hexagons represent the sampling locations and the number of samples indicated by the depth of black. This figure was cited from Akiyama et al. (2017a).

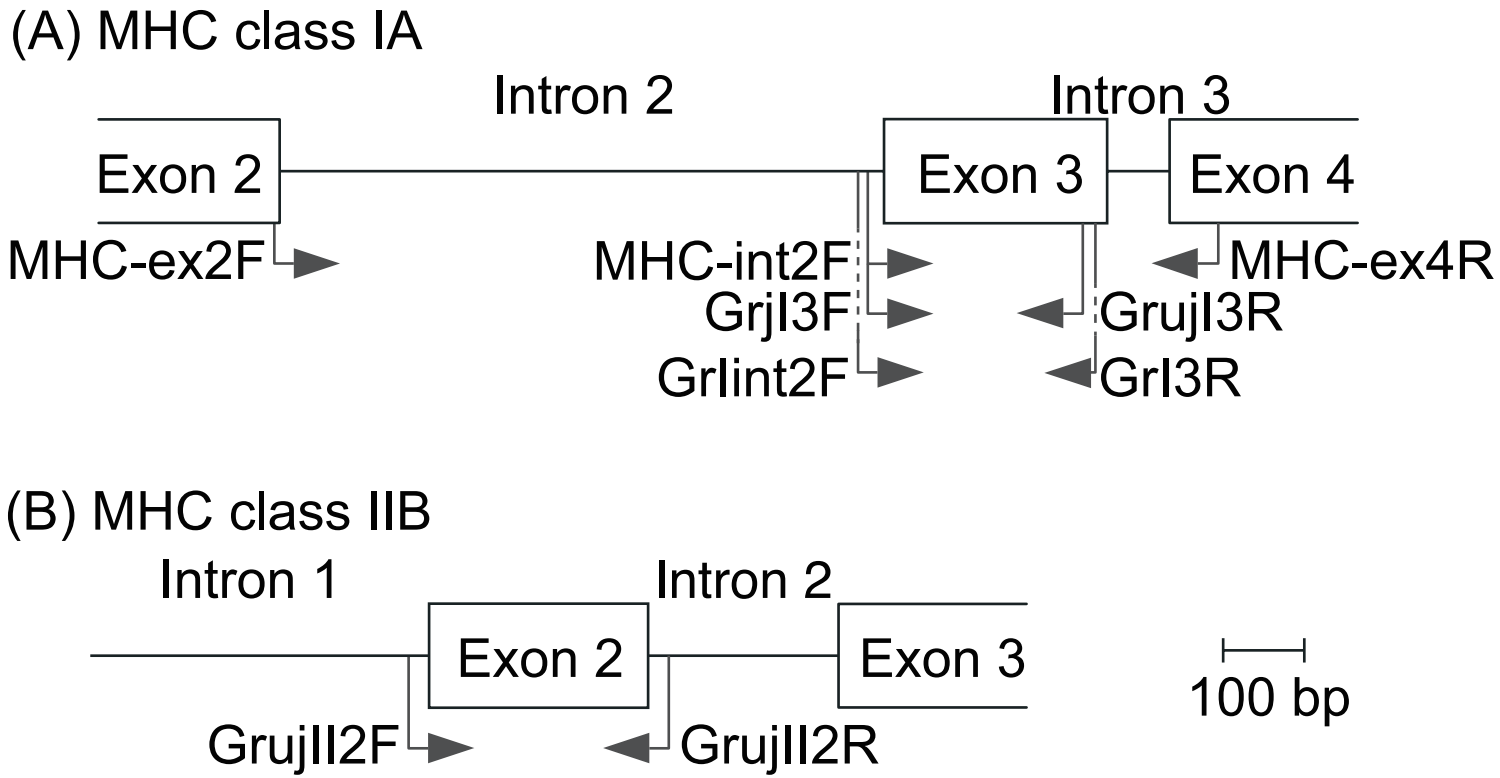

Fig. III-2 Primers for MHC class IA (A) and MHC class IIB genes (B). Arrowheads indicate the positions of primers. This figure was partially cited from Akiyama et al. (2017a).

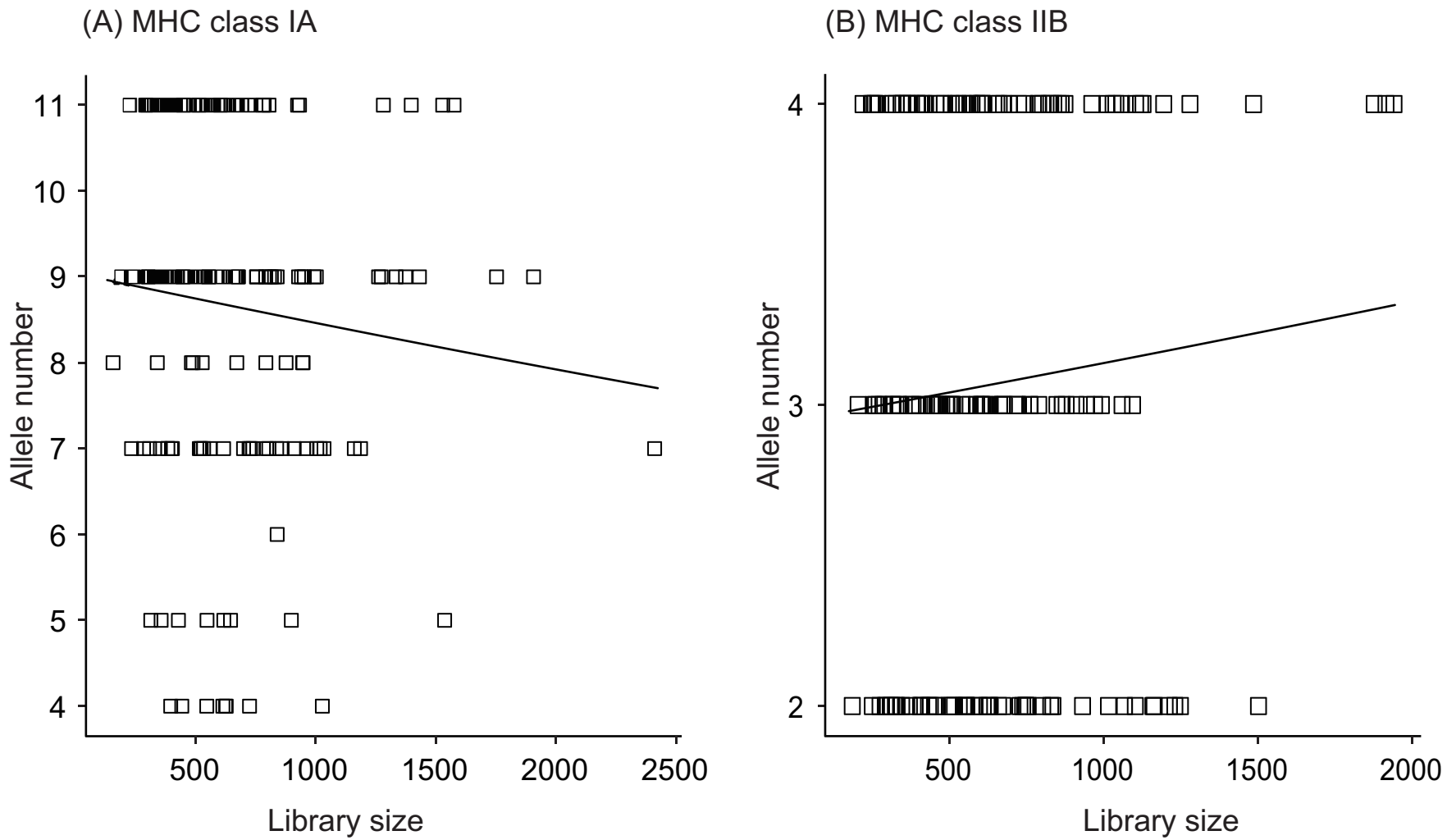

Fig. III-3 Relationships between the library size (number of reads) and the allele number per amplicon. Each square indicates one amplicon. No correlation between the library size and allele number was found using GLM (explanatory variable: library size, response variable: allele number, poisson-distributed, link function: log; class I A: $\chi^2 = 0.772$; $P = 0.38$, class II B: $\chi^2 = 0.221$; $P = 0.63$). This figure was cited from Akiyama et al. (2017a).

(A) MHC class IA

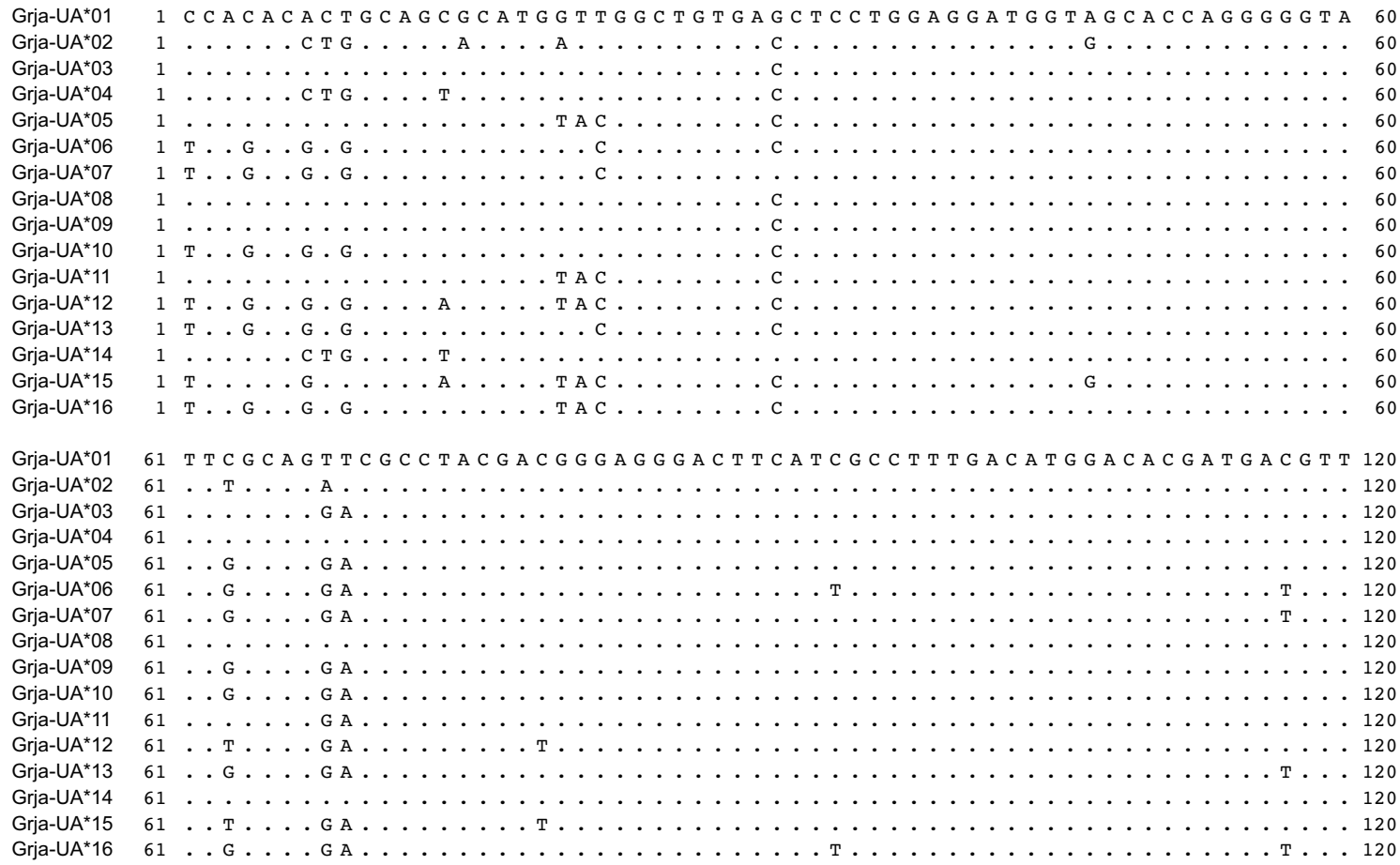

Fig. III-4 Alignment of the nucleotide sequences of MHC alleles identified from the red-crowned crane in Hokkaido. The sequences of MHC class IA exon 3 and class IIB exon 2 were shown in (A) and (B), respectively. Dots indicate identity with the consensus nucleotides on the top of the alignment. This figure was cited from Akiyama et al. (2017a).

(A) MHC class IA

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Grja-UA*01  121  C A C T G C G G C G G A C G C A G C A G C G C A A A T C A C C A A G A G G A A G T G G G A A G C G G A C G G G A C T G A  180
Grja-UA*02  121  . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*03  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*04  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*05  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T  180
Grja-UA*06  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*07  121  . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*08  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*09  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*10  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*11  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . T  180
Grja-UA*12  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . T  180
Grja-UA*13  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*14  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . T  180
Grja-UA*15  121  . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180
Grja-UA*16  121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .  180

Grja-UA*01  181  A G C T G A G A G A C A G A A G C A C T A C C T G C A G A A C A C C T G C G T C G A  222
Grja-UA*02  181  . . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*03  181  . . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*04  181  . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*05  181  C . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*06  181  . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*07  181  . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*08  181  . . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*09  181  . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*10  181  . . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*11  181  C . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*12  181  C . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*13  181  . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*14  181  C . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*15  181  . . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
Grja-UA*16  181  . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  222
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(B) MHC class IIB

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Grja-DAB*01   1 CGGTTTTCCAGGATTGGTTTGTGGCCGAGTGTCATTACCTCAACGGCACCGAGCGGGTGA  60
Grja-DAB*02   1 ........................GT........G.........................  60
Grja-DAB*03   1 ........................A.........G.........................  60
Grja-DAB*04   1 ........................................................................  60
Grja-DAB*05   1 ............C....T......AGT.......................................  60
Grja-DAB*06   1 ........................................G.........................  60

Grja-DAB*01  61 GGTTTGTGGAAGGGTACAGCTACAACCGGGTGCAGTACGTGCACTTTGACAGCGATGTGG 120
Grja-DAB*02  61 ..C......TGC.......................................CA.....C.............. 120
Grja-DAB*03  61 ...................................C...CA.....C.............. 120
Grja-DAB*04  61 ...A.....TGC.......................................CA....A............... 120
Grja-DAB*05  61 ...A.....TGC.......................................CA....A............... 120
Grja-DAB*06  61 ...................................................CA.....C.............. 120

Grja-DAB*01 121 GGCACTATGTGGGCGACACCCCCCTGGGTGAGTACCAAGCCAAGTACAGGAACAGCCAGC 180
Grja-DAB*02 121 ...G.........................C.....................T............. 180
Grja-DAB*03 121 ...................................C.....................T............. 180
Grja-DAB*04 121 ...................................C.....................T............. 180
Grja-DAB*05 121 ...................................C.....................T............. 180
Grja-DAB*06 121 ...................................................................T............. 180

Grja-DAB*01 181 CAGAAATACTGGAAAATGCACAGGCTGCAGTGGACGCGTACTGCCGACACAACTACGGGG 240
Grja-DAB*02 181 ..............GCGGAA........AG......A..GT...................A.. 240
Grja-DAB*03 181 ..............GT.CAA..G..G.CAG.......................................  240
Grja-DAB*04 181 ..............GT.CAG..G..G.CAG.....T.........................  240
Grja-DAB*05 181 ..............GT.CAG..G..G.CAG.....T.........................  240
Grja-DAB*06 181 ..............GT.CAA..G..G.CAG.......................................  240

Grja-DAB*01 241 TGGTCCGGTCTTTCACCGTGGAGAGGAGAG 270
Grja-DAB*02 241 ..T.GA.........................  270
Grja-DAB*03 241 ..TGGA.........................  270
Grja-DAB*04 241 ..TGGA.........................  270
Grja-DAB*05 241 ..TGGA.........................  270
Grja-DAB*06 241 ...GGA.........................  270
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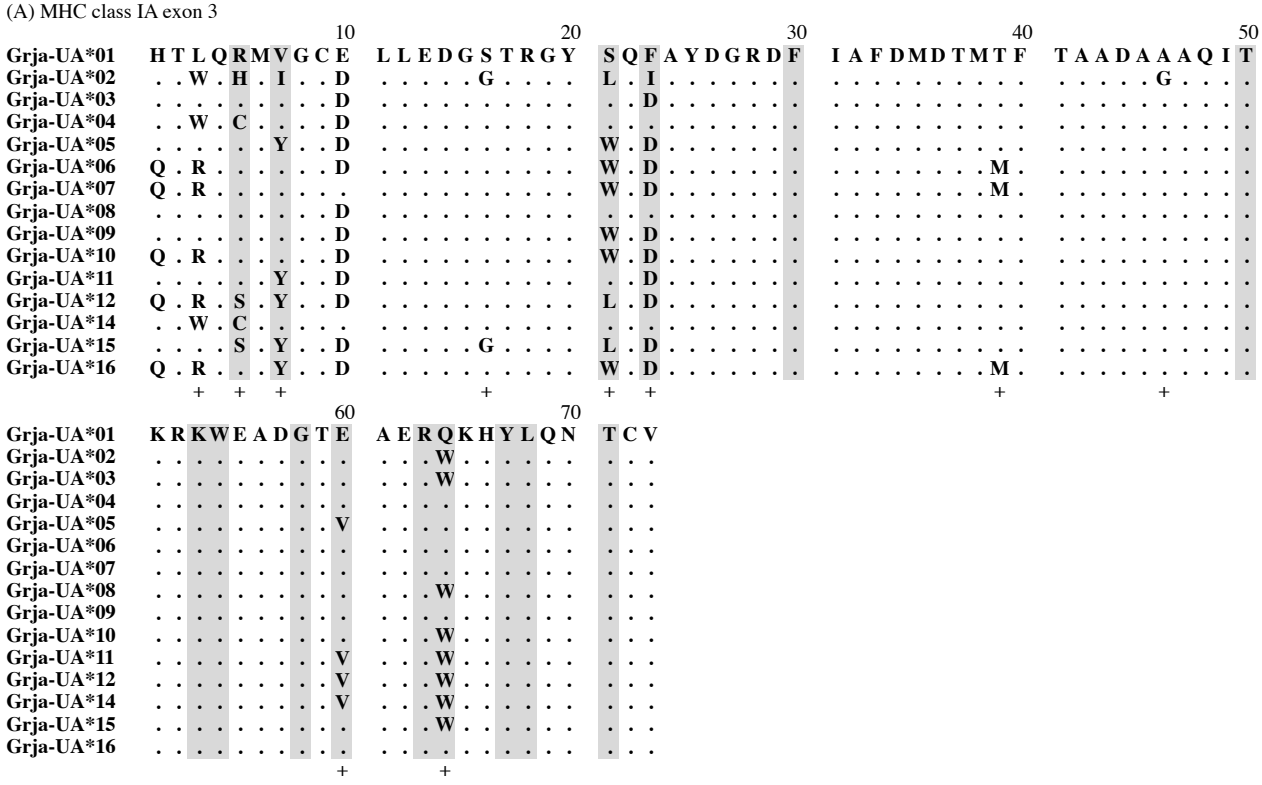
Fig. III-4 Alignment of the nucleotide sequences of MHC alleles identified from the red-crowned crane in Hokkaido. The sequences of MHC class IA exon 3 and class IIB exon 2 were shown in (A) and (B), respectively. Dots indicate identity with the consensus nucleotides on the top of the alignment. This figure was cited from Akiyama et al. (2017a).

(A) MHC class IA exon 3



(B) MHC class IIB exon 2

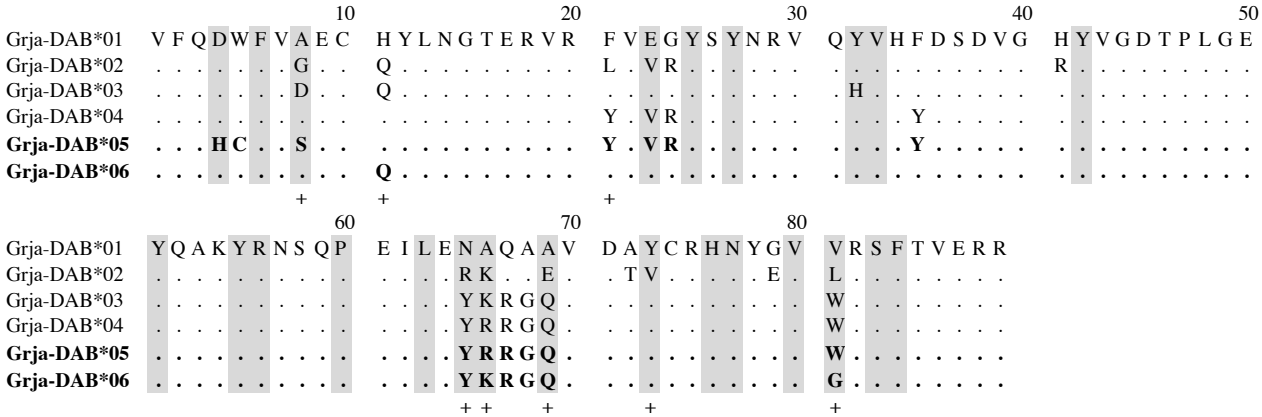


Fig. III-5 Amino acid alignments of the MHC class IA (A) and MHC class IIB (B) identified from red-crowned cranes in Hokkaido. The alleles newly identified in Akiyama et al. (2017a) were denoted with bold letters. Numbers above alignments indicate codon positions. The amino acid sequence of Grja-UA*06 was the same as that of Grja-UA*13, but the nucleotide sequences were different from each other (Fig III-4). Dots denote consensus with the amino acids on the top line. Putative antigen-binding sites predicted from chicken and human MHC are shaded, and the positions under positive selection revealed by the omegaMap (Fig. III-8) are indicated with plus in the bottom line.

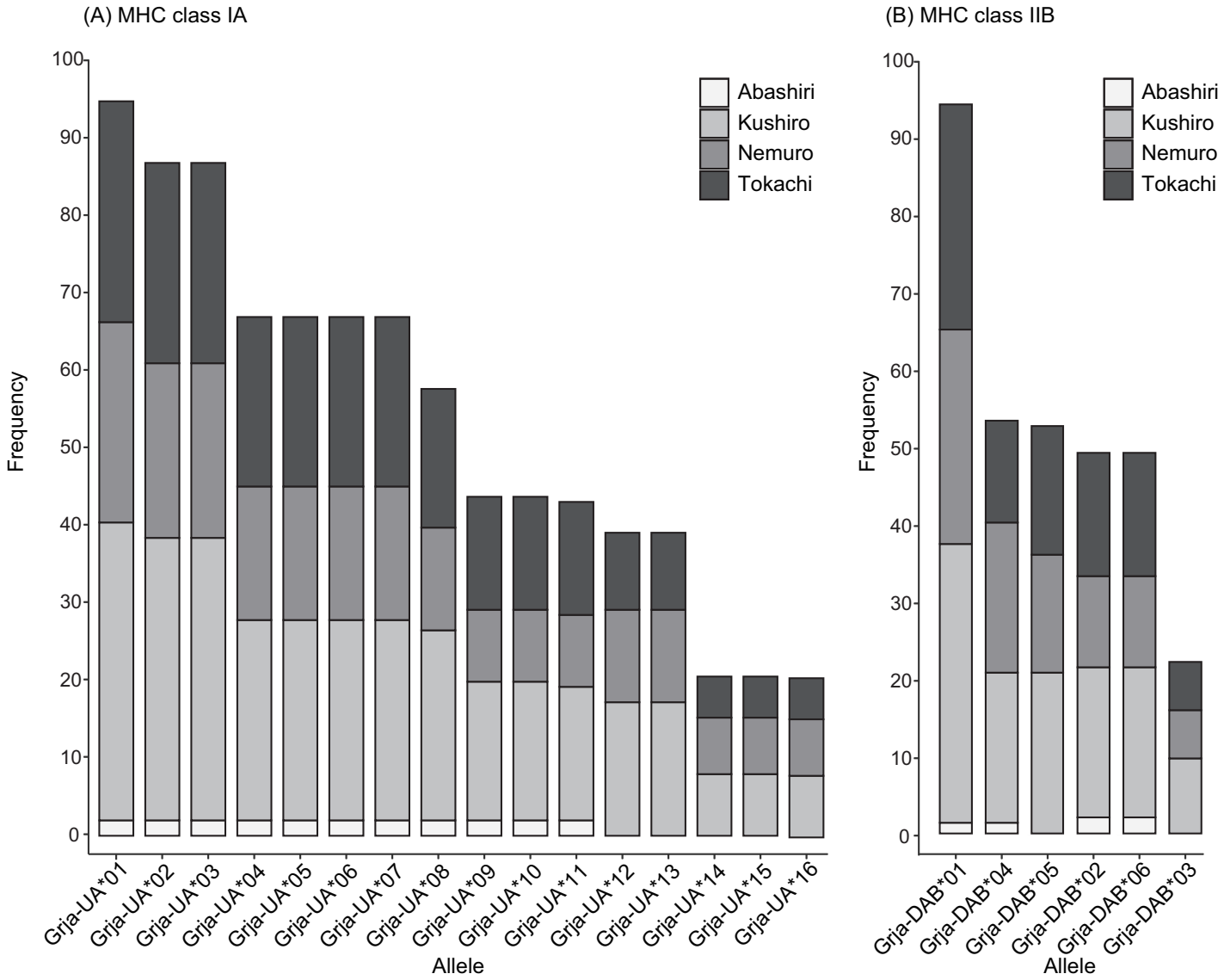


Fig. III-6 A stacked bar chart of the frequency of red-crowned crane individuals per allele of MHC class IA exon 3 (A) and MHC class IIB exon 2 (B). Each bar shows the proportion of subpopulations of Abashiri, Kushiro, Nemuro and Tokachi. This figure was cited from Akiyama et al. (2017a).

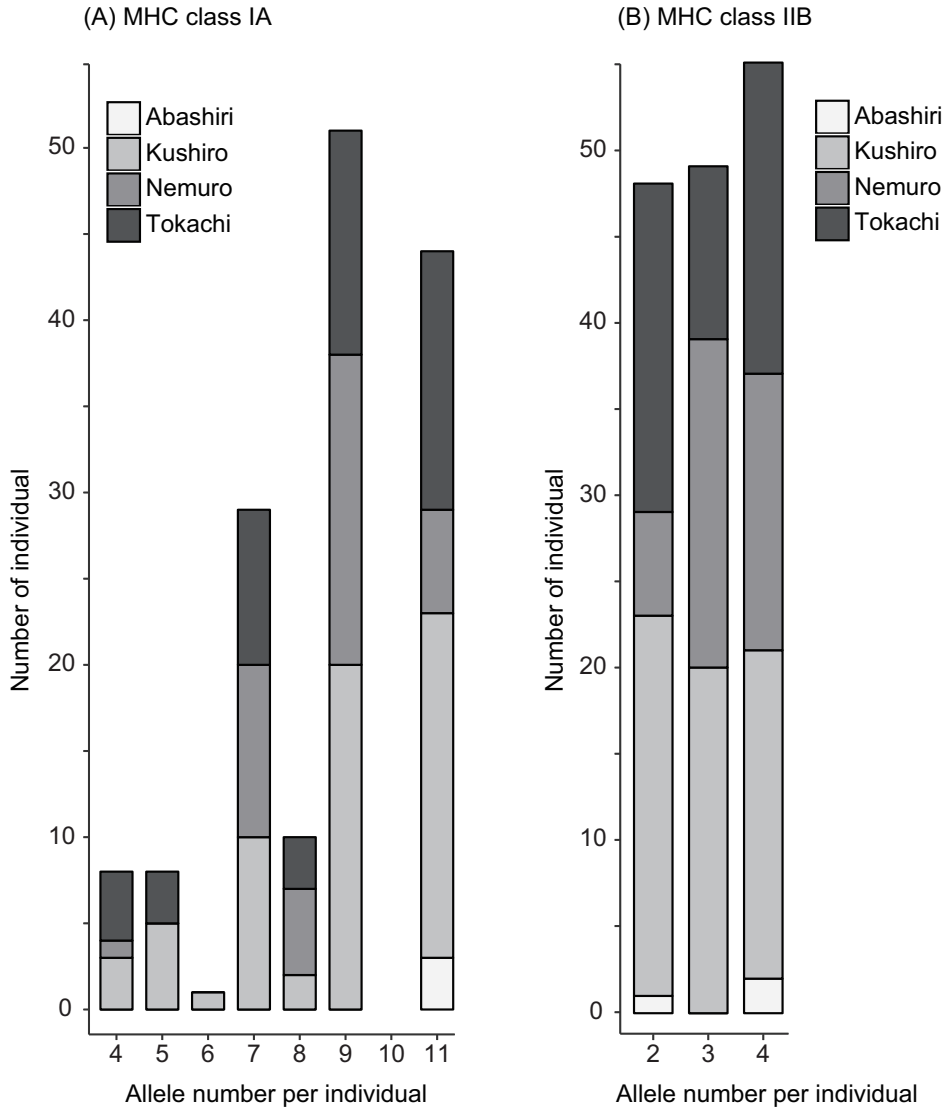


Fig. III-7 A stacked bar chart of the number of red-crowned crane individuals with the number of alleles of MHC class IA exon 3 (A) and MHC class IIB exon 2 (B). Each bar shows the proportion of subpopulations of Abashiri, Kushiro, Nemuro and Tokachi. This figure was cited from Akiyama et al. (2017a).

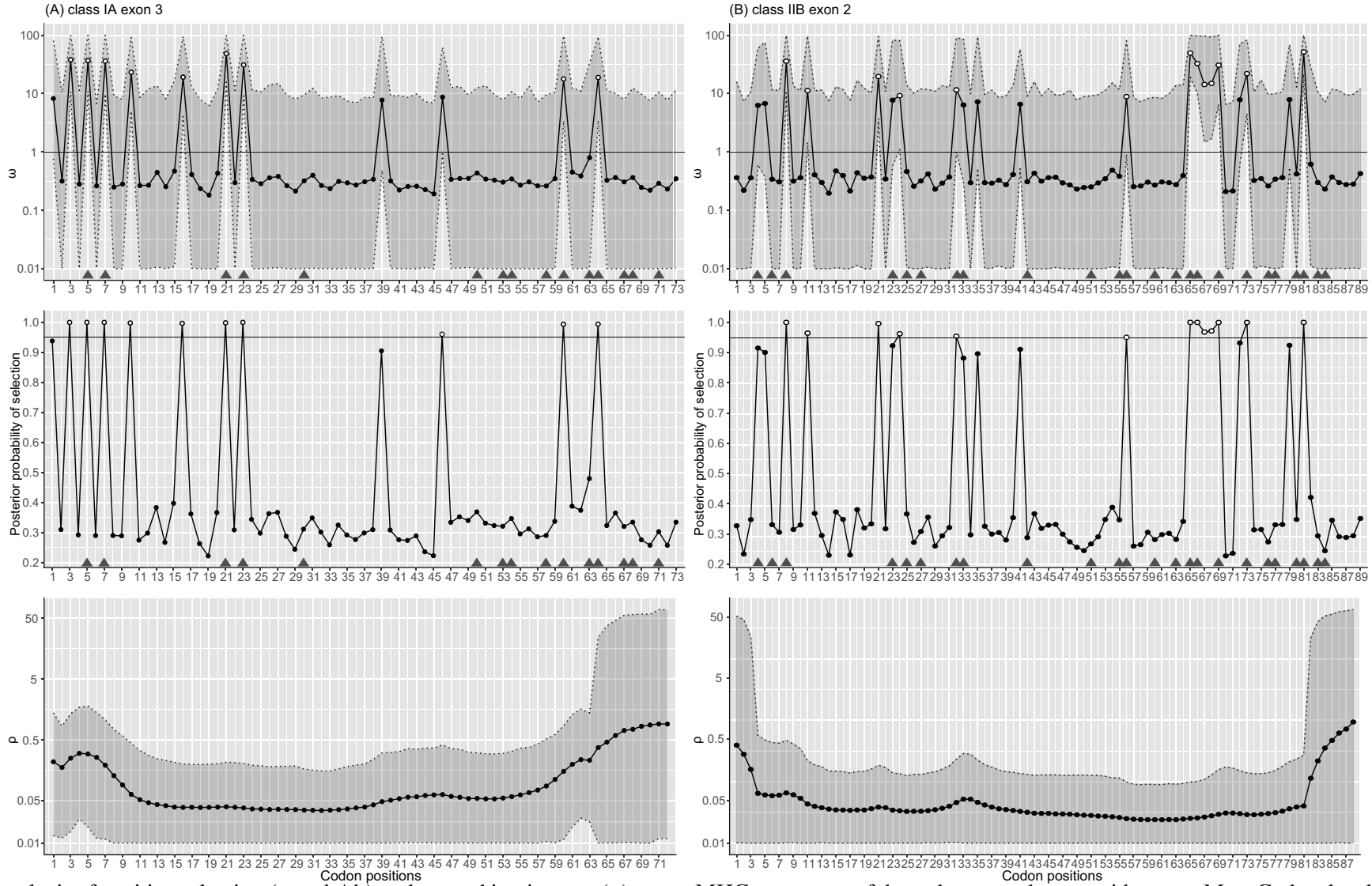


Fig. III-8 Analysis of positive selection ($\omega = d_N/d_S$) and recombination rate (ρ) across MHC sequences of the red-crowned crane with omegaMap. Codon-level variations of the ω value (upper), the posterior probability of positive selection (middle) and ρ (bottom) along the MHC class IA exon 3 (A) and MHC class IIB exon 2 (B). The black straight lines in upper and middle panels indicate the neutral ω value (=1) and the 95% posterior probability, respectively. Dashed lines and gray backgrounds in upper and lower panels indicate maximum and minimum, and the intervals of 95% highest posterior probability density, respectively. Triangles in upper and center plots show the putative ABSs. Open circles mean codons under positive selection (posterior probability for $\omega > 1$ exceeds 0.95). This figure was cited from Akiyama et al. (2017a).

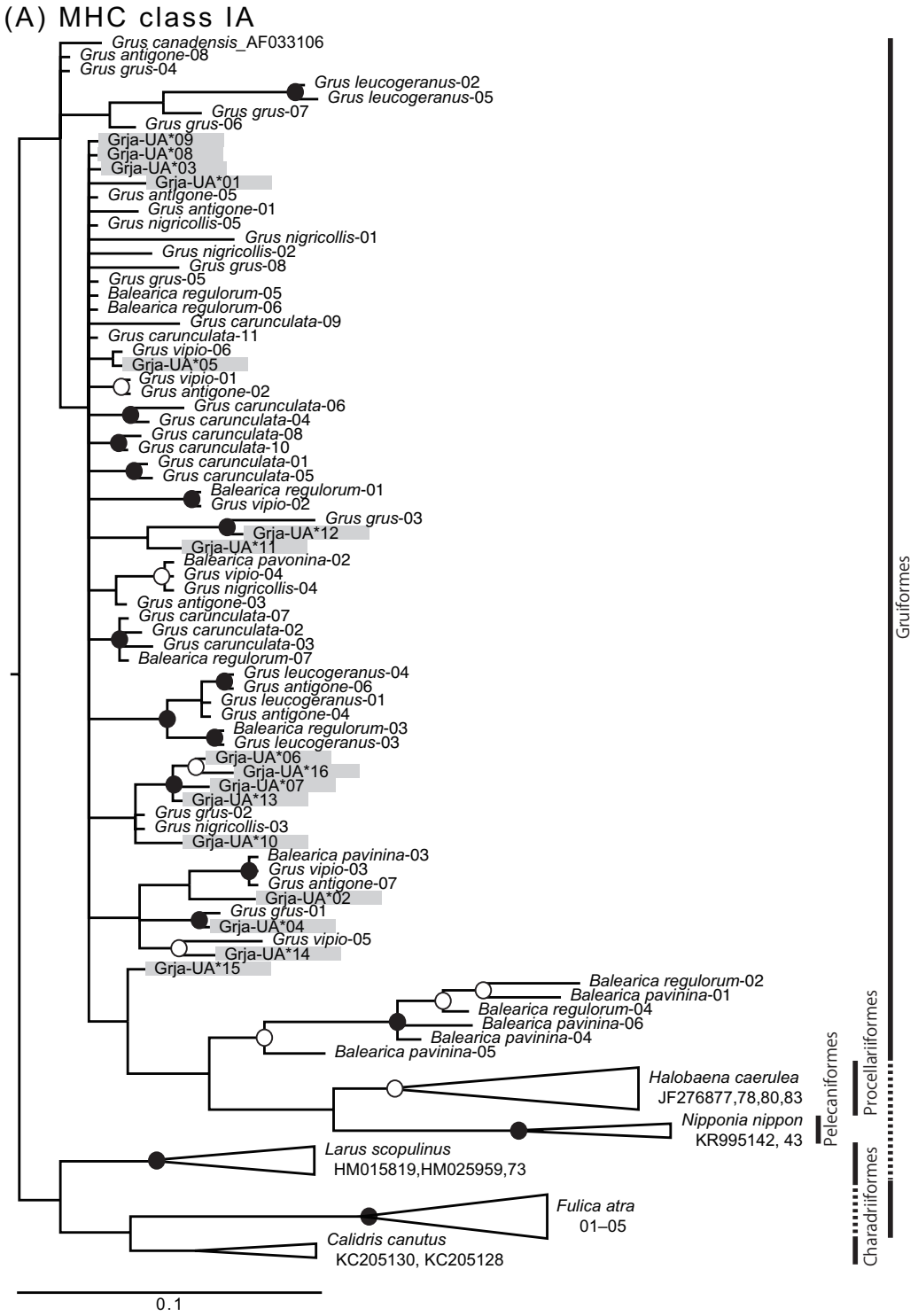
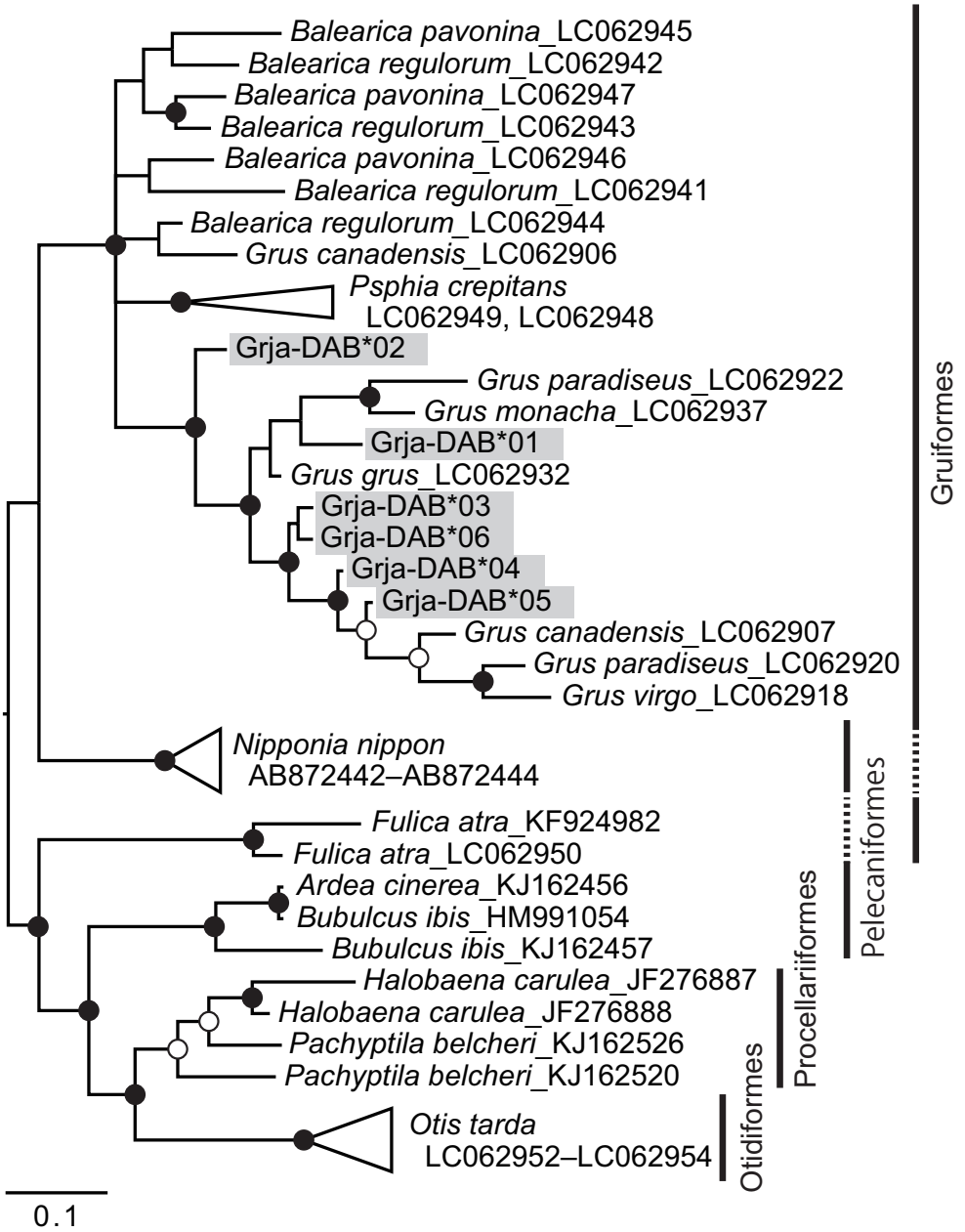


Fig. III-9 Bayesian trees of MHC class IA exon 3 (A) and IIB exon 2 (B) sequences from the red-crowned crane and some other species. Fifty percent majority-rule trees were inferred from: (A) 219 bp sequences from Akiyama et al. (2017a) within MHCIA exon 3, including the sequences from the Gruiformes, Pelecaniformes, Charadriiformes, and Procellariiformes; and (B) 267 bp sequences of complete MHCIIB exon 2, including the sequences from Gruiformes (family Gruidae; Rallidae; Psophiidae) Pelecaniformes, Procellariiformes, and Otidiformes. Taxon names indicate species names with GenBank accession numbers of published nucleotide sequences. Node support is given as Bayesian posterior probability (BPP): closed circles denote $0.95 \leq$ BPP, open circles denote $0.9 \leq$ BPP < 0.95, unlabeled nodes denote BPP ≤ 0.9. The alleles obtained in this study were indicated with the gray backgrounds. The scale bar indicates branch length in substitutions per site.

(B) MHC class IIB



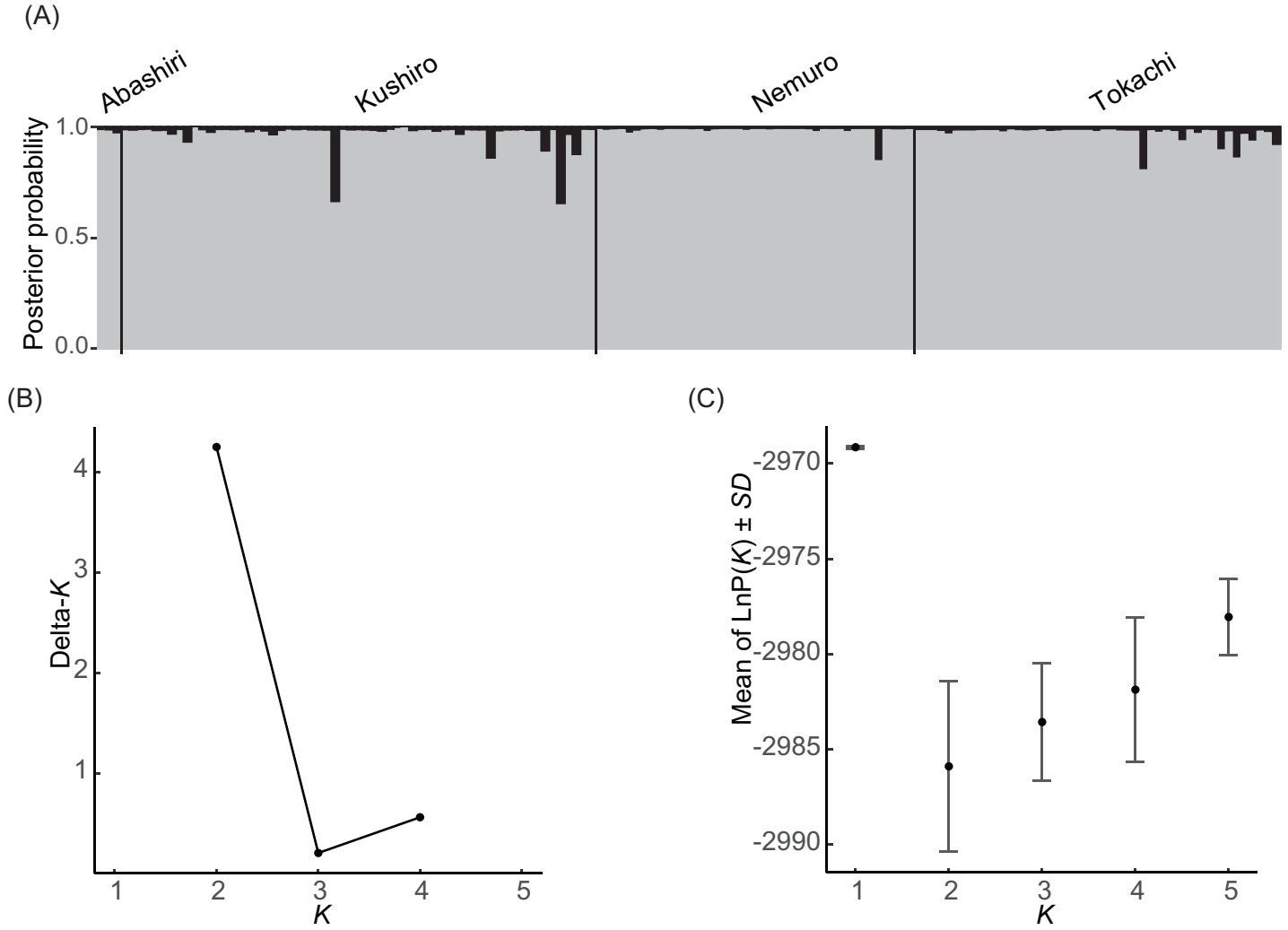


Fig. III-10 Bayesian clustering analysis performed in STRUCTURE (cited from Akiyama et al. 2017a). The results were obtained for K = 2 (A). Each sample is represented by a thin vertical bar, which was classified, based on the posterior probability of each cluster (black and gray). Black vertical lines separate four pre-detected subpopulations (Fig. III-7). Mean values of delta-K (B) and LnP(K) (C), which equals the average log probability of K, across ten runs detected in the analysis. Error bars show standard deviations.

Table I-1 Profiles of samples examined (cited from Akiyama et al. 2017c)

Species	Common name	Accession no. of GenBank	Sample type (N)	Source
Balearica pavonina	Black-crowned crane	LC114470	Fibroblasts	Maruyama Zoo
Balearica regulorum	Gray-crowned crane	LC114471	Fibroblasts	Nogeyama Zoo
Grus virgo	Demoiselle crane	LC114472	Feather shafts	Tennoji Zoo
Grus paradisea	Blue crane	LC114473	Feather shafts	Hirakawa Zoological Park
Grus antigone	Sarus crane	LC114474	Fibroblasts	Maruyama Zoo
Grus canadensis	Sandhill crane	LC114475	Feather shafts	Hirakawa Zoological Park
Grus carunculata	Wattled crane	LC114476	Blood	Sayama Chikozan Park Children's Zoo
Grus grus	Eurasian crane	LC114477	Fibroblasts	Kushiro Zoo
Grus leucogeranus	Siberian crane	LC114478	Blood	Tama Zoological Park
Grus monacha	Hooded crane	LC114479	Blood	Inokashira Park Zoo
Grus nigricollis	Black-necked crane	LC114480	Blood	Ueno Zoological Gardens
Grus vipio	White-naped crane	LC114481	Blood	Fukuoka City Zoological Garden
Grus japonensis	Red-crowned crane	LC114482-114484	Liver (2), lung (1)	National Institute for Environmental Studies
		LC114485	fibroblasts (1)	Nogeyama Zoo

Table I-2 Primers used for PCR and sequencing (cited from Akiyama et al. 2017c)

Primer	Nucleotide sequences (5'-3')	Location in mtDNA	Position no. in Fig. I-1	Source
LCO3g	CCT TCT CAA TCC GAT TCT TCC	3' end of CO III		This study
HCO3g	TGA TTG CTC TTC TCT GGG GCT	5' end of CO III		This study
Lcfb58	CAC ACC GGA CTA ATC AAA ACC T	3' end of ND5	1	This study
Lcyt200	ACT AAT CCG CAA CCT ACA TG	Middle of cyt b1	2	This study
H15655	GGG TTT GCT GGR GTR AAG TTT TC	3' end of cyt b1 and 2	3	Omote et al. (2015)
LtrnaP	ATC TCC ARC TCC CAA AGC TG	tRNA-Pro	4	This study
HtrnaP	GTA AAA TAC CAG CTT TGG GAG	tRNA-Pro	5	This study
LtrnaG	TAA GTC ATC AGA AAT TTC TG	tRNA-Glu	6	This study
H463cr	TAG GGG GAA AGA ATG GTC CT	Middle of CR1 and 2	7	This study
Hbirdsb	CAA AGT GCA TCA GTG	Middle of CR1 and 2	8	This study
Ldbox	CCT CTG GTT CCT ATG TCA GG	Middle of CR1 and 2	9	This study
H01251	TCT TGG CAT CTT CAG TGC CAT GC	tRNA-Phe	10	Omote et al. (2015)

Table I-3 Alignment of the duplicated CR sequences used for phylogenetic analyses of 13 crane species (cited from Akiyama et al. 2017c). Boxes indicate conserved sequence blocks: F-box, D-box, C-box, B-box, Bird similarity box and the conserved sequence block-1 (CSB-1). Dots show identical to nucleotides on the top of alignment, and dashes (-) indicate deletions. Abbreviations for species names are as follow: BP, *Balearica pavonina*; BR, *Balearica regulorum*; GVR, *Grus virgo*; GP, *Grus paradisea*; GA, *Grus antigone*; GCN, *Grus canadensis*; GCR, *Grus carunculata*; GG, *Grus grus*; GL, *Grus leucogeranus*; GM, *Grus monacha*; GN, *Grus nigricollis*; GVP, *Grus vipio*; GJ, *Grus japonensis*. The letters (a to d) of *G. japonensis* indicates the different red-crowned cranes.

```
BP_CR1   : TAAACGACAGTGCTCTAGAACATAACATGAATGGCTCAGTCCATAG-CATGCAACCCTCTCTCGACGTGCCGGTAGCTGTCGGACCAGGTTATTTATTAA 100
BR_CR1   : .....CC.T.....T..G.....A-A.....
GVR_CR1  : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TAC.....AT.....A.....---C.....G
GP_CR1   : C.T...G.TA..T.TCGA.G..A.T.....T....GT.....TAC.....A.....--T.C.....G
GA_CR1   : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TAG.....AT.....---C.....G
GCN_CR1  : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TA.....AT.....GA.....---C.....G.....G
GCR_CR1  : C.T...G.....T.TCGA.G..A.TT.....T....GT.....TAC.....A.....---C.....G
GG_CR1   : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TA.....A.....---C.....G
GL_CR1   : C.T...G.C...T.TCGA.G..A.TT.....T..G.AT.....TAG....G.AT.....--T.C.....G.....G
GM_CR1   : C.T...G.....T.TCGA.G..A.TT.....T....GT.....TA.....A.....---C.....G
GN_CR1   : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TA.....A.....---C.....
GVP_CR1  : C.T...G.....T.TCGA.G..A.TT.....T..G.AA.....TA.....AT.....--T.C.....G
GJ_a_CR1 : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TA.....A.....---C.....G
GJ_b_CR1 : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TA.....A.....---C.....G
GJ_c_CR1 : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TA.....A.....---C.....G
GJ_d_CR1 : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TA.....A.....---C.....G
BP_CR2   : .....-
BR_CR2   : .....C..T.....T..G.....A-A.....
GVR_CR2  : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TAC.....AT.....---C.....G
GP_CR2   : C.T...G.....T.....AGT.....T....GT.....TAC.....A.....--T.C.....G
GA_CR2   : C.T...G.....T.TCGA.G..A.TT.....T..G.AT.....TAG.....AT.....---C.....G
GCN_CR2  : C.T...G.....G...G.C.....T..G.AT.....TA.....AT.....GA.....---C.....G.....G
GCR_CR2  : C.T.....G.TT.....T....GT.....TAC.....A.....---C.....G
GG_CR2   : ..T...G.....C.....A.CT.....GT....ACA.....A.....---C.....G
GL_CR2   : C.T...G.....A.C.....T....A.....ACA.....G.....---C.....G.....G
GM_CR2   : ..T.....C.....A.TT.....T....GT.....TA.....A.....---C.....
GN_CR2   : ..T...G.....CT.....A.C.....TC...GT....ACA.....TA.....---C.....
GVP_CR2  : C.T...G.....T.TCGAGG..A.C.....T....AA.....TA.....AT.....---C.....G
GJ_a_CR2 : C.T.....C.AG...AGT.....T....GT....ACA.....A.....---C.....
GJ_b_CR2 : C.T.....C.A....AGT.....T....GT....ACA.....A.....---C.....G
GJ_c_CR2 : C.T.....C.C.AG...AGT.....T....GT....ACA.....A.....---C.....
GJ_d_CR2 : C.T.....C.AG...AGT.....T....GT....ACA.....A.....---C.....
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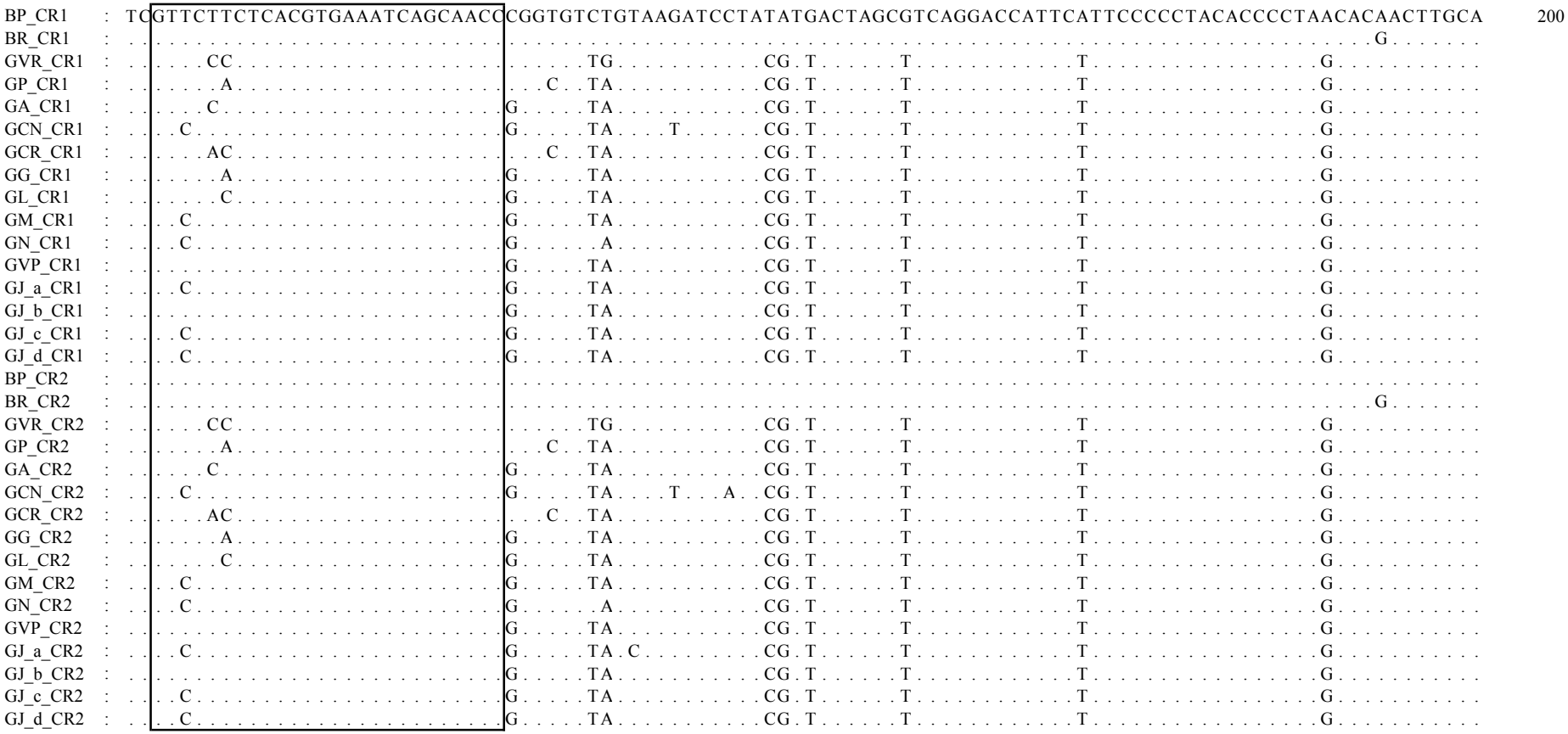
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BR_CR1 : G
GVR_CR1 : CC TG CG T T T G
GP_CR1 : A C TA CG T T T G
GA_CR1 : C G TA CG T T T G
GCN_CR1 : C G TA T CG T T T G
GCR_CR1 : AC C TA CG T T T G
GG_CR1 : A G TA CG T T T G
GL_CR1 : C G TA CG T T T G
GM_CR1 : C G TA CG T T T G
GN_CR1 : C G A CG T T T G
GVP_CR1 : G TA CG T T T G
GJ_a_CR1 : C G TA CG T T T G
GJ_b_CR1 : G TA CG T T T G
GJ_c_CR1 : C G TA CG T T T G
GJ_d_CR1 : C G TA CG T T T G
BP_CR2 :
BR_CR2 : G
GVR_CR2 : CC TG CG T T T G
GP_CR2 : A C TA CG T T T G
GA_CR2 : C G TA CG T T T G
GCN_CR2 : C G TA T A CG T T T G
GCR_CR2 : AC C TA CG T T T G
GG_CR2 : A G TA CG T T T G
GL_CR2 : C G TA CG T T T G
GM_CR2 : C G TA CG T T T G
GN_CR2 : C G A CG T T T G
GVP_CR2 : G TA CG T T T G
GJ_a_CR2 : C G TA C CG T T T G
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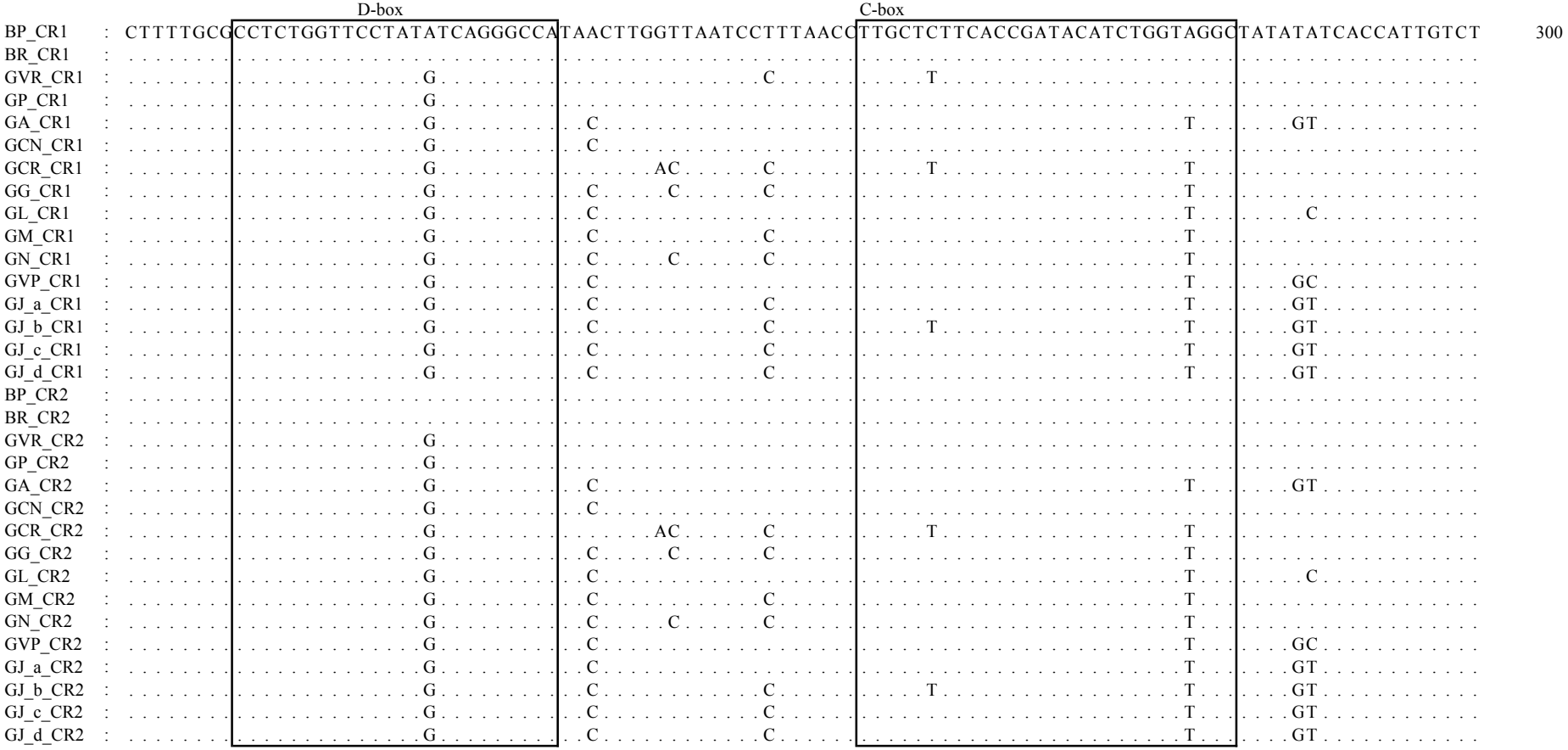
GJ_d_CR2 : C G TA CG T T T G


D-box
C-box
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BR_CR1 :
GVR_CR1 :
GP_CR1 :
GA_CR1 :
GCN_CR1 :
GCR_CR1 :
GG_CR1 :
GL_CR1 :
GM_CR1 :
GN_CR1 :
GVP_CR1 :
GJ_a_CR1 :
GJ_b_CR1 :
GJ_c_CR1 :
GJ_d_CR1 :
BP_CR2 :
BR_CR2 :
GVR_CR2 :
GP_CR2 :
GA_CR2 :
GCN_CR2 :
GCR_CR2 :
GG_CR2 :
GL_CR2 :
GM_CR2 :
GN_CR2 :
GVP_CR2 :
GJ_a_CR2 :
GJ_b_CR2 :
GJ_c_CR2 :
GJ_d_CR2 :

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BP_CR1    : CTCTTAATCGCGGCATCTTTCCTTTTTAGCGCTGCTGGTTCCCTTTTTTTTTCTGGGCGTCTTCAGGCTACCCCCCCGTGCGACGGGAACTTACAATCTA   400
BR_CR1    : ..T.................................................T.......................G....................T..
GVR_CR1   : ................T..C......CG..A..TT.................TCT..G.......AC..G...T..G....AG....TG........T..
GP_CR1    : ................T..C......CG..A..TT.................TCT..G.......AC..G...T..G....AG....TG........T..
GA_CR1    : .....................T.....G....CTT.................TCT..G.......AT..G...T..G....AG....TGTA......T..
GCN_CR1   : .....................T.....G....CTT.................TCT..G.......A...G...T..G....AG....TGTA......T..
GCR_CR1   : ................T..C......CG..A..TT.................TCT..G.......ATT.G...T..G.....G....TG........T..
GG_CR1    : ................T..C......CG..A..TT.................TCT..G.......TC..G...T..G....AG....TGTA......T..
GL_CR1    : ...........................G....CTT.................TCT..G..........G...........AG....TGTA......T..
GM_CR1    : ................T..C......CG..A..TT.................TCT..G.......CT..G...T..G....AG....TG........T..
GN_CR1    : ................T..C......CG..A..TT.................TCT..G.......CT..G...T..G....AG....TG........T..
GVP_CR1   : ...................C......CG..A..TT.................TCT..G.......AT..G...T..G....AG....TG.C......T..
GJ_a_CR1  : ................T..C......CG..A..TT.................TCT..G.......AC..G...T..G....AG....TG........T..
GJ_b_CR1  : ................T..C......CG..A..TT.................TCT..G.......AC..G...T..G....AG....TG........T..
GJ_c_CR1  : ................T..C......CG..A..TT.................TCT..G.......AC..G...T..G....AG....TG........T..
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BP_CR2    : ...........................G........................................................................
BR_CR2    : ....................................................T.......................G....................T..
GVR_CR2   : ................T..C.......G..A..TT.................TCT..G.......AC..G...T..G....AG....TG........T..
GP_CR2    : ................T..C.......G..A..TT.................TCT..G.......AC..G...T..G....AG....TG.C......T..
GA_CR2    : .....................T........A..TT................CTCT..G.......AT..G...T..G....AG....TGTA......T..
GCN_CR2   : .....................T.....G..A..TT................CTCT..G..........G...T..G....AG....TGTA......T..
GCR_CR2   : ................T..C......CG..A..TT.................TCT..G.......AT..G...T..G.....G....TG........T..
GG_CR2    : ................T..C......CG..A..TT.................TCT..G.......TC..G...T..G....AG....TGTA......T..
GL_CR2    : ...................C.T........A..TT................CTCT..G..........G...........AG....TGTA......T..
GM_CR2    : ................T..C......CG..A..TT.................TCT..G.......CT..G...T..G....AG....TG........T..
GN_CR2    : ................T..C......CG..A.CTT................-TCT..G.......CT..G...T..G....AG....TG........T..
GVP_CR2   : ..........................CG..A..TT................-TCT..G.......AT..G...T..G....AG....TG........T..
GJ_a_CR2  : ................T..C.......G..A..TT.................TCT..G.......AC..G...T..G....AG....TG........T..
GJ_b_CR2  : ................T..C.......G..A..TT.................TCT..G.......AC..G...T..G....AG....TG........T..
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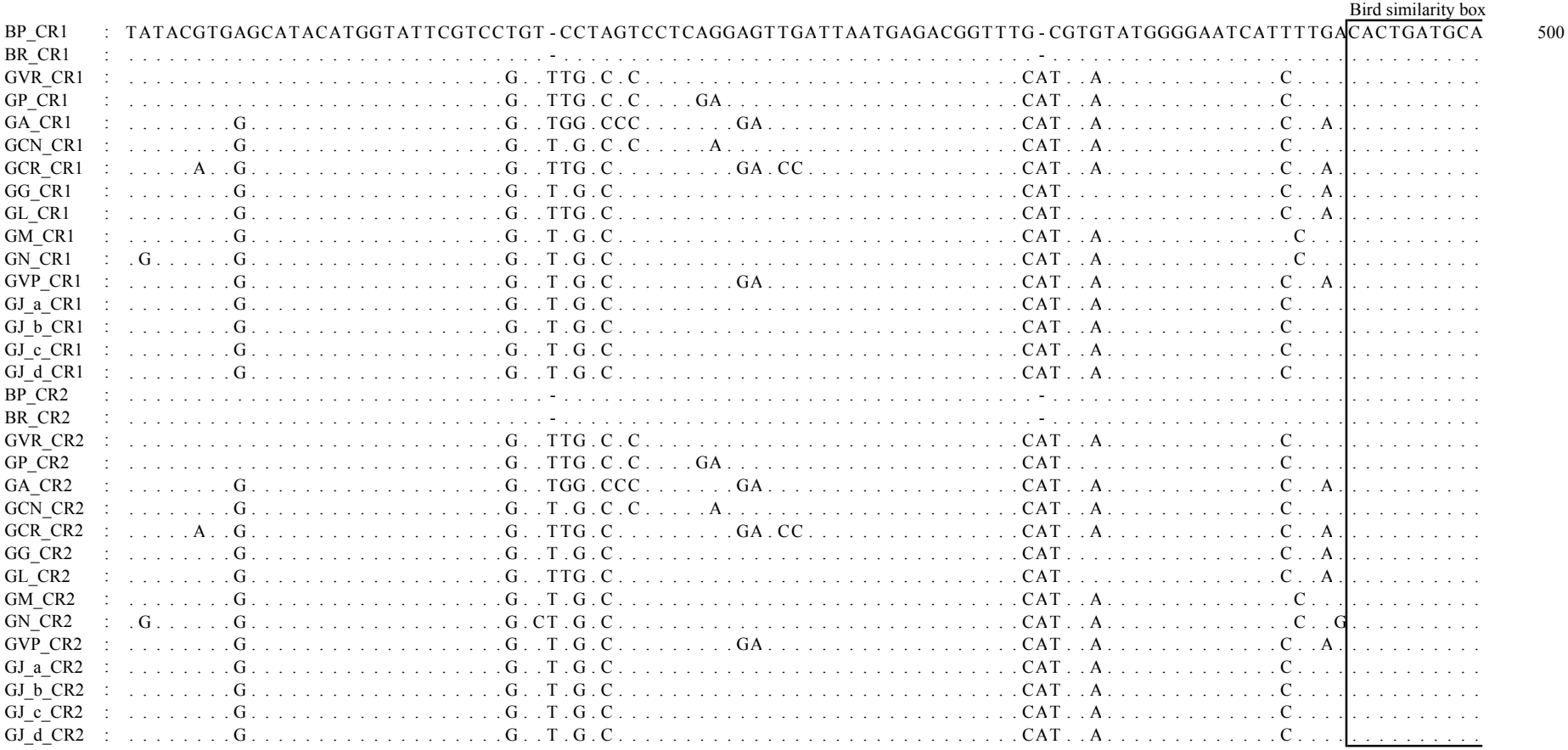
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BR_CR1 :
GVR_CR1 :
GP_CR1 :
GA_CR1 :
GCN_CR1 :
GCR_CR1 :
GG_CR1 :
GL_CR1 :
GM_CR1 :
GN_CR1 :
GVP_CR1 :
GJ_a_CR1 :
GJ_b_CR1 :
GJ_c_CR1 :
GJ_d_CR1 :
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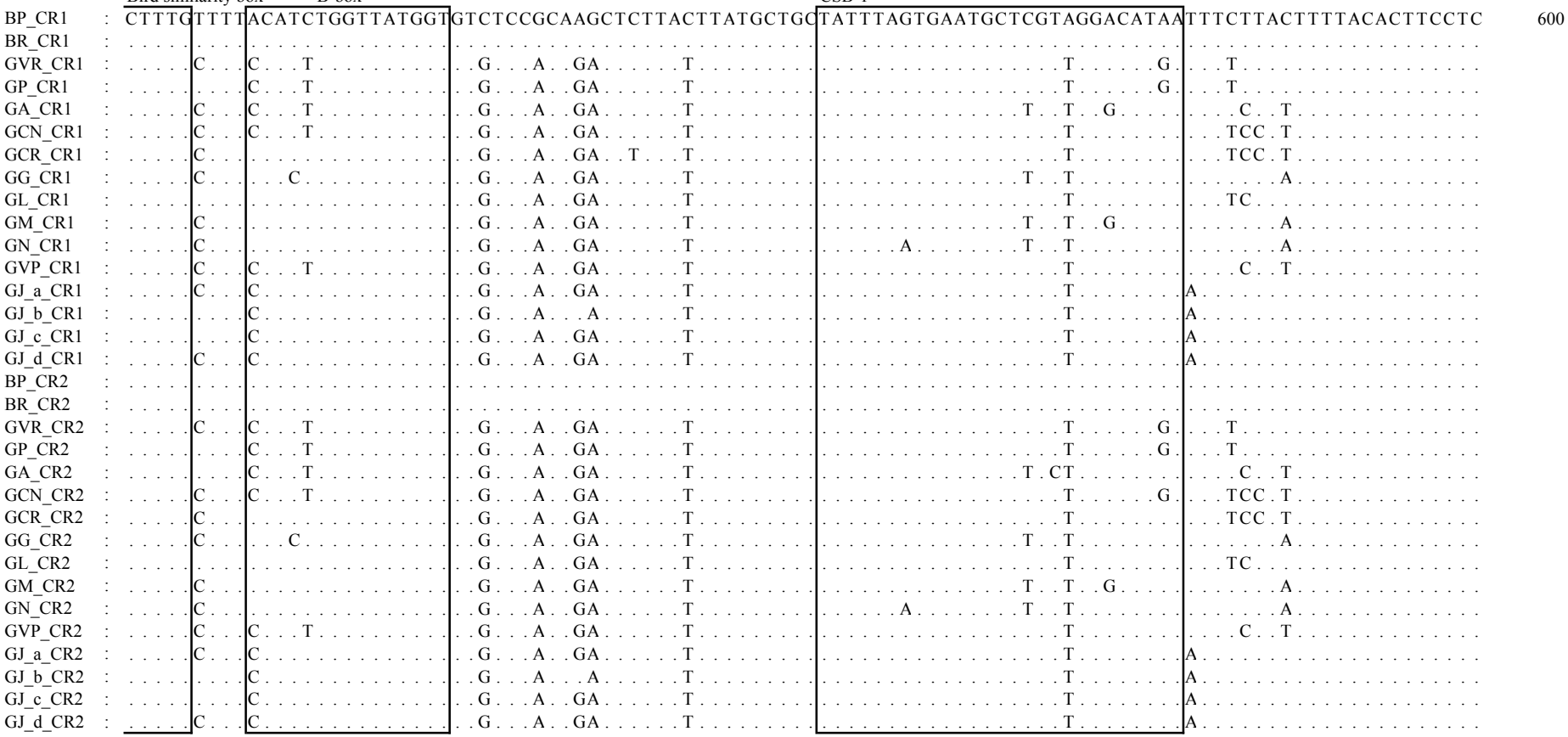
Bird similarity box
B-box
CSB-1
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BR_CR1 :
GVR_CR1 :
GP_CR1 :
GA_CR1 :
GCN_CR1 :
GCR_CR1 :
GG_CR1 :
GL_CR1 :
GM_CR1 :
GN_CR1 :
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GJ_a_CR1 :
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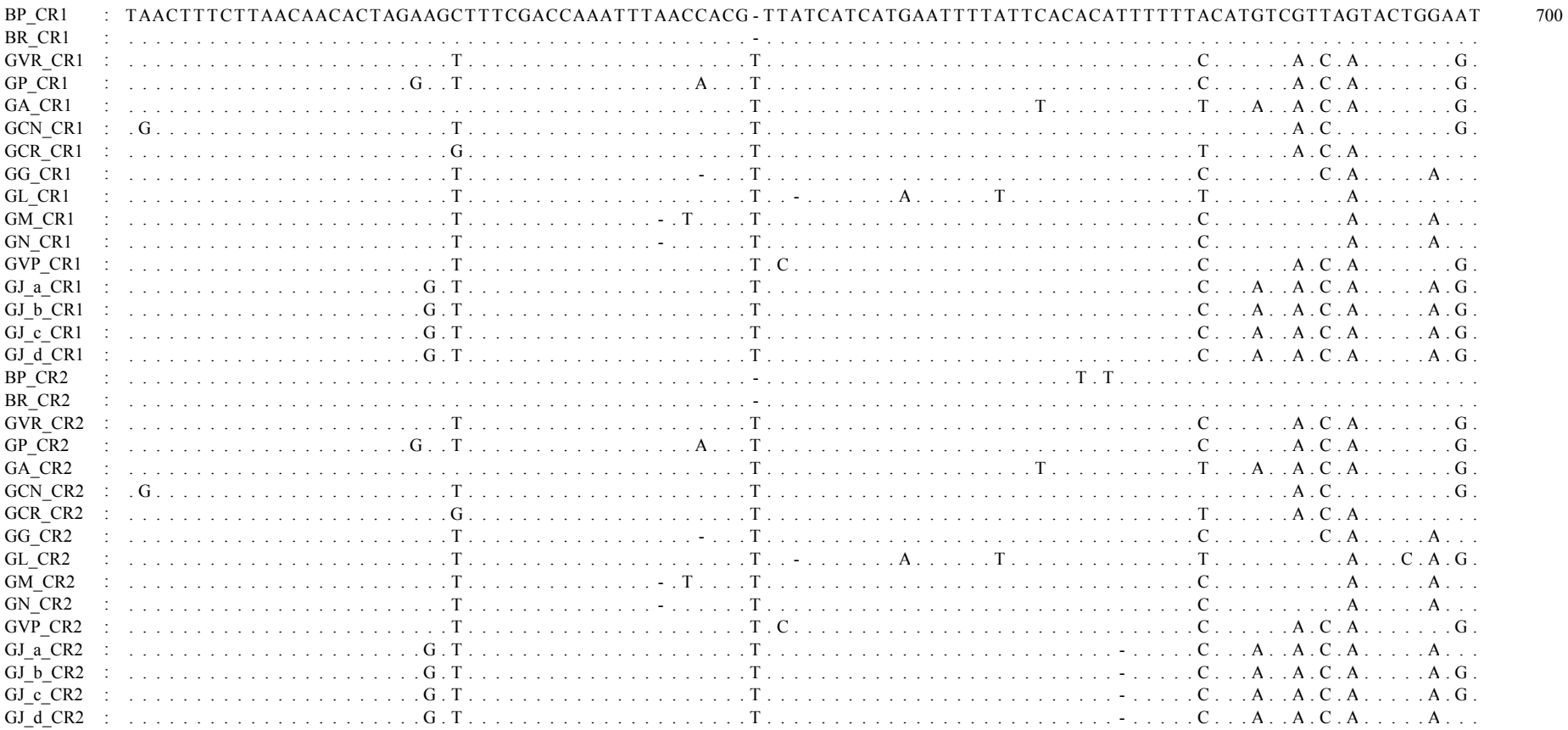
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BR_CR1 :
GVR_CR1 :
GP_CR1 :
GA_CR1 :
GCN_CR1 :
GCR_CR1 :
GG_CR1 :
GL_CR1 :
GM_CR1 :
GN_CR1 :
GVP_CR1 :
GJ_a_CR1 :
GJ_b_CR1 :
GJ_c_CR1 :
GJ_d_CR1 :
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GCN_CR2 :
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GG_CR2 :
GL_CR2 :
GM_CR2 :
GN_CR2 :
GVP_CR2 :
GJ_a_CR2 :
GJ_b_CR2 :
GJ_c_CR2 :
GJ_d_CR2 :

```
BP_CR1    : TACATTAATAAA      712
BR_CR1    : ............
GVR_CR1   : ............
GP_CR1    : ............
GA_CR1    : ............
GCN_CR1   : ............
GCR_CR1   : C...........
GG_CR1    : .G..........
GL_CR1    : ............
GM_CR1    : .G..........
GN_CR1    : .G..........
GVP_CR1   : .G..........
GJ_a_CR1  : .G..........
GJ_b_CR1  : .G..........
GJ_c_CR1  : .G..........
GJ_d_CR1  : .G..........
BP_CR2    : ............
BR_CR2    : ............
GVR_CR2   : ............
GP_CR2    : ............
GA_CR2    : ............
GCN_CR2   : ............
GCR_CR2   : C...........
GG_CR2    : .G..........
GL_CR2    : .........C...
GM_CR2    : .G..........
GN_CR2    : .G..........
GVP_CR2   : .G..........
GJ_a_CR2  : .G..........
GJ_b_CR2  : .G..........
GJ_c_CR2  : .G..........
GJ_d_CR2  : .G..........
```

Table II-1 Profiles of feather samples collected from stuffed specimens examined (cited from Akiyama et al. 2017b)

Sample ID	Year of sampling	Location of sampling[a]	mtDNA haplotype	Supplier
1C2	1878	Chitose	Gj2	Field Science Center for Northern Biosphere, Hokkaido University
2K2	1927	Kushiro	Gj2	Field Science Center for Northern Biosphere, Hokkaido University
3K2	1960	Kushiro	Gj2	Field Science Center for Northern Biosphere, Hokkaido University
4K2	1971	Kushiro	Gj2	Historical Museum of Hokkaido
5ND2	1983	N.A.	Gj2	Tsurui Village Furusato Information Center "Minakuru"
6ND2	1983	N.A.	Gj2	Tsurui Village Furusato Information Center "Minakuru"
7ND1	1983	N.A.	Gj1	Tsurui Village Furusato Information Center "Minakuru"
8ND2	1983	N.A.	Gj2	Tsurui Village Furusato Information Center "Minakuru"
9K2	1986	Kushiro	Gj2	Center for Molecular Biodiversity Research, National Museum of Nature and Science, Tokyo
10B2	1987	Bekkai	Gj2	Betsukai Town Historical Museum
11N2	1987	Nemuro	Gj2	Nemuro City Museum of History and Nature
12A2	1988	Akan	Gj2	Abiko City Museum of Birds
13K2	1991	Kushiro	Gj2	Center for Molecular Biodiversity Research, National Museum of Nature and Science, Tokyo
14A2	1992	Akan	Gj2	Miyazaki Prefectural Museum of Nature and History
15ND1	1997	N.A.	Gj1	Tsurui Village Furusato Information Center "Minakuru"
16S13	1997	Shibecha	Gj13	Abiko City Museum of Birds
17K2	2001	Kushiro	Gj2	Tomakomai City Museum

[a]N.A., not available.

Table II-2 Alignment of control region 2 (CR2; 1165 bp) haplotypes of the red-crowned crane (cited from Akiyama et al. 2017b). Only substitutions are shown. Nine haplotypes (Gj1–Gj9) were reported by Hasegawa et al. (1999), and three haplotypes (Gj10–Gj12) were reported by Miura et al. (2013b). Haplotype Gj13 (bold) was newly identified in the present study. Nucleotides identical to those in Gj1 are indicated by dots; nucleotides different from Gj1 are shown by letters.Dashes show no sequence information (not gaps.) Sequence data were not available at positions 550–1000 for haplotypes Gj5-Gj9.

Haplotype	23	24	25	49	74	130	166	190	237	239	247	257	274	318	323	402	407	440	550	562	622	808	836	1000	Reference	accession nos.
Gj1	T	A	C	A	A	T	C	C	C	G	G	A	C	T	G	A	C	C	T	C	C	C	G	A	Hasegawa et al. 1999	AB714136
Gj2	.	.	.	.	.	.	T	.	T	A	A	.	.	.	A	G	T	T	C	T	.	T	A	G	Hasegawa et al. 1999	AB714137
Gj3	.	.	.	.	.	C	T	.	.	.	A	G	.	C	.	.	.	T	C	.	T	T	.	G	Hasegawa et al. 1999	AB714138
Gj4	.	.	.	G	.	.	T	.	.	.	A	.	.	.	.	.	.	T	C	.	.	.	.	G	Hasegawa et al. 1999	AB714139
Gj5	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	G	.	T	C	.	.	T	.	G	Hasegawa et al. 1999	AB714140
Gj6	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	.	.	T	-	-	-	-	-	-	Hasegawa et al. 1999	-
Gj7	.	.	.	.	.	.	T	.	.	A	A	.	.	.	A	G	.	T	-	-	-	-	-	-	Hasegawa et al. 1999	-
Gj8	.	.	.	.	.	.	T	.	.	.	A	.	T	.	.	G	.	T	-	-	-	-	-	-	Hasegawa et al. 1999	-
Gj9	.	.	.	.	.	.	T	.	.	.	A	G	.	C	.	.	.	T	-	-	-	-	-	-	Hasegawa et al. 1999	-
Gj10	C	T	A	.	.	.	T	.	.	.	A	.	.	.	.	G	.	T	C	.	.	T	.	G	Miura et al. 2013a	AB669093
Gj11	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	G	.	T	C	T	.	T	.	G	Miura et al. 2013a	AB669094
Gj12	.	.	.	.	G	.	T	.	.	A	A	.	.	.	A	G	.	T	C	T	.	T	.	G	Miura et al. 2013a	AB669095
Gj13	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**T**	**C**	**.**	**.**	**.**	**.**	**.**	**This study**	**LC129041**

Table III-1 Gruiformes samples and number of MHCIA alleles detected in one individual per species

Order and Family	Species	Common name	No. Alleles	Source
Gruiformes				
Gruidae	*Balearica pavonina*	Black-crowned crane	5	Maruyama Zoo
	Balearica regulorum	Gray-crowned crane	7	Nogeyama Zoo
	Grus antigone	Sarus crane	8	Maruyama Zoo
	Grus carunculata	Wattled crane	11	Sayama Chikozan Park Children's Zoo
	Grus grus	Eurasian crane	8	Kushiro Zoo
	Grus leucogeranus	Siberian crane	5	Tama Zoological Park
	Grus nigricollis	Black-necked crane	5	Ueno Zoological Gardens
	Grus vipio	White-naped crane	6	Fukuoka City Zoological Garden
Ralidae	*Fulica atra*	Eurasican coot	5	Tama Zoological Park

Table III-2 Number of MHC class IA exon 3 (A) and MHC class IIB exon 2 (B) sequences found in individuals. Detected and undetected alleles are indicated by 1 and 0, respectively (cited from Akiyama et al. 2017a)

		MHC class IA																		MHC class IIB							
Sample.id	Regions	Grja-UA*01	Grja-UA*02	Grja-UA*03	Grja-UA*04	Grja-UA*05	Grja-UA*06	Grja-UA*07	Grja-UA*08	Grja-UA*09	Grja-UA*10	Grja-UA*11	Grja-UA*12	Grja-UA*13	Grja-UA*14	Grja-UA*15	Grja-UA*16	Total number	Library size	Grja-DAB*01	Grja-DAB*02	Grja-DAB*03	Grja-DAB*04	Grja-DAB*05	Grja-DAB*06	Total number	Library size
id001	Kushiro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	1907	1	1	0	0	1	1	4	602
id002	Nemuro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	781	1	1	0	1	0	1	4	260
id003	Kushiro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	332	1	1	0	0	1	1	4	344
id004	Kushiro	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	5	1537	1	1	0	0	1	1	4	422
id005	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	354	1	0	0	1	0	0	2	660
id006	Kushiro	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	392	1	0	1	1	0	0	3	337
id007	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	525	1	1	0	0	1	1	4	1040
id008	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	1273	1	0	0	1	1	0	3	507
id009	Kushiro	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	4	725	0	1	0	0	0	1	2	633
id010	Nemuro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	665	1	1	0	0	1	1	4	788
id011	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	520	1	1	0	1	0	1	4	567
id012	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	452	0	1	0	0	0	1	2	582
id013	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	400	1	0	0	1	0	0	2	412
id014	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	613	1	1	0	0	1	1	4	1941
id015	Nemuro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	410	1	1	0	1	0	1	4	247
id016	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	700	1	0	0	1	0	0	2	346
id017	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	1753	1	0	0	1	1	0	3	318
id018	Kushiro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	237	1	0	1	0	0	0	2	622
id019	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	376	1	1	0	0	1	1	4	484
id020	Kushiro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	993	1	0	1	1	0	0	3	291
id021	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	1019	1	0	0	1	0	0	2	511
id022	Kushiro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	808	1	0	1	1	0	0	3	723
id023	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	560	1	1	0	1	0	1	4	822
id024	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	361	0	1	0	0	0	1	2	803
id025	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	418	1	1	0	0	1	1	4	802
id026	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	309	1	1	0	1	0	1	4	529
id027	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	691	1	1	0	1	0	1	4	547
id028	Tokachi	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	614	1	0	1	0	1	0	3	273
id029	Kushiro	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	4	397	1	1	0	1	0	1	4	378
id030	Kushiro	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	5	899	0	1	0	0	0	1	2	459
id031	Kushiro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	351	1	1	0	0	1	1	4	725
id032	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	351	1	1	0	1	0	1	4	457
id033	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	796	1	0	0	1	1	0	3	1060
id034	Nemuro	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	4	1877
id035	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	1005	1	0	0	1	1	0	3	589
id036	Nemuro	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	490	1	1	0	1	0	1	4	854
id037	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	385	1	0	0	1	1	0	3	679
id038	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	585	1	0	0	0	1	0	2	756
id039	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	380	1	0	0	1	1	0	3	605
id040	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	829	1	0	0	1	1	0	3	469
id041	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	2411	1	0	0	1	0	0	2	309
id042	Kushiro	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	630	1	0	0	1	0	0	2	330
id043	Nemuro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	518	1	0	0	1	1	0	3	393
id044	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	317	1	1	0	0	1	1	4	839
id045	Tokachi	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	402	1	0	1	0	1	0	3	509
id046	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	516	1	1	0	1	0	1	4	863
id047	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	941	1	1	0	1	0	1	4	609
id048	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	754	1	0	0	0	1	0	2	729
id049	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	838	1	0	1	1	0	0	3	790
id050	Kushiro	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	553	1	0	0	1	1	0	3	671
id051	Nemuro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	727	1	1	0	1	0	1	4	694
id052	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	320	1	1	0	0	1	1	4	1195
id053	Nemuro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	1576	1	1	0	1	0	1	4	662
id054	Tokachi	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	1398	1	0	0	1	0	0	2	297
id055	Nemuro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	461	1	1	0	0	1	1	4	412
id056	Kushiro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	1003	1	1	0	0	1	1	4	367
id057	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	309	1	0	0	1	1	0	3	720
id058	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	336	1	0	0	1	1	0	3	377
id059	Tokachi	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	4	1028	0	1	0	0	0	1	2	276
id060	Kushiro	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	1282	1	0	1	1	0	0	3	443
id061	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	717	1	1	0	0	1	1	4	745
id062	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	620	1	1	0	0	1	1	4	829
id063	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	493	1	0	1	0	1	0	3	614
id064	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	860	1	0	0	1	1	0	3	866
id065	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	414	1	0	0	0	1	0	2	439
id066	Nemuro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	1374	1	0	1	0	1	0	3	463
id067	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	673	1	0	1	0	1	0	3	205
id068	Kushiro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	193	1	0	1	0	1	0	3	285
id069	Nemuro	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	672	1	1	1	0	0	1	4	258
id070	Tokachi	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	5	314	1	0	0	0	1	0	2	517
id071	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	617	1	1	0	1	0	1	4	328
id072	Nemuro	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	877	1	1	1	0	0	1	4	221
id073	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	1035	1	0	0	1	0	0	2	293
id074	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	523	1	0	1	0	1	0	3	445
id075	Kushiro	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	4	625	0	1	0	0	0	1	2	674
id076	Nemuro	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	524	1	0	1	1	0	0	3	501
id077	Kushiro	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	528	1	1	1	0	0	1	4	473
id078	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	357	1	0	1	1	0	0	3	713
id079	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	674	1	0	0	0	1	0	2	598
id080	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	1529	1	1	0	1	0	1	4	317
id081	Kushiro	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	157	0	1	0	0	0	1	2	407
id082	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	385	1	1	0	0	1	1	4	364
id083	Nemuro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	300	1	1	0	1	0	1	4	574
id084	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	374	1	0	0	1	1	0	3	383
id085	Tokachi	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	946	1	0	1	0	1	0	3	262
id086	Abashiri	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	933	1	1	0	1	0	1	4	396
id087	Tokachi	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	483	1	1	1	0	0	1	4	608
id088	Nemuro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	676	1	0	0	1	1	0	3	633
id089	Tokachi	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	454	1	0	1	1	0	0	3	568
id090	Tokachi	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	5	429	1	0	1	1	0	0	3	608
id091	Kushiro	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	5	646	0	1	0	0	0	1	2	1502
id092	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	655	1	0	0	0	1	0	2	546
id093	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	739	1	0	0	1	1	0	3	255
id094	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	806	1	1	0	1	0	1	4	350
id095	Nemuro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	1432	1	1	0	0	1	1	4	350
id096	Nemuro	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	341	1	1	1	0	0	1	4	521
id097	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	593	1	1	0	1	0	1	4	316
id098	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	1187	1	0	0	1	0	0	2	314
id099	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	1262	1	0	0	0	1	0	2	507
id100	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	729	1	0	0	1	0	0	2	500
id101	Tokachi	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	948	1	1	1	0	0	1	4	606
id102	Nemuro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	511	1	0	1	0	1	0	3	466
id103	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	792	1	0	1	0	1	0	3	490
id104	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	840	1	0	0	1	1	0	3	761
id105	Kushiro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	416	1	0	0	1	1	0	3	542
id106	Kushiro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	310	0	1	0	0	0	1	2	453
id107	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	816	1	1	0	0	1	1	4	1915
id108	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	962	1	0	0	1	0	0	2	1016
id109	Kushiro	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	0	11	786	1	0	0	0	1	0	2	829
id110	Tokachi	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	4	547	0	1	0	0	0	1	2	749
id111	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	571	1	1	0	0	1	1	4	419
id112	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	385	1	0	0	1	0	0	2	1213
id113	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	953	1	0	0	0	1	0	2	1068
id114	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	404	1	0	0	1	0	0	2	553
id115	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	243	1	0	0	0	1	0	2	747
id116	Nemuro	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	8	793	1	1	0	1	0	1	4	1031
id117	Abashiri	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	718	1	1	0	1	0	1	4	654
id118	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	723	1	0	0	1	0	0	2	1160
id119	Nemuro	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	9	541	1	0	1	0	1	0	3	762
id120	Tokachi	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	590	1	0	0	0	1	0	2	326
id121	Kushiro	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	5	357	0	1	0	0	0	1	2	556
id122	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	371	1	1	0	0	1	1	4	789
id123	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	1161	1	0	0	1	1	0	3	946
id124	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	368	1	0	0	1	0	0	2	408
id125	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	284	1	0	0	1	1	0	3	315
id126	Tokachi	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	755	1	1	0	0	1	1	4	1120
id127	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	911	1	0	0	1	1	0	3	911
id128	Kushiro	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	5	548	1	0	1	0	0	0	2	1231
id129	Kushiro	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	11	511	1	0	1	0	1	0	3	341
id130	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	305	1	0	0	1	1	0	3	971
id131	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	534	1	0	0	1	0	0	2	836
id132	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	343	0	1	0	0	0	1	2	629
id133	Tokachi	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	929	1	1	0	0	1	1	4	962
id134	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	309	1	0	0	1	0	0	2	750
id135	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	228	1	1	0	0	1	1	4	268
id136	Tokachi	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	679	1	1	0	0	1	1	4	1104
id137	Abashiri	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	925	0	1	0	0	0	1	2	1249
id138	Tokachi	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	4	614	0	1	0	0	0	1	2	932
id139	Kushiro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	524	1	0	0	1	1	0	3	673
id140	Kushiro	1	1	1	0	0	0	0	1	0	0	0	1	1	0	0	0	6	840	1	0	1	0	1	0	3	879
id141	Nemuro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	480	1	0	0	1	1	0	3	447
id142	Nemuro	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	234	1	0	0	1	0	0	2	186
id143	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	1335	1	0	0	1	1	0	3	1094
id144	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	309	1	0	0	1	1	0	3	404
id145	Tokachi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	310	1	1	0	0	1	1	4	591
id146	Tokachi	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	4	628	0	1	0	0	0	1	2	1103
id147	Tokachi	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7	805	1	0	1	1	0	0	3	663
id148	Kushiro	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11	450	1	1	0	1	0	1	4	676
id149	Tokachi	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	5	618	1	0	0	0	1	0	2	557
id150	Kushiro	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	9	303	1	1	0	0	1	1	4	1011
id151	Kushiro	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	9	518	1	0	1	1	0	0	3	335
id152	Nemuro	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	4	443	0	1	0	0	0	1	2	738

Table III-3 Nucleotide diversities and substitution rates in the MHC class IA exon 3 and MHC class IIB exon 3 (cited from Akiyama et al. 2017a)

Genes	Sites	bp	S	π	θ	D (P-value)	Nc	d_N (±SE)	d_S (±SE)	ω	Z (P-value)
MHC class IA	All	222	26	0.048	0.035	1.486 (P>0.10)	73	0.04 (0.01)	0.07 (0.03)	0.571	-0.815 (P=1.0)
	ABS		13	0.136			15	0.16 (0.06)	0.14 (0.09)	1.143	0.400 (P=0.25)
	non-ABS		13	0.023			58	0.02 (0.01)	0.05 (0.02)	0.400	-1.198 (P=1.0)
MHC class IIB	All	270	39	0.065	0.063	0.219 (P>0.10)	89	0.07 (0.01)	0.08 (0.03)	0.875	-0.539 (P=1.0)
	ABS		23	0.146			24	0.17 (0.05)	0.16 (0.08)	1.063	0.550 (P=0.29)
	non-ABS		16	0.037			65	0.03 (0.01)	0.05 (0.03)	0.600	-1.026 (P=1.0)

Abbreviations are as follows: bp, length of the nucleotide sequences in base-pairs; S, segregating sites; π, nucleotide diversity; θ, Watterson's θ; D, Tajima's D; Nc, number of codons;d_N, non-synonymous substitution rate; d_S,synonymous substitution rate; ω, d_N/d_S. Standard errors for d_N and d_S obtained through 1,000 bootstrap replicates and P-values for θ and Z are indicated in parentheses.

Table III-4 Genetic diversity based on nine microsatellite locus (cited from Akiyama et al. 2017a)

Area	N	N_A	A_P	A_R	H_O (±s.d.)	H_E (±s.d.)	F_{IS}
Kushiro	61	5.00	6	4.70	0.57 (±0.17)	0.58 (±0.14)	0.03
Tokachi	47	4.22	2	4.22	0.55 (±0.15)	0.58 (±0.09)	0.05
Nemuro	41	4.22	0	4.16	0.55 (±0.13)	0.56 (±0.09)	0.02
Abashiri	3	2.56	0	-	0.59 (±0.40)	0.62 (±0.17)	0.06
overall	152	5.33	8	4.44	0.56 (±0.15)	0.58 (±0.11)	0.03

The following parameters are given: N, number of samples analyzed; N_A, mean number of alleles; A_P, number of private alleles; A_R, mean allelic richness at the minimum sample size 41; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient.

Table III-5 Population pairwise G_{ST} values (lower matrices) and P-values (upper matrices) between four regions within Hokkaido (cited from Akiyama et al. 2017a). No significant G_{ST} values ($P < 0.05$) were estimated.

	MHC class IIB				MHC class IA				Microsatellite			
	Abashiri	Kushiro	Nemuro	Tokachi	Abashiri	Kushiro	Nemuro	Tokachi	Abashiri	Kushiro	Nemuro	Tokachi
Abashiri		1.000	1.000	1.000		0.999	0.999	1.000		0.989	0.994	0.989
Kushiro	-0.0072		1.000	1.000	-0.0030		1.000	1.000	0.0085		0.954	0.993
Nemuro	-0.0086	-0.0023		1.000	-0.0034	-0.0008		1.000	0.0087	0.0025		0.983
Tokachi	-0.0080	-0.0012	-0.0025		-0.0036	-0.0004	-0.0009		0.0086	-0.0014	0.0005	