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Duplication and Variation in the Major Histocompatibility Complex Genes in Blakiston's Fish Owl, *Bubo blakistoni*

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The major histocompatibility complex (MHC) includes many genes that are essential for the adaptive immune system, and variation in the antigen binding site (ABS) is related to resistance against pathogens. In the present study, quantitative real-time PCR indicated a larger number of MHC gene copies in the endangered population of Blakiston's fish owl (*Bubo blakistoni*) than in five other owl species, and massively parallel pyrosequencing detected more MHC class IIβ per individual alleles in *B. blakistoni* than in the other species. A chromosomal fluorescence in situ hybridization (FISH) analysis showed that the MHC class I and class IIβ loci are closely linked on a single pair of microchromosomes, indicating that the MHC genes were tandemly duplicated in a limited chromosomal region. Because *B. blakistoni* has twice as many MHC genes as its sister species, the tawny fish owl (*Bubo flavipes*), the duplication of MHC genes occurred after these species diverged by speciation. A Bayesian molecular phylogenetic analysis showed that the *DAB1* and *DAB2* lineages of MHC class IIβ alleles from various strigid species each formed a separate clade, indicating that the two allelic lineages preceded the radiation of Strigidae and evolved as paralogs. By contrast, the ABS sequences did not form distinct clades between *DAB1* and *DAB2* alleles but were intermixed, presumably due to gene conversion. Despite the low diversity of alleles per locus, *B. blakistoni* had many lineages of MHC class IIβ alleles. Gene duplication increases variation in the MHC genes in this species, and could have facilitated adaptation in small populations.

Key words: gene duplication, fluorescence in situ hybridization, MHC class I, MHC class Iiβ, quantitative real-time PCR

INTRODUCTION

The major histocompatibility complex (MHC) is a polymorphic genomic region that contains many genes and plays a role in the adaptive immune system of jawed vertebrates. In humans, the MHC is large and has a complex genomic structure, consisting of over 200 genes that include class I, class II, and class III gene families (MHC sequencing consortium, 1999). The MHC class I (MHCI) genes encode cell-surface proteins that present antigen peptides from pathogens in the cytoplasm or nucleus. MHC class II (MHCII) molecules, which consist of α and β chains, are expressed on subsets of cells such as B cells and macrophages to present antigen peptides from pathogens in intracellular vesicles and extracellular spaces.

Among birds, MHC organization is best characterized in the chicken (*Gallus gallus*). The chicken MHC has a highly streamlined organization that contains only 19 genes, including two class I and two class IIβ genes (Kaufman et al., 1999), all located on a single microchromosome. By contrast, while the Japanese quail (*Coturix japonica*) MHC is similar in overall organization to that in *G. gallus*, it is more diverse, having seven class I and 10 class IIβ genes (Shiina et al., 2004). More complex MHC organizations, with highly duplicated genes, have been found in several songbirds, which are the most diverse group of birds and are included in a clade of landbirds, along with a variety of other groups such as parrots, falcons, raptors, woodpeckers, and owls. In the great reed warbler (*Acrocephalus arundinaceus*), Westerdahl et al. (2000) found seven different sequences for exon 3 of MHCI, and seven divergent sequences for the antigen-binding site (ABS) of MHCIIβ per individual, and suggested that the class I and class IIβ restriction fragments were linked. The zebra finch (*Taeniopygia guttata*) has highly duplicated MHCI and MHCIIβ genes that are dis-

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persed among four chromosomes (Balakrishnan et al., 2010). This indicates that gene duplications and chromosome rearrangements have caused the evolution of complex MHC organization in birds.

Blakiston's fish owl (*Bubo blakistoni*) is an endangered fish-eating owl endemic to northeastern Asia. The population of *B. blakistoni* on Hokkaido Island, Japan, decreased in the 20th century, resulting in low genetic diversity at present (Omote et al., 2015). Recently, Kohyama et al. (2015) reported up to 16 different MHCIIβ alleles per individual in *B. blakistoni* based on the next-generation sequencing approach. This indicates that the *B. blakistoni* genome contains at least eight copies of MHCIIβ genes, the highest number of gene copies reported in a non-passerine bird. We speculate that the large number of MHCIIβ genes in *B. blakistoni* arose through recent gene duplication events, but the sequencing-based approach is insufficient to reveal accurately the number of gene copies and the gene organization at the chromosomal level.

The aims of the present study were fourfold: (1) estimate the copy numbers of MHCI and MHCIIβ genes in *B. blakistoni*, (2) map the locations of the loci on chromosomes, (3) reconstruct the phylogenetic relationships among alleles, and (4) discuss evolutionary patterns and variation in the MHC genes in *B. blakistoni* and closely related species.

MATERIALS AND METHODS

Sampling, and RNA and DNA extraction

Samples for RNA extraction were obtained from two individuals each of *B. blakistoni* and the Eurasian eagle owl (*Bubo bubo*), and one individual of the Ural owl (*Strix uralensis*). Blood samples were collected with the anticoagulant heparin and precipitated at 4°C within 12 hours after sampling. Leukocytes were isolated from the fresh blood and cultured at 39°C for 72 hours under 5% CO_2 in RPMI1640 medium supplemented with 20% fetal bovine serum (FBS), 90 μg/ml of phytohemagglutinin, 3 μg/ml of concanavalin A, and 10 μg/ml of lipopolysaccharide as mitogens. RNA extractions were performed with the RNeasy Plus Mini Kit (Qiagen). Extracted RNA samples were stored at –80°C until use.

Samples for genomic DNA extraction were obtained from 10 individuals of *B. blakistoni*; two individuals each of *B. bubo*, the great horned owl (*Bubo virginianus*), and the snowy owl (*Bubo scandiacus*); and one individual each of the tawny fish owl (*Bubo flavipes*) and *S. uralensis*. DNA extractions were performed with the DNeasy Blood & Tissue Kit (Qiagen).

Sequencing of MHC class I and II genes

To determine long sequences from RNA transcripts from MHCI and MHCII genes, we synthesized complimentary DNA (cDNA) libraries from total RNA from *B. blakistoni*, *B. bubo*, and *S. uralensis* by reverse transcription with the 3'-Full RACE Core Set (TaKaRa). Target genes were amplified by PCR using unique forward primers and a common reverse primer (the 3′-site adaptor primer; 3′-Full RACE Core Set), and PrimeSTAR GXL DNA Polymerase (TaKaRa). The forward primer MHCI-ex2F (5′-CGC TAC AAC CAG ASC RRS G-3′) (Alcaide et al., 2008) was used to amplify MHCI alleles, and the forward primers AvesEx1-F1 (5′-ACT GGT GGC ACT GGT GGY GC-3′) and AvesEx1-F2 (5′-GCA CTG GTG GYG CTG GGA GC-3′) (Burri et al., 2014) were used for semi-nested PCR to amplify MHCIIβ alleles. PCR products were subjected to agarose-gel electrophoresis and visualized by ethidium bromide staining under ultraviolet illumination, and candidate bands were excised and purified with the QIAEX II Gel Extraction Kit (Qiagen). Purified PCR products were cloned into the plasmid vector pBluescript II SK (+), and then transformed into *Escherichia coli* strain DH5α. Plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen) and used as templates for Sanger sequencing. Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3730 DNA automated sequencer (Thermo Fisher Scientific) using M13 forward and reverse primers. Nucleotide sequences were aligned by using MEGA 5.0 software (Tamura et al., 2011).

We utilized sequence data from Kohyama et al. (2015), who obtained partial sequences (203 bp) of MHCIIβ exon 2 from 174 *B. blakistoni* individuals sampled on Hokkaido Island, by massively parallel pyrosequencing. In addition, sequences were obtained from one individual of *B. flavipes*, and two individuals each of *B. bubo*, *B. virginianus*, and *B. scandiacus*, by the same method as in Kohyama et al. (2015). Briefly, pyrosequencing was performed on PCR products obtained from genomic DNA by using the primer set BublIIb2F (5′-GAG TGT CAG YAC CTY RAY RG-3′) and BublIIb2R (5′-CTT TCY TCT SCS TGA YGW AGG-3′). The amplicon library was commercially sequenced on a 1/4 Titanium Pico-Titer Plate with the GS FLX Titanium Sequencing Kit XLR70 (Roche) at Hokkaido System Science Co. (Sapporo, Japan).

We reconstructed phylogenetic relationships among MHCIIβ alleles by Bayesian inference using BEAST 1.6.2 software (Drummond et al., 2006; Drummond and Rambaut, 2007). Phylogenetic trees of MHCIIβ alleles were reconstructed separately for exon 2 and exon 3 sequences. In owl species, MHCIIβ exon 3 alleles show two distinct lineages, *DAB1* and *DAB2* (Burri et al., 2014). The cloned sequences, whose allelic lineages were defined, were used for phylogenetic analysis, which included MHCIIβ sequences from 14 other owl species obtained from GenBank (Figs. 1A and 1B).

Quantifying copy numbers of MHC genes

To estimate the copy numbers of MHCI and MHCIIβ genes, quantitative real-time PCR (qPCR) was used on genomic DNA, with a double-strand DNA-binding dye as the reporter. Primer sets were designed for MHCI exon 2 (MHCI-Ex2-F3, 5′-CGC AAA TCA CCA AGA GGA AG-3′; MHCI-Ex2-R1, 5′-GCC CGT AGC TCA CGT ATT TC-3′) and MHCIIβ exon 3 (MHCII-Ex3-F5, 5′-CGA GGT GAA GTG GTT CCA GA-3′; MHCIIEx3-R1, 5′-TGG TTT CCA GCA TCA CCA G-3′), based on sequences conserved in *B. blakistoni*, *B. bubo*, and *S. uralensis*. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a single-copy reference-gene control for the qPCR, based on copy number validation in avian species (Criscuolo et al., 2009). The primer set GAPDH-F2 (5′-CCA TCA CAG CCA CAC AGA AG-3′) and GAPDH-R2 (5′-TTA GCA GCC CCA GTA GAT GC-3′) was designed for GAPDH exon 3, based on sequences from GenBank: *Strix aluco* (KF201575), *Taeniopygia guttata* (NM_001198610), *Falco cherrug* (XM_014287574), *Nipponia nippon* (XM_009461700), and *Gallus gallus* (V00407). The three genes were analyzed by qPCR for *B. blakistoni*, *B. flavipes*, *B. bubo*, *B. virginianus*, *B. scandiacus*, and *S. uralensis*; qPCR reactions were performed with the KAPA SYBER Fast qPCR Kit (Kapa Biosystems). Reactions were performed forth times with primer sets MHCI, MHCIIβ, and GAPDH and 10 μl of KAPA SYBER FAST qPCR Master Mix. Real-time amplifications were performed with the 7300 Real-Time PCR System (Applied Biosystems); cycling conditions were 95˚C for 10 min; 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec; and a following dissociation stage. To generate a reference curve for estimating the amplification efficiency, qPCR was run on serial dilutions (4.8 ng, 2.4 ng, 1.2 ng, 0.6 ng, 0.3 ng, and 0.15 ng of template DNA per reaction).

The number of MHC genes was estimated by comparing the threshold cycle value (Ct) with that of the internal single-gene control (GAPDH) measured from the same DNA sample, using the following formula: gene number = $2^{-\Delta Ct}$ where $\Delta Ct = Ct^{\text{target gene}} - Ct^{\text{control}}$.

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Mapping of MHC loci on chromosomes

To determine the locations of *B. blakistoni* and *B. bubo* MHC genes on chromosomes, fluorescence in situ hybridization (FISH) was performed as described by Matsuda and Chapman (1995). Cloned plasmids containing MHCI (1035–1036 bp) or MHCIIβ (990 bp) sequences were used as probes. In addition, human 18 S and 28 S rRNA gene sequences, pHr21Ab (5.8 kbp) and pHr14E3 (7.3 kbp), respectively, provided by the Japanese Cancer Research Resource Bank, Japan, were used as probes, as these rRNA genes are closely linked with one another and with MHC genes on the same microchromosome in some bird species, such as chicken (Delany et al., 2009) and turkey (Chaves et al., 2007). In a preliminary study, we confirmed that the human 18 S and 28 S rDNA probes could detect the microchromosomes on which the nucleolus organizer region is located in *B. blakistoni*. Each probe was labeled with Cy3-dUTP by using the Nick Translation Kit (Roche Applied Science) and ethanol-precipitated along with salmon sperm DNA and *E. coli* tRNA.

Single-color FISH mapping was performed for three types of probes on the same chromosome samples by triplicate hybridization and washing in the order MHCIIβ, MHCI, and 18 S/28 S. Chromosome samples were prepared on slides from cultured fibroblasts of *B. blakistoni* and *B. bubo*. After FISH, the slides were washed with 3:1 methanol/acetic acid, rinsed in distilled water, and air-dried. The chromosomal locations of the genes were examined on the same metaphase spreads for each probe by using a Nikon Eclipse E800 fluorescence microscope.

RESULTS

Sequence variation and phylogeny of the MHC alleles

One MHCI allele was identified from more than 10 clones in both *B. blakistoni* and *B. bubo* partial sequences (1008 bp). Some sequence variants were also detected in each species, but we could not rule out PCR and/or sequencing errors as the cause, as each variant was identified from only a single clone. More than 10 MHCIIβ alleles were found in multiple clones of both *B. blakistoni* and *B. bubo* (792–1003 bp), which included part of exon 1 and complete exons 2–6. Two distinct allelic lineages for MHCIIβ, *DAB1*, and *DAB2*, based on exon 3 sequences (Burri et al., 2014), were detected among the sequences obtained by cloning. The newly identified allele sequences were deposited in the DDBJ (DNA Data Bank of Japan) database under accession numbers LC169500–LC169503.

In the Bayesian tree of MHCIIβ alleles based on exon 3 sequences (215 bp) (Fig. 1A), *DAB1* and *DAB2* alleles from strigid species each form a distinct clade, whereas those from the barn owl (*Tyto alba*; Tytonidae) group together in yet another clade. By contrast, in the Bayesian tree based on MHCIIβ exon 2 sequences (268 bp) (Fig. 1B), in the strigid clade *DAB1* alleles form an unresolved polytomy with *DAB2* alleles, i.e., the distinct allelic lineages seen in Fig. 1A are completely intermixed. Some clades consist of alleles from a single strigid species, but most include alleles from two or more strigid species.

Copy numbers of the MHC genes

The Ct values (Ct^{MHCI}, $Ct^{MHCII\beta}$, and $Ct^{control}$) obtained by qPCR varied only slightly among replicates from the same samples, and so mean Ct values were used to estimate the copy numbers of the genes. The qPCR results indicate there are two to four MHCI genes: $2^{-\Delta Ct} = 3.9$ in *B. blakistoni*, 2.0 in *B. flavipes*, 2.6 in *B. bubo*, 3.7 in *B. virginianus*, 3.7 in *B. scandiacus*, and 2.1 in *S. uralensis* (Fig. 2). The number of MHCIIβ genes was much more variable among these species: $2^{-\Delta Ct} = 11.9$ in *B. blakistoni*, 6.0 in *B. flavipes*, 4.1 in *B. bubo*, 5.9 in *B. virginianus*, 3.6 in *B. scandiacus*, and 4.1 in *S. uralensis* (Fig. 2). *Bubo blakistoni* had the largest number of genes for both MHCI and MHCIIβ, and in each case twice

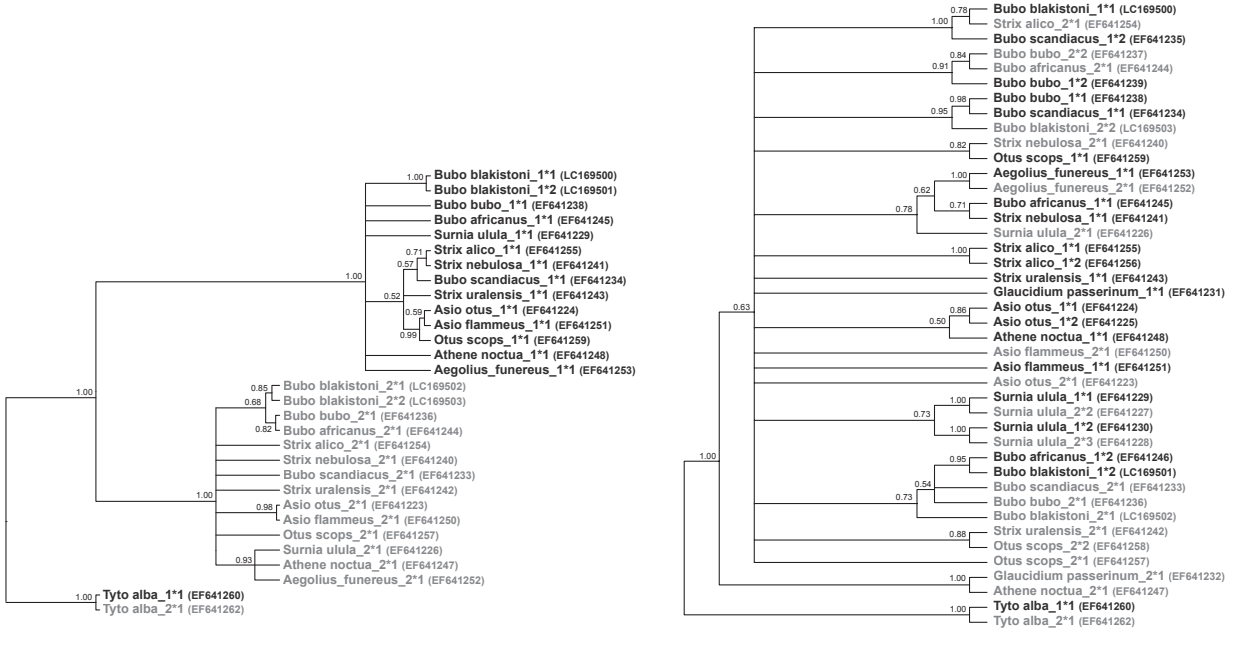

Fig. 1. Phylogenetic relationships among MHC class IIβ exon 3 **(A)** and MHC class IIβ exon 2 **(B)** alleles from owl species, reconstructed by Bayesian analysis. Black font, *DAB1* alleles; gray font, *DAB2* alleles. Numbers near nodes are posterior probability values. Accession numbers for allele sequences are in parentheses.

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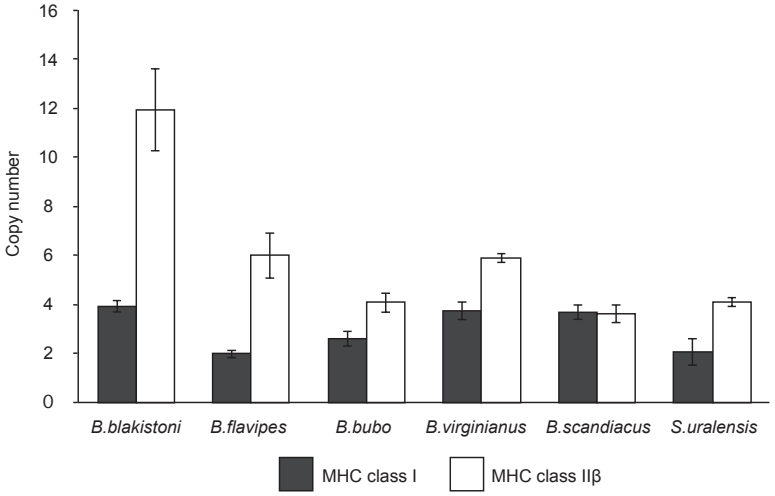

Fig. 2. Number of gene copies in six owl species, estimated by quantitative real-time PCR. Black and white bars refer to MHC class I and class IIβ genes, respectively. The error bars show standard deviations.

as many as in *B. flavipes*.

Table 1 shows the number of alleles identified from each individual by massively parallel pyrosequencing of MHCIIβ exon 2. The maximum number of alleles per individual was larger in *B. blakistoni* (16) than in the other species: 12 *B. flavipes*, seven in *B. bubo*, four in *B. virginianus*, and seven in *B. scandiacus* (Table 1). Two sequences in MHCIIβ exon 2 were shared by *Bubo flavipes* and *B. blakistoni*.

Location of the MHC genes on chromosomes

MHCI, MHCIIβ, and 18 S/28 S were mapped onto chromosomes for *B. blakistoni* and *B. bubo*. The FISH mapping (Fig. 3) shows that the MHCI and MHCIIβ loci are closely linked on a single pair of microchromosomes in both species, whereas the 18 S/28 S loci are on a different pair of microchromosomes from the MHC loci in both species (Fig. 3).

Table 1. Sample sizes, chromosome number (Rebholz et al., 1993) and numbers of MHC class IIβ alleles per individual detected by massively parallel pyrosequencing. The pyrosequencing data for *B. blakistoni* are from Kohyama et al. (2015).

Species	Chromosome number (2 n)	No. of samples for qPCR	No. of samples for pyrosequencing	No. of MHC class IIβ allele per individual			Tissue	Source
				Minimum	Mean	Maximum		
B. blakistoni	82	10	174	8	11.7	16	Blood, fibroblasts	Wildlife Center, wild-caught on Hokkaido Island
B. flavipes	82	1	1	–	12	–	Fibroblasts	Ueno Zoological Gardens
B. bubo	80	2	2	5	6	7	Blood, fibroblasts	Kushiro City Zoo, Tama Zoological Park
B. virginianus	82	2	2	3	3.5	4	Fibroblasts	Sapporo Maruyama Zoo
B. scandiacus	82	2	2	5	6	7	Fibroblasts	Kushiro City Zoo
S. uralensis	82	1	–	–	–	–	Blood	Wildlife Center

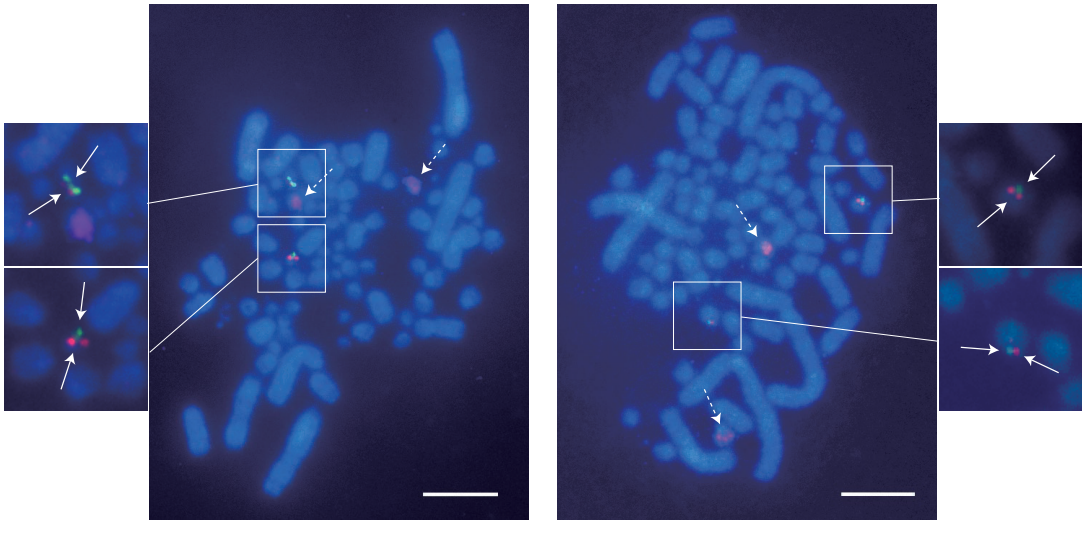

Fig. 3. FISH mapping of MHC class I (red), MHC class II (green) and the 18 S/28 S locus (purple) on chromosomes of *B. blakistoni* **(A)** and *B. bubo* **(B)**. Solid arrows indicate the MHC loci; dashed arrows indicate the 18 S/28 S locus. Scale bars, 10 μm.

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DISCUSSION

Duplication patterns of MHC genes in *Bubo* species

The MHC region contains the most diverse set of coding genes in vertebrates because variation in the MHC genes is necessary to recognize many types of antigens in immune responses. The chicken (*Gallus gallus*) has the minimum essential MHC complement (Kaufman et al., 1999), whereas the MHC of the quail (*Coturnix japonica*) and some passerine birds contains many more genes (Shiina et al., 2004; Westerdahl et al., 2000; Balakrishnan et al., 2010). Most genes in the MHC family are assumed to have evolved by gene duplication, which is mediated mainly by recombination, replication slippage, retrotransposition, or chromosome duplication. The number of retroelements in *C. japonica* and some passerine birds is high for birds, but lower than in humans, whereas no retroelements are present in *G. gallus* (Hess et al., 2002). Balakrishnan et al. (2010) reported highly duplicated MHC genes and interspersed among many chromosomes in the zebra finch (*Taeniopygia guttata*).

In our study, qPCR-based copy number validation revealed high variation in the numbers of MHCI and MHCIIβ genes among *Bubo* species. The number of MHCIIβ genes was more variable, and that of *B.* blakistoni (12 ± 2) was markedly larger than in the other *Bubo* species (fewer than six) (Fig. 2). Massively parallel pyrosequencing detected the largest number of MHCIIβ alleles in *B. blakistoni*, up to 16 per individual. This result indicates that *B. blakistoni* has more than eight copies of the MHCIIβ gene. The pyrosequencing results for the other *Bubo* species are congruent with the number of MHCIIβ genes estimated by qPCR (Fig. 2). These results indicate frequent duplications of MHCIIβ genes have occurred in *Bubo* species.

Bubo blakistoni had twice as many MHCI and MHCIIβ genes as *B. flavipes*. According to a phylogenetic study based on mitochondrial DNA (Omote et al., 2013), *B. flavipes* is closely related to *B. blakistoni*. The two sequences shared by the two species for the ABS in MHCIIβ exon 2 also support a close relationship between *B. blakistoni* and *B. flavipes*. This finding suggests that duplications of the MHC genes occurred in *B. blakistoni* after speciation; probably the entire complement MHC gene underwent duplication simultaneously.

To reveal the pattern of gene duplication, we investigated the location of the MHC gene loci on chromosomes of *B. blakistoni* and *B. bubo*, whose MHCI and MHCIIβ genes were less than half as numerous of those of *B. blakistoni*. *Bubo blakistoni* and *B. bubo* have 82 and 80 chromosomes, respectively, including microchromosomes (Rebholz et al., 1993) (Table 1). Although the MHC gene family is linked with 18 S/28 S on a microchromosome in chicken and turkey (Delany et al., 2009; Chaves et al., 2007), our FISH results showed that the MHCI and MHC IIβ genes were on another microchromosome than 18 S/28 S in *B. blakistoni* and *B. bubo*. The FISH results also showed that the MHCI and MHCIIβ loci are located in the same sites on a pair of chromosome, indicating that duplication of the MHC genes was not at the chromosomal level, but was the result of tandem duplications within the gene family region. To clarify the detailed duplication history of MHC genes in *B. blakistoni*, it will be necessary to survey the whole-genomic structure in the MHC region, using such methods as BAC clone sequencing.

Variation in MHCIIβ alleles in *Bubo blakistoni*

Among strigid species, the *DAB1* and *DAB2* alleles of MHCIIβ exon 3 formed completely separate clades (Fig. 1A), indicating that the two allele lineages diverged before radiation of the Strigidae and evolved as paralogs. In contrast, the *DAB1* and *DAB2* alleles with respect to MHCIIβ exon 2, including the ABS, did not form distinct clades (Fig. 1B). This might be due to gene conversion among *DAB1* and *DAB2* alleles, resulting in rearrangements of sequences and an increase in allelic variation. The ABS sequences of *DAB1* and *DAB2* alleles of strigid species formed multi-genus clades (Fig. 1B). The similarities in ABS sequences among species could have been caused by convergent or parallel evolution. Because MHC variation affects the adaptation of populations through having functional importance such as parasite resistance (Sommer, 2005), the trans-species polymorphism may be maintained by heterozygote advantage or frequency-dependent selection.

In general, the high level of variation in MHC genes often observed in wild animals is related to resistance to diseases, but it is difficult to maintain this variation in small populations because of the strong effect of genetic drift and inbreeding. *Bubo blakistoni* preys on freshwater fishes in the subarctic region and needs large ranges along rivers. Other fish owl species, including *B. flavipes*, inhabit tropical or subtropical regions in Southeast Asia. The *B. blakistoni* populations could have experienced severe bottlenecks during glacial periods, which could have reduced genetic variation. In addition, recent population fragmentation and decline owing to human activities has resulted in decreased genetic variation (Omote et al., 2015), and the level of MHCIIβ variation has decreased in the *B. blakistoni* population on Hokkaido Island during the past decades (Kohyama et al., 2015). However, the number of MHCIIβ alleles per individual remains large among owl species (Table 1), probably because of multiple loci. When loci are duplicated, the number of alleles per individual can markedly increase. Duplications of MHC genes could have prevented a decline in immunity in the small *B. blakistoni* population and contributed to its successful range expansion into sever subarctic habitats.

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COMPETING INTERESTS

The authors have no competing interests to disclose.

AUTHOR CONTRIBUTIONS

KS and SF collected samples. CN cultured leukocytes, prepared chromosome samples, and mapped loci on chromosomes. KO conducted PCR, qPCR, cloning, sequencing, and phylogenetic

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analyses. TIK conducted massively parallel pyrosequencing. RM and KO wrote the paper, and all authors read and approved the final manuscript.

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