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Characterization of Major Histocompatibility Complex Genes in Jungle Fowls, Genus *Gallus*.

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Chicken major histocompatibility complex (*Mhc*) is smaller in size and contains fewer genes than does mammal *Mhc*. To clarify the characters of *Mhc* genes in junglefowls as the ancestral species of domestic chickens, we analyzed their *Mhc* class I *B–F* and *Mhc* class IV *B–G* genes using the restriction fragment length polymorphisms (RFLPs) method in addition to comparing the B blood types of Red junglefowls and Green junglefowls. The B blood types were quite different between the two species of junglefowls.Green junglefowl had small variation of *B* blood types, almost Green junglefowls reacted with all of the *B* antisera, $B^{ABCDEGIKLMT}$.

Using the *B–G* gene probe *gene* 8.5 in RFLPs, many bands were observed in the junglefowls. The RFLPs patterns based on *B–G* genes as the probes of Red junglefowls and Green junglefowls were quite different patterns within the same *B* blood type as determined by hemagglutination. On the contrary, the RFLPs of *B–F* regions of Green junglefowls showed the same patterns regardless of chicken *B* blood types. We suggest that the *Mhc* class I *B–F* and *Mhc* class IV *B–G* genes might not be affected by their history of domestication from junglefowls, and artificial selection only reduced the number of *B–G* genes.

Key words : *B* blood type, domestication, junglefowl, major histocompatibility complex gene, restriction fragment length polymorphisms

Introduction

The major histocompatibility complex (*Mhc*) is encoded by a number of multigene families. Chicken *Mhc* genes are arranged into two genetically independent clusters, the *B* system and *Rfp-Y*. One of the clusters, the *B* system, was defined initially as a blood group system (Briles *et al.,* 1950 ; Okada, 1992). It is a compact chromosomal segment on the long arm of microchromosome 16 and is a 92 kilo base pairs (kbp) region of the *B* locus containing only 19 genes, classical *Mhc* class I, class IIβ, antigen processing gene (*TAP*), natural killer receptor gene (*NKr*), and several other genes (Kaufman *et al.,* 1999a). The cluster is roughly 20-fold smaller than the human *Mhc*, HLA (MHC Sequencing Consortium, 1999). These 19 genes have smaller introns than their mammalian counterparts, and most of the genes are homologues to the mammalian

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Mhc, suggesting a "minimal *Mhc*" (Kaufman *et al.,* 1999b). The other cluster, *Rfp-Y,* was found to reside on the short arm of microchromosome 16 in the same manner as the *B* system by a two color fluorescent *in situ* hybridization (Fillon *et al.,* 1996 ; Miller *et al.,* 1996). It contained two class I and three class II genes which had lower expression and seemed to be less polymorphic than the genes in the *Mhc* B system cluster (Miller *et al.,* 1996 ; Juul-Madsen *et al.,* 1997 ; Afanassieff *et al.,* 2000 ; Afanassieff *et al.,* 2001).

The *Mhc* region of the Japanese quail (*Coturnix japonica*) named as *'Coja',* corresponding to the chicken *B* complex was recently characterized and shown to be larger (156kbp) and less streamlined than that of the chicken (Shiina *et al.,* 1999). Thirty-five genes containing several duplications of four class I, seven class IIβ, six lectin-like, and four *NKr* genes were found in the *Coja* region. It was suggested that the *Coja* is constructed with more complicated duplication than is chicken *Mhc* region. On the other hand, it was reported that chicken *Mhc* has been conserved by defining a minimal essential set of *Mhc* genes by artificial selection and breeding over a period of 10,000 years (Shiina and Inoko, 2001). Nishibori *et al.* (2000) showed that the number of *Mhc* class IV *B–G* genes of the inbred and selected chickens based on several immunological characters were reduced from those of random mating chickens affected by inbreeding and selections.

Chickens were domesticated from junglefowls approximately 5,000～7,000 years ago. Chicken and quail would have diverged 36 million years ago (Tuinen and Hedge, 2001) and their genetical distance was greater than that between chicken and junglefowls (Nishibori *et al.,* 2001a ; Nishibori *et al.,* 2001b). Therefore, it would be difficult to evaluate the effect of artificial selection and breeding by comparing chicken and Japanese quail.

In this study, in order to clarify the characters of *Mhc* genes in junglefowls as the ancestors of domestic chickens, we analyzed *Mhc* class I *B–F* and *Mhc* class IV *B–G* genes of Red junglefowl and Green junglefowl using the methods of restriction fragment length polymorphisms (RFLPs).

Materials and Methods

Birds and blood collection

The two species of junglefowls, Red junglefowl (*Gallus gallus,* RJF) and Green junglefowl (*Gallus varius,* GJF) and inbred and selected chickens surveyed in this study are listed in Table 1. Blood samples from RJF and GJF were collected at Jakarta and Bali, and at Bali and Lombok in Indonesia in 1990, respectively (Yamamoto *et al.,* 1996). The two lines of White Leghorn, GVHR–HG (HG), and -LG (LG), were established by selection for high and low competencies of splenomegaly in graft-versus-host reaction (GVHR) (Okada and Mikami, 1974). The IgG-H (GH) and IgG-L (GL) lines of White Plymouth Rock were developed by selection for high and low levels, respectively, of immunoglobulin G (IgG) at 10 weeks of age (Tamaki, 1980). The *B* blood types of HG, LG, GH, and GL were B^9, B^9, B^{31}, and B^{32} homozygous, respectively (Nishibori *et al.,* 2000).

Table 1. List of species of jumglefowls and lines of chickens surveyed

Species/lines	Abbreviations of species/lines	No. of birds	Place and year of sampling
Junglefowls ;			
Red junglefowl (*Gallus gallus*)	RJF	5	Jakarat and Bali, Indonesia (1990)
Green junglefowl (*Gallus varius*)	GJF	5	Bali and Lombok, Indonesia (1990)
Chickens (*Gallus gallus* var. *domesticus*) ;			
White Leghorn CB line	CB	1	Hiroshima University (1999)
White Leghorn HG line	HG	1	Hiroshima University (1999)
White Leghorn LG line	LG	1	Hiroshima University (1999)
White Plymouth Rock GH line	GH	1	Hiroshima University (1999)
White Plymouth Rock GL line	GL	1	Hiroshima University (1999)

All blood samples were heparinized and separated into plasma and erythrocytes by centrifugation (450×g for 5 min). Erythrocytes were washed three times with physiological saline by centrifusion. A portion of the erythrocytes of junglefowls were used for blood typing, while remaining erythrocytes were stored at -20℃ until DNA analysis.

Blood typing

Serological *B* blood types were determined for junglefowls using the hemagglutination test employing eleven antisera for *B* systems (B^A, B^B, B^C, B^D, B^E, B^G, B^I, B^K, B^L, B^M, and B^T) *prepared from chickens at the laboratory of Animal Breeding and Genetics, Hiroshima University.*

Genomic DNA extraction

Genomic DNAs from junglefowls and chickens were prepared from peripheral red blood cells according to the methods applied by Nishibori *et al.* (1997). Concentration and purity of DNAs were measured by a spectrophotometer (GeneQuant, Amersham Biosciences).

B–G and B–F cDNA probes

The cDNA clones of *gene 8.5* (600 bp, Kaufman *et al.,* 1989 ; Nakaki *et al.,* 1997) and *B–F*10, (1,286 bp, Guillemot *et al.,* 1988) were used as a probe for the region of chicken *Mhc* class IV *B–G* and *Mhc* class I *B–F*, respectively. These two cDNA clones were kindly provided by Dr. C. Auffray, CNRS, France. The probes were labeled with [α-^{32}P] dCTP using Multiprime DNA labeling system (Amersham Bioscience).

Southern blot analysis

Genomic DNAs (20 ng) of each sample were digested with the restriction endonucleases of *Bgl*II, *Hin*dIII and *Pvu*II, respectively. Southern blotting and hybridization were performed according to the method described previously (Nishibori *et al.,* 2000), except that the final washing for *B–F* cDNA probe was carried out in 0.1×SSC buffer (3 M sodium chloride, 0.3 M sodium citrate) with 0.1% SDS for 15 min at 65 ℃ twice.

Results

The *B* blood types of RJFs and GJFs are shown in Table 2. Three of the RJFs

(RJF #2, #3 and #5) collected at Bali in Indonesia were the same type as B^{GKM}, and other two junglefowls (RJF #1 and #4) were shown different types each other. Four out of five GJFs reacted with all of the allo-antisera prepared from domesticated chicken for the *B* locus used in this study, while the rest (GJF #1) did not react with B^I *and* B^L.

Typical RFLP patterns were digested with *Bgl*II and hybridized with *B-G* cDNA probe, *gene8.5* are shown in Fig. 1. In junglefowls shown in Fig. 1A, although the *B* blood types were same within RJF #2 and #3, or GJF #2, #3 and #4, their observed RFLP patterns did not correspond to their *B* blood types (lines 2 and 3, and lines 6, 7 and 8, respectively). Whereas, in the inbred and selected chickens in Fig. 1B, the RFLPs of LG (line 4) were same as those of HG (line 5) because both of them were derived from the same population by two-way selection and had the same *B* blood type, and B^9B^9 homozygotes. The RFLP patterns of GH and GL lines (lanes 2 and 3) were distinctly different, despite that the lines were derived from the same population by two-way selection. The number of RFLP bands in junglefowls (4–7 bands) was more than that in chicken (2–5 bands). Correspondingly, a similar correlation between the B blood type and RFLP patterns digested with *Hind*III and hybridized with *gene8.5,* as shown in Fig. 2A. However, in the inbred and selected chickens, the RFLP patterns corresponded with the *B* genotypes like as HG (lane 4) and LG (lane 5). The HG and LG were homozygous for B^9. In the case of RFLP analysis digested with *Hin*dIII and hybridized with *gene 8.5* was almost the same between junglefowls (6–10 bands) and inbred and selected chickens (4–9 bands).

RFLP analysis using a chicken class I *B-F* cDNA probe *B-F*10 was performed

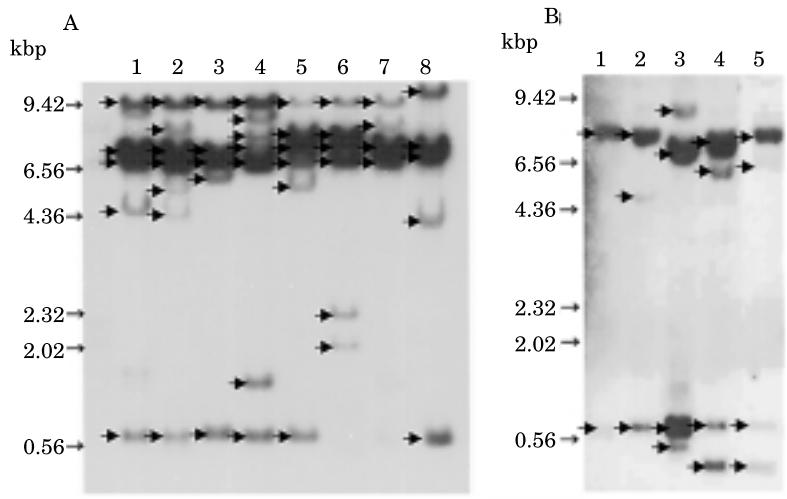

Fig. 1. RFLPs patterns of *B-G* genes in junglefowls, and inbred and selected chickens. Genomic DNAs were digested with *Bgl*II and hybridize with *B-G* cDNA probe, *gene8.5,* of junglefowls (A) and inbred and selected chickens (B). (A) Lanes 1–4, RJF #1–℃4 ; lanes 5–8, GJF #1–#4 ; (B) Lane 1, CB ; 2, GH ; 3, GL ; 4, LG ; 5, HG. Molecular size markers are based on λDNA digested with *Hin*dIII. The arrow indicates a RFLP band.

with *Pvu*II digested genomic DNAs of RJFs and GJFs, and those of inbred and selected chickens (Fig. 3). The same patterns of RFLPs were observed between two B blood types, $B^{ABCDEGKMT}$ and $B^{ABCDEGIKLMT}$ in GJFs. The RFLP patterns were different, however, between RJF #2 and #3 in that were observed the same *B* blood types (B^{GKM}). On the other hand, RFLP patterns of inbred and selected chickens were dependent on the *B* blood types observed. The 0.6 kbp and 0.5 kbp bands were conserved with weak

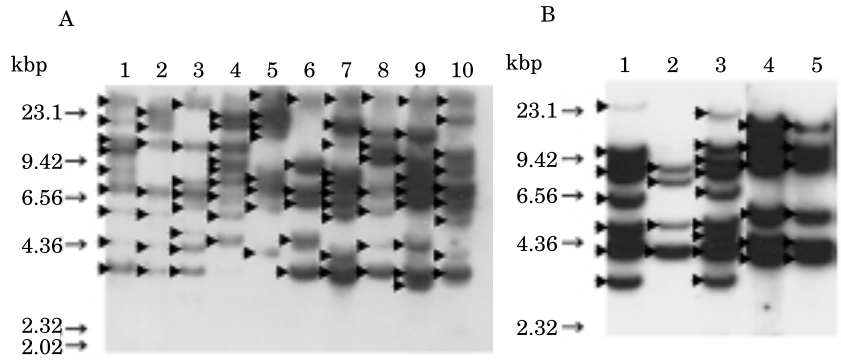

Fig. 2. RFLPs patterns of *B-G* genes in junglefowls, and inbred and selected chickens. Genomic DNAs were digested with *Hin*dIII and hybridize with B-G cDNA probe, *gene8.5,* of junglefowls (A) and inbred and selected chickens (B). (A) Lanes 1–5, RJF #1–#5 ; lanes 6–10, GJF #1–#5 ; (B) Lane 1, CB ; 2, GH ; 3, GL ; 4, HG ; 5, LG. Molecular size markers are based on λDNA digested with *Hin*dIII. The arrow indicates a RFLP band.

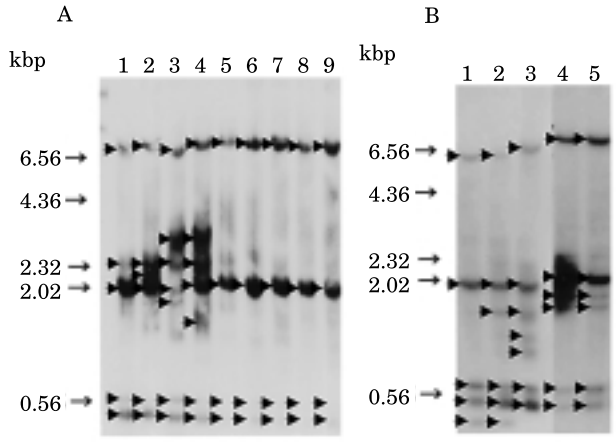

Fig. 3. RFLPs patterns of *B-F* genes in junglefowls, and inbred and selected chickens. Genomic DNAs were digested with *Pvu*II and hybridize with *B-F* cDNA probe, *B-F10,* of junglefowls (A) and inbred and selected chickens (B). (A) Lanes 1–4, RJF #–#4 ; lanes 5–9, GJF #1–#5 ; (B) Lane 1, CB ; 2, GH ; 3, GL ; 4, HG ; 5, LG. Molecular size markers are based on λDNA digested with *Hin*dIII. The arrow indicates a RFLP band.

intensity in all junglefowls and chickens. The number of *B–F* RFLP bands hybridized with the *B–F*10 was smaller as compared to that of the *B–G* RFLP bands hybridized with the *gene 8.5*.

Discussion

In this study, we compared RFLP patterns using probes of *Mhc* genes in junglefowls and inbred and selected chickens in order to reveal the effects of domestication and artificial selection on the *B* locus in the *Mhc* region. The present study indicates that the number of *Mhc* class IV *B–G* genes of junglefowls is greater than that of the inbred and selected chickens, but that the number of *Mhc* class I *B–F* genes of junglefowls is similar to inbred and selected chickens (Figs. 1, 2 and 3). Shiina *et al.* (1999) reported that the *Coja* occupied larger regions (158 kbp) and was composed of a larger number of genes than those of the domesticated chicken. They suggested that chicken *Mhc* genes would be smaller and simpler due to domestication and artificial selection as compared with Japanese quail. Unexpectedly, however, the *Mhc* class I *B–F* genes of chickens were almost same as RJFs and GJFs. Nevertheless, the GJF was the most distant from domesticated chicken in the genetical relationship among four species of junglefowls (Nishibori *et al.,* 2001b). To clarify the structure of the *Mhc* class I *B–F* region in junglefowls, we must determine the full length of the sequence of this region. The present work shows that the *Mhc* class I *B–F* genes should be almost the same number as in the genus *Gallus*.

In the *Mhc* class IV-coding *B–G* antigens on erythrocytes, these results (Fig. 1) suggest that the number of genes in junglefowls should be more than that of inbred and selected chickens. Furthermore, the GJFs reacted with all or almost all anti-sera (Table 2). Alloantibodies to *B–G* antigens on erythrocytes were responsible for discovery of the chicken *Mhc* class IV (Kaufman and Lamont, 1996). The GJF may be assigned to the cross-reactions by having made the antiserum for *B* locus from domesticated

Table 2. *B* blood types of junglefowls

Junglefowl	Antisera for *B* locus											*B* bllod type	Place of sampling
	A	B	C	D	E	G	I	K	L	M	T		
Red junglefowl-#1	1[a)]	0[b)]	1	0	0	1	0	1	0	1	0	B^{ACGKM}	Jakarta, Indonesia
#2	0	0	0	0	0	1	0	1	0	1	0	B^{GKM}	Bali, Indonesia
#3	0	0	0	0	0	1	0	1	0	1	0	B^{GKM}	Bali, Indonesia
#4	1	0	0	0	0	1	0	1	0	1	0	B^{AGKM}	Bali, Indonesia
#5	0	0	0	0	0	1	0	1	0	1	0	B^{GKM}	Bali, Indonesia
Green junglefowl-#1	1	1	1	1	1	1	0	1	0	1	1	$B^{ABCDEGKMT}$	Lombok, Indonesia
#2	1	1	1	1	1	1	1	1	1	1	1	$B^{ABCDEGIKLMT}$	Lombok, Indonesia
#3	1	1	1	1	1	1	1	1	1	1	1	$B^{ABCDEGIKLMT}$	Lombok, Indonesia
#4	1	1	1	1	1	1	1	1	1	1	1	$B^{ABCDEGIKLMT}$	Bali, Indonesia
#5	1	1	1	1	1	1	1	1	1	1	1	$B^{ABCDEGIKLMT}$	Bali, Indonesia

a), b) 1 and 0 indicated coagulation and non-coagulation for the antisera, respectively.

chickens because the GJF is genetically far distance from domesticated chicken among four species of junglefowls (Nishibori *et al.,* 2001b). On the other hand, Nishibori *et al.* (2000) showed that the randomly mating and crossbred population of White Leghorn and White Plymouth Rock had many RFLP bands (6–11 bands) when *gene 8.5* was used as a probe and digested with *Bgl*II have the similar results to those shown in Fig. 1. They suggested that a random mating population should have more *Mhc* class IV *B–G* genes than would an inbred line. Kaufman *et al.* (1995) stated that at least twenty *B–G* genes existed in the *B* locus of the inbred chicken, the B^{12} homozygote. *Mhc* genes were created by repeated gene duplication, and some duplicate genes were maintained in the genome for a long time, but others were deleted or became non-functional due to deleterious mutations (Nei *et al.,* 1997 ; Nei and Kumar, 2000). We consider that the number of *B–G* genes in the inbred and selected chickens may have been reduced in a short time by artificial selection for only a *B* blood type.

In conclusion, we suggest that the *Mhc* class I *B–F* and *Mhc* class IV *B–G* genes would not be affected by domestication from junglefowls, while only the numbers of *B–G* genes might have reduced in a short time due to artificial selection.

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