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BRIEF COMMUNICATION

Allelic diversity of the MHC class II *DRB* genes in brown bears (*Ursus arctos*) and a comparison of *DRB* sequences within the family Ursidae

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Abstract

The allelic diversity of the *DRB* locus in major histocompatibility complex (MHC) genes was analyzed in the brown bear (*Ursus arctos*) from the Hokkaido Island of Japan, Siberia, and Kodiak of Alaska. Nineteen alleles of the *DRB* exon 2 were identified from a total of 38 individuals of *U. arctos* and were highly polymorphic. Comparisons of non-synonymous and synonymous substitutions in the antigen-binding sites of deduced amino acid sequences indicated evidence for balancing selection on the bear *DRB* locus. The phylogenetic analysis of the *DRB* alleles among three genera (*Ursus, Tremarctos,* and *Ailuropoda*) in the family Ursidae revealed that *DRB* allelic lineages were not separated according to species. This strongly shows trans-species persistence of *DRB* alleles within the Ursidae.

The major histocompatibility complex (MHC) plays a key role in the initiation of immune response and transmits information of pathogens invading the body to T-cells (1). It is one of the most important genetic systems for infectious disease resistance in vertebrates (2–5). The high allelic diversity and high levels of sequence divergences among alleles of the MHC class I and class II genes have been reported in the populations of natural and domesticated animals: the domestic cat (6–7), red wolf (8), island fox (9), sheep (10), finless porpoise (11), and vole (12). Conversely, a loss of genetic diversity in the MHC genes, as shown in endangered species or isolated populations (13, 14), reduces the ability of species or populations to evolve to cope with new and changed diseases (15). As the genetic diversity of MHC genes is thus likely to be involved in the susceptibility to diseases, the study of the MHC diversity in populations of wildlife could provide a considerable insight into a view of their conservation genetics. The high levels of MHC polymorphisms have been mainly exhibited in the functionally important antigen-binding sites (ABS) (16–17), indicating that high genetic variations at MHC genes are maintained because of a role of pathogen-driven balancing selection concentrating on the ABS (18, 19) and that the encoded MHC proteins can bind to various foreign peptides (20). Because the balancing selection acting on MHC genes also maintains allelic lineages over long periods of time, even across speciation events, the gene trees for MHC loci show the pattern that alleles found within a species are often more closely related to alleles of other species than to other alleles within the same species (trans-species evolution) (21). Trans-species polymorphisms among genera have been reported in many species of mammals, such as primates (22), rodents (12, 23, 24), lagomorphs (25), and carnivores (8, 26). We sought to investigate molecular diversity of MHC class II *DRB* of *Ursus arctos*, and then discuss their molecular evolution, compared with other bear species in the family Ursidae.

The bear family Ursidae is one of the major families of the order Carnivora and distributed widely in Eurasia and North and South America. This family consists of three extant genera and eight species: the brown bear (*U. arctos*), polar bear (*Ursus*

maritimus), Asiatic black bear (*Ursus thibetanus*), American black bear (*Ursus americanus*), sun bear (*Ursus malayanus*), sloth bear (*Ursus ursinus*), spectacled bear (*Tremarctos ornatus*), and giant panda (*Ailuropoda melanoleuca*). The MHC variations in Ursidae species were examined in *A. melanoleuca* (14, 27, 28) and *U. arctos* (29) and reported to be relatively low in both species. In *U. arctos*, however, information on only the class II *DQA* gene is available (29), and there are no previous studies about molecular evolution of the MHC genes among the family Ursidae.

In the present study, we obtained the nucleotide sequences derived from *DRB* exon 2 alleles from seven species in the family Ursidae by cloning and sequencing of polymerase chain reaction (PCR) products (Table 1 and Figure 1). PCR

Table 1 Profiles of bear samples and numbers of *DRB* alleles

Species	Common name	Source of sample	Tissue profile	No. of individuals	No. of *DRB* alleles
Ursus arctos	Brown bear	Hokkaido: Hokkaido Institute of Environmental Science, Japan	Liver or spleen	32	11
		Siberia: Institute of Plant and Animal Ecology, RAS	Muscle	5	9
		Alaska: Sapporo Maruyama Zoo, Japan	Blood	1	1
Ursus maritimus	Polar bear	Tennoji Zoo, Japan	Blood	1	2
Ursus thibetanus	Asiatic black bear	Hunting, Honshu, Japan	Muscle	7	3
Ursus americanus	American black bear	Ikeda Zoo, Japan	Blood	1	3
Ursus malayanus	Sun bear	Ueno Zoological Garden, Japan	Hair roots	1	2
Ursus ursinus	Sloth bear	Kobe Municipai Oji Zoo, Japan	Liver	3	4
Tremarctos ornatus	Spectadled bear	Yokohama Zoo, Japan	Blood	3	2

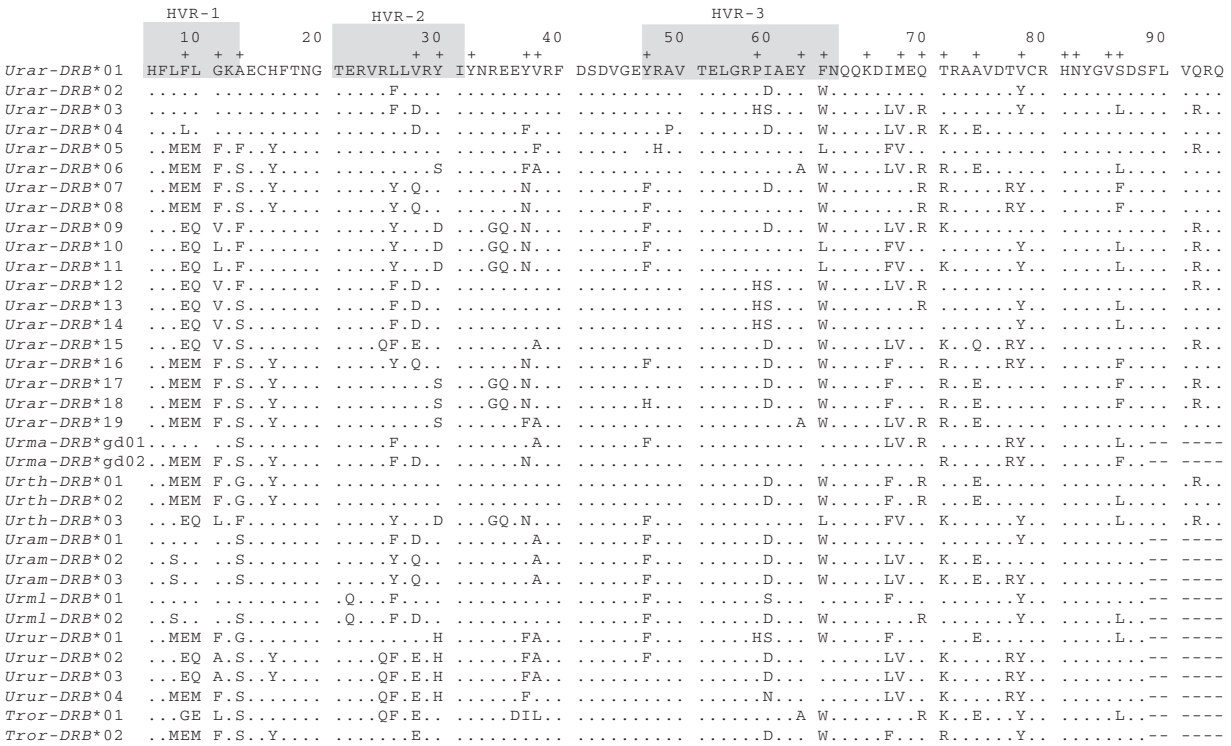

Figure 1 Alignments of amino acid sequences of the *DRB* exon 2 alleles of seven species in Ursidae: *Urar*, the brown bear (*Ursus arctos*); *Urma*, the polar bear (*Ursus maritimus*); *Urth*, the Asiatic black bear (*Ursus thibetanus*); *Uram*, the American black bear (*Ursus americanus*); *Urml*, the sun bear (*Ursus malayanus*); *Urur*, the sloth bear (*Ursus ursinus*); and *Tror*, the spectacled bear (*Tremarctos ornatus*). Amino acid residues are numbered according to Brown et al. (30). Dots indicate identity with the amino acid sequence of the *Urar-DRB**01 and dashes show no sequence data available. Pluses correspond to the amino acid residues identified as the functionally important antigen-binding sites (ABS) by X-ray crystallography in human leukocyte antigen (HLA) (30). The positions of hyper variable regions (HVR) shown by shaded boxes are indicated as defined for canine *DRB*1 (41).

primers amplifying the *DRB* exon 2 of the seven Ursidae species were designed as follows: *DRB*-UinF (5′-TCG CCT CTA TCC CCA CAG-3′) and *DRB*-UinR (5′-CAC ACG CCC TCC CCC AAT-3′) for amplification of the 326-bp fragment including most regions of the entire exon 2 (267 bp); and *DRB*-UrF1 (5′-TGA GCG GCT CGC CTC T(A/G)T CCC CAC A-3′) and *DRB*-UrR1 (5′-GCT CAC CTT GTC GC(C/T) GCA CCA GGA A-3′) for amplification of the 301-bp fragment.

The nucleotide sequences obtained in the present study were deposited in the DNA databases of DDBJ/EMBL/GenBank with the following accession numbers: AB490457–AB490490. In *U. arctos*, 11 alleles (*Urar-DRB**01 to *Urar-DRB**11) were identified from 32 individuals of the Hokkaido Island, Japan (Figure 1). In addition, eight alleles were found from five individuals of Western Siberia and one individual of Alaska (Figure 2). The nucleotide sequences were translated into the amino acid sequences, which consisted of 89 residues and started with codon position 6 and ended with position 94, compared with the complete sequence of the human *DRB* exon 2 region [according to Brown et al. (30)]. Nine of eleven alleles (*Urar-DRB**01, *02, *03, *04, *05, *06, *08, *09, and *10) were identified uniquely in the population of Hokkaido, whereas seven alleles (*Urar-DRB**12, *13, *14, *15, *16, *17, and *18) were unique to Western Siberia. Two alleles (*Urar-DRB**07 and *11) common between Japan and Western Siberia were found (Figure 2). One Alaskan brown bear had a unique allele (*Urar-DRB**19); the sequence differed in one nucleotide from *Urar-DRB**06 identified in Hokkaido. Nucleotide distances between *DRB* exon 2 alleles of *U. arctos* ranged from 0.37% to 13.86% (9.08 ± 1.06% on average; Table 2). Although it was relatively lower than that in different rodents (9.20%–14.71%) (24), it was higher than those in other mammals previously reported for the *DRB* locus [e.g. 4.4% for the alpine chamois (31); 5.00%–7.80% for various canids (26)].

Of the 38 *U. arctos*, however, seven individuals had three alleles and the other individuals had one or two alleles (Table 3). In addition, two of three individuals of *U. ursinus* had three or four alleles, and one individual of *U. americanus* had three alleles. One or two alleles were identified from *U. maritimus*, *U. thibetanus,* and *T. ornatus* (Table 3). These results indicate the existence of at least two *DRB* loci in the bear genome. Two expressed loci were reported in *DRB* of the domestic cat (6, 7), sheep (32), and red deer (33). Furthermore, the chimpanzees have four expressed *DRB* genes (34). Although more than two alleles found from single bears in the present study were not assigned to each distinct locus, it may indicate that the multiple *DRB* loci were generated recently. For example, one of *U. arctos* individuals examined in the present study had four distinct alleles, *Urar-DRB**01, *03, *04, and *11 (Table 3). The percentage differences of nucleotides among the four alleles ranged from 5.2% to 12.7% (8.1%, on average) and the phylogenetic position in

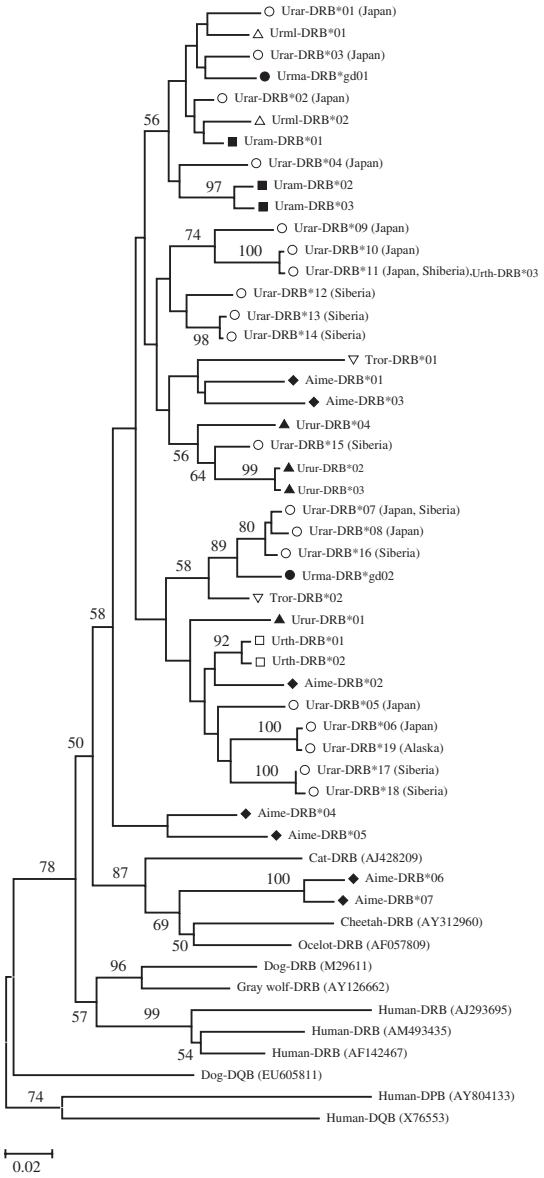

Figure 2 Neighbor-joining relationships among *DRB* exon 2 nucleotide sequences (228 bp) of species in the family Ursidae. *Urar*, *Ursus arctos* (open circle); *Urma*, *Ursus maritimus* (filled circle); *Urth*, *Ursus thibetanus* (open spuare); *Uram*, *Ursus americanus* (filled square); *Urml*, *Ursus malayanus* (open triangle); *Urur*, *Ursus ursinus* (filled triangle); *Tror*, *Tremarctos ornatus* (inverted open triangle); and *Aime*, *Ailuropoda melanoleuca* [filled diamond shape: AY895155-AY895161(14)]. As outgroup sequences, the *DRB* exon 2 sequences of human [AF142467 (42); AJ293695: Wu and Shiao (no information of publication); AM493435: Corell et al. (no information of publication)], dog [M29611 (43)], gray wolf [AY126662 (26)], domestic cat [AJ428209 (7)], cheetah [AY312960 (44)], ocelot [AF057809: Yuhki et al. (no information of publication)], the *DPB* exon 2 sequence of human [AY804133 (45)] and the *DQB* exon 2 sequences of human [X76553 (46)], dog [EU605811 (47)] were used. Bootstrap values >50 are indicated (1000 replications). The scale bar indicates evolutionary distances in units of the number of nucleotide substitutions per site. The nucleotide sequence of *Urar-DRB**11 was identical with that of *Urth-DRB**03.

Table 2 Mean nucleotide distances, amino acid distances, d_S and d_N for ABS and non-ABS in class II alleles of Ursidae

		Distance		ABS			Non-ABS		
	No. of alleles	Nucleotide	Amino acid	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
Within *U. arctos*	19	9.08 (1.06)	18.03 (3.21)	32.42 (6.90)	16.56 (7.74)	1.96*	4.82 (1.50)	4.26 (2.31)	1.13 ns
Within *Ursus*	33	9.74 (1.23)	19.90 (3.72)	32.65 (7.48)	14.89 (6.34)	2.19**	4.81 (1.60)	5.73 (2.48)	0.84 ns
Ursus vs T. ornatus	35	11.12 (1.35)	20.91 (3.88)	33.3 (7.5)	17.0 (7.9)	1.96**	5.1 (1.7)	10.3 (4.2)	0.50 ns
Ursus vs A. melanoleuca	40	14.51 (1.55)	26.41 (4.51)	41.7 (11.0)	34.7 (13.9)	1.20*	8.0 (2.1)	12.0 (4.1)	0.67 ns
T. ornatus vs A. melanoleuca	9	14.89 (1.53)	28.63 (4.94)	42.3 (11.7)	32.2 (13.4)	1.38 ns	8.7 (2.2)	13.3 (4.2)	0.65 ns
Within Ursidae	42	12.38 (1.48)	21.94 (3.69)	35.2 (7.7)	20.8 (7.6)	1.69*	6.1 (1.8)	7.0 (2.5)	0.87 ns

d_S, Synonymous substitution; d_N, non-synonymous substitutution; ABS, antigen-binding sites; non-ABS, nonantigen-binding sites.
Standard errors (in parentheses) were estimated by bootstrapping with 1000 replicates.
Significant levels: $*P \leq 0.05$, $**P \leq 0.01$, ns not significant. *P* values were calculated by codon-based *Z*-test of selection.
The values are shown in percentage.

the constructed gene tree was relatively closely related to each other (Figure 2), suggesting that the alleles between multiple *DRB* loci were not so distinct. In addition, the existence of multiple *DRB* loci was not reported in *A. melanoleuca* (14). These findings suggest the following two hypotheses: (1) the gene duplication event of the *DRB* gene occurred after the divergence of *A. melanoleuca* from the remaining bears at least on some haplotypes or in some individuals, or (2) the primer pair used by Wan et al. (14) did not amplify the second locus as estimated in the present study. Thus, the higher value of allelic sequence diversity in *U. arctos DRB* may have resulted from PCR amplification of multiple *DRB* loci as described earlier. In addition, Goda et al. (29) examined the *DQA* gene, which is another gene of the MHC class II of *U. arctos*, and reported that only three unique *DQA* alleles were found from the 32 individuals of *U. arctos* of Hokkaido

Table 3 Genotypes of *DRB* alleles of each species in the family Ursidae

U. arctos		*U. maritimus*		*U. thibetanus*		*U. americanus*		*U. malayanus*		*U. ursinus*		*T. ornatus*	
Genotype	No. of individuals	Genotype	No. of individuals	Genotype	No. of individuals	Genotype	No. of individuals	Genotype	No. of individuals	Genotype	No. of individuals	Genotype	No. of individuals
01/01	1	01/02	1	01	1	01/02/03	1	01/02	1	01/02	1	01/01	1
04/04	1			03	3					01/02/03	1	01/02	2
05/05	1			01/02	1					01/02/03/04	1		
07/07	6			01/03	1								
08/08	1			02/03	1								
12/12	1												
19/19	1												
01/11	1												
02/09	2												
03/04	1												
04/07	1												
05/06	1												
05/07	2												
05/10	3												
05/11	1												
06/07	2												
07/08	1												
07/09	1												
07/11	1												
07/14	1												
11/18	1												
03/04/06	1												
04/06/10	1												
05/07/10	1												
11/15/17	1												
12/13/16	1												
01/03/04/11	1												
01/05/10/11	1												
Total	38		1		7		1		1		3		3

Table 4 Polymorphic amino acid positions for *DRB* exon 2 sequences of each genus

Position	*Ursus* (33)	*T. ornatus* (2)	*A. melanoleuca* (7)
8	LMS	LM	LM
9*	EFL	EG	EGNV
10	LMQ	EM	LMQ
11*	AFGLV	FL	AFGLW
13*	AFGS	S	GST
16	HY	HY	HY
22	EQ	E	E
25	QR	QR	R
26	FLY	FL	FLY
28*	DEQV	E	DQR
30*	DHSY	Y	CHY
31	I	I	FI
34	GR	R	GR
35	EQ	E	E
36	E	DE	E
37*	FNY	IY	FY
38*	AV	LV	V
42	S	S	GS
43	D	D	DE
47*	FHY	Y	FY
51	AP	A	AP
56*	HP	P	P
57	DINS	DI	DT
59	E	E	EK
60*	AY	AY	Y
61*	FLW	W	W
63	Q	Q	GQ
65*	K	K	EK
67	FIL	FI	FY
68*	MV	M	LM
70*	QR	QR	GQ
71*	KRT	KR	QRT
73	A	A	AT
74*	AEQ	AE	A
77	RT	T	TW
78*	VY	Y	VY
84	G	G	GV
86*	FLS	LS	G
92	QR	—	—

Genus *Ursus* includes *U. arctos*, *U. martimus*, *U. thibetanus*, *U. americanus*, *U. malayanus*, and *U. ursinus*.

Amino acid position and ABS (shown as asterisks) are according to Brown et al. (30).

Numbers in parentheses show the numbers of alleles observed in each genus.

Dashes show that there are no sequence data available.

identical to the present study and that the level of polymorphisms among them (1.98% on average of nucleotide distances) was lower than that of the *DRB* alleles identified in the present study. The results indicate that the polymorphisms at the *DQA* locus occurred more recently than at the *DRB* locus in *U. arctos* and the *DRB* gene has diversified for a longer time than the *DQA* gene in multiple loci through gene duplication, providing contrastive features between the two genes of *U. arctos* MHC class II.

Among 33 alleles of genus *Ursus*, 30 of 83 amino acid residues were polymorphic and their distribution was concentrated on ABS and/or sites adjacent to them (Table 4). The similar distribution of polymorphic codons was observed in both *T. ornatus* and *A. melanoleuca*. In addition, nonsynonymous substitutions occurred significantly more frequently than synonymous substitutions at ABS both within *U. arctos* and within genus *Ursus* ($d_N/d_{S\ Urar} = 1.96$, $z = 2.252$, $P = 0.013$; $d_N/d_{S\ Ursus} = 2.19$, $z = 2.946$, $P = 0.002$: Table 2), whereas such significant differences between d_N and d_S values were not found at non-ABS. This suggests that the ABS of the bear *DRB* gene have been selectively under balancing selection, maintaining the MHC allelic diversity (18, 19). Such kinds of features are also shown in MHC loci of other species (8, 24, 35–37).

In order to analyze the phylogenetic relationships among Ursidae species based on *DRB* allelic sequences, a gene tree of *DRB* exon 2 sequences (228 bp) obtained from all species was constructed (Figure 2) using neighbor-joining method (38). The result firstly showed that alleles of a species are often more closely related to alleles of other species than to other alleles of the same species (i.e. trans-species evolution) (21). The trans-species persistence of allelic lineages among genera have been reported as one of evidence for balancing selection in many species of mammals, such as primates (22), rodents (12, 23, 24), lagomorphs (25), and carnivores (8, 26). Secondly, the gene tree showed that the alleles of Ursidae species, except for the four alleles of *A. melanoleuca* (*Aime-DRB**04 and *05 formed a single cluster, and *Aime-DRB**06 and *07 were included within the group of felids), were not clustered according to species. This suggests that allelic lineages of *DRB* gene in Ursidae diverged before speciation of *A. melanoleuca* from the other six Ursidae species. According to the fossil record and the complete mitochondrial genome data of the family Ursidae (39, 40), *A. melanoleuca* first diverged around 12 million years ago (MYA). Therefore, the divergence of the *DRB* allelic lineages in Ursidae is estimated to have occurred more than 12 MYA. Finally, an identical allele was shared between *U. arctos* and *U. thibetanus* (*Urar-DRB**11 and *Urth-DRB**03, respectively). Within genus *Ursus*, *U. ursinus* first diverged at 6.34 MYA, followed by a bifurcation forming two clades: one leading to the ancestor of *U. arctos* and *U. maritimus* and the other leading to the ancestor of two species of black bears (*U. americanus* and *U. thibetanus*) and *U. malayanus* at 6.13 MYA (40). Therefore, the allele (*Urar-DRB**11 or *Urth-DRB**03) shared by both *U. arctos* and *U. thibetanus* is purported to have persisted for more than 6 million years, and is implicated in the persistence of the species up to the present time.

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