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Diversity and evolution of MHC class II *DRB* gene in the Eurasian badger genus *Meles* (Mammalia: Mustelidae)

SHAMSHIDIN ABDURIYIM[1], YOSHINORI NISHITA[1,2], PAVEL A. KOSINTSEV[3], EVGENIY RAICHEV[4], RISTO VÄINÖLÄ[5], ALEXEY P. KRYUKOV[6], ALEXEI V. ABRAMOV[7], YAYOI KANEKO[8] and RYUICHI MASUDA[1,2]*

[1]*Department of Natural History Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*
[2]*Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan*
[3]*Institute of Plant and Animal Ecology, Ural Branch, Russian Academy of Sciences, Ekaterinburg 620144, Russia*
[4]*Agricultural Faculty, Trakia University, Stara Zagora 6000, Bulgaria*
[5]*Finnish Museum of Natural History, University of Helsinki, PO Box 17, Helsinki FI-00014, Finland*
[6]*Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, Institute of Biology and Soil Science, Russian Academy of Sciences, Vladivostok 690022, Russia*
[7]*Zoological Institute, Russian Academy of Sciences, Saint Petersburg 199034, Russia*
[8]*Carnivore Ecology and Conservation Research Group, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan*

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Major histocompatibility complex (MHC) genes encode proteins that play a key role in the adaptive immune system of vertebrates and are generally highly polymorphic for defence against various pathogens. To understand the diversity and evolution of MHC variation in badgers in the genus *Meles* (Carnivora, Mustelidae), we analysed sequence variation of the MHC class II *DRB* gene exon 2 in the Japanese (*Meles anakuma*), Asian (*Meles leucurus*), European (*Meles meles*) and Southwest Asian (*Meles canescens*) badgers. Variation was higher in the *Meles* species than in other species in Mustelidae, and altogether 60 alleles were isolated from 28 individuals. The variable number of three to eight putative alleles per individual was observed, indicating the presence of two to four *DRB* loci per haploid genome. Non-synonymous substitutions exceeded synonymous substitutions at putative antigen-binding sites. Selection analyses of PAML models, fixed-effect likelihood and mixed-effect model evolution, together with the single breakpoint recombination, indicated that recombination and selection could be responsible for driving and maintaining the diversity of *Meles DRB*s. In a phylogenetic analysis, the *DRB* sequences from *Meles* were distributed in several clusters, which were dispersed among sequences of other mustelid family, even five alleles comprised a monophyletic group of *Meles DRB*s within a canid clade. The data demonstrate trans-species polymorphisms at different taxonomic and temporal scales, transgressing family-, genus- and species-level splits. Some allele sequences were shared by two to four of the *Meles* species, in line with a close phylogenetic relationship among these species.

ADDITIONAL KEYWORDS: balancing selection – Eurasian badgers – genetic diversity – genus *Meles* – MHC class II *DRB* gene – trans-species polymorphism.

INTRODUCTION

Major histocompatibility complex (MHC) genes encode proteins that play a key role in the adaptive immune system of vertebrates and are generally highly polymorphic for defence against various pathogens. Based on their molecular structure and function, MHC proteins are generally divided into classes I, II and III. The MHC classes I and II proteins both work as heterodimers. The MHC class I heterodimer consists of a MHC

*Corresponding author. E-mail: masudary@sci.hokudai.ac.jp

protein and a non-MHC β_2 microglobulin molecule. In contrast, MHC class II proteins work as dimers of two class II proteins, comprising a complex of α and β chains; they present antigens, exogenously derived from invading pathogens, to CD4+ helper T cells in order to activate antibody production by B cells, resulting in the destruction of the pathogens (Edwards & Hedrick, 1998; Cresswell *et al.*, 2005). MHC class II allele variation has been studied in humans (Doxiadis *et al.*, 2007; Shams *et al.*, 2009), several non-human primate species (Kriener, O'hUigín & Klein, 2001; Go *et al.*, 2002; Doxiadis *et al.*, 2007; de Groot *et al.*, 2017) and further non-model mammalian species (e.g. Sin *et al.*, 2012a, 2014, 2015; Rico *et al.*, 2015, 2016; Nishita *et al.*, 2015, 2017). These studies show that MHC class II genes are generally highly polymorphic, although they are subject to functional and/or structural constraints (Reche & Reinherz, 2003). The MHC variability within and between populations reflects evolutionarily processes, such as selection and adaptation, and should provide a fruitful system for addressing questions in evolutionary ecology and conservation (Sommer, 2005). The *DRB* variability is further complicated by the potential presence of multiple (tightly linked) copies of the gene within the MHC complex. The number of expressed *DRB* copies varies with the taxon, but it can also vary among haplotypes (i.e. chromosomes) within a species. In addition, non-functional pseudogenes may be present (Aguilar, Smith & Wayne, 2005; Sommer, 2005; Sin *et al.*, 2012a; Nishita *et al.*, 2015).

Members of the genus *Meles* (Melinae, Mustelidae, Carnivora) are opportunistic foragers with an omnivorous diet (Pigozzi, 1991; Li *et al.*, 2013); they are widespread across Eurasia, from the British Isles eastward to Japan. The taxonomy of *Meles* has been convoluted and controversial. The Eurasian badger was once considered to be subdivided into 23 geographical populations of unclear taxonomic rank, loosely termed 'subspecies' (Ellerman & Morrison-Scott, 1951), but later was treated as a single species comprising three subspecies (Heptner, 1967). More recently, some researchers have proposed two species within the genus on the basis of dental and cranial characters (Baryshnikov & Potapova, 1990; Lynch, 1994), while others recognize three species (Wozencraft, 2005) on the basis of morphological characters (Abramov, 2002; Baryshnikov, Puzachenko & Abramov, 2003; Abramov & Puzachenko, 2005, 2006) or external parasites (Abramov & Medvedev, 2003).

Genetic analyses have begun to clarify the taxonomy of *Meles*. From analyses of autosomal, Y-chromosomal and mitochondrial markers, evidence for four distinct species in *Meles* has been concluded (Marmi, López-Giráldez & Domingo-Roura, 2004; Del Cerro *et al.*, 2010; Tashima *et al.*, 2011a, b): the European badger (*Meles meles*), distributed across most of Europe; the Asian badger (*Meles leucurus*) in continental Asia; the Japanese badger (*Meles anakuma*) in Japan; and the Southwest Asian badger (*Meles canescens*) in southwestern Asia and the mountains of central Asia. The species status of *M. canescens* (Blanford, 1875) was confirmed through an analysis of cranial data (Abramov & Puzachenko, 2013). The complete cytochrome *b* gene sequenced from badgers from Turkey and from other Eurasian badgers (Ibiş *et al.*, 2015) has subsequently supported the results of the previous studies.

Recently, *DRB* genes encoding the β chain of MHC class II proteins are extensively studied in diverse species for various attempts, for instance, heterozygous advantage against pathogen pressures (Sin *et al.*, 2014; Osborne *et al.*, 2015), MHC association with infection intensity (Sin *et al.*, 2014), pathogen-driven balancing selection (Nishita *et al.*, 2015, 2017), MHC polymorphism influenced by balancing selection and genetic drift (Rico *et al.*, 2015), gene flow shaping MHC polymorphism (Rico *et al.*, 2016), MHC variability for the management of captive breeding programmes (Cai *et al.*, 2015) and the importance of MHC class II for mate preference (Sin *et al.*, 2015). Moreover, an analysis of genomic and complementary DNA from seven European badger individuals demonstrated that among MHC class II genes, *DRB*, *DQB* and *DQA* (but not *DRA*) show high variation in exon 2, with the highest variation occurring in *DRB* (Sin *et al.*, 2012a). Therefore, to further understand the genetic variation and evolution of MHC class II genes in *Meles*, we focussed in the present study on the MHC class II *DRB* gene in European, Asian, Southwest Asian and Japanese badgers. Here, we present results on genetic variation and phylogenetic relationships among *DRB* alleles, assess evidence for selection and discuss the observed pattern of trans-species polymorphism (TSP).

MATERIAL AND METHODS

Samples and DNA extraction

We obtained tissue and skin samples from a total of 28 individuals of four *Meles* species from throughout their natural range in Eurasia, including Japan. Sampling locations and sample sizes are shown in Figure 1 and Table 1. Small pieces of tissue samples were obtained from incidental roadkills and preserved for each individual in 99% ethanol and stored at either 4 °C or −20 °C. Dried skin samples were obtained from old museum specimens. Total genomic DNA was extracted from samples using the DNeasy Blood and Tissue Kit (Qiagen, Hiden, Germany) following the manufacturer's protocol, and DNA extracts were stored in TE buffer at 4 °C until use.

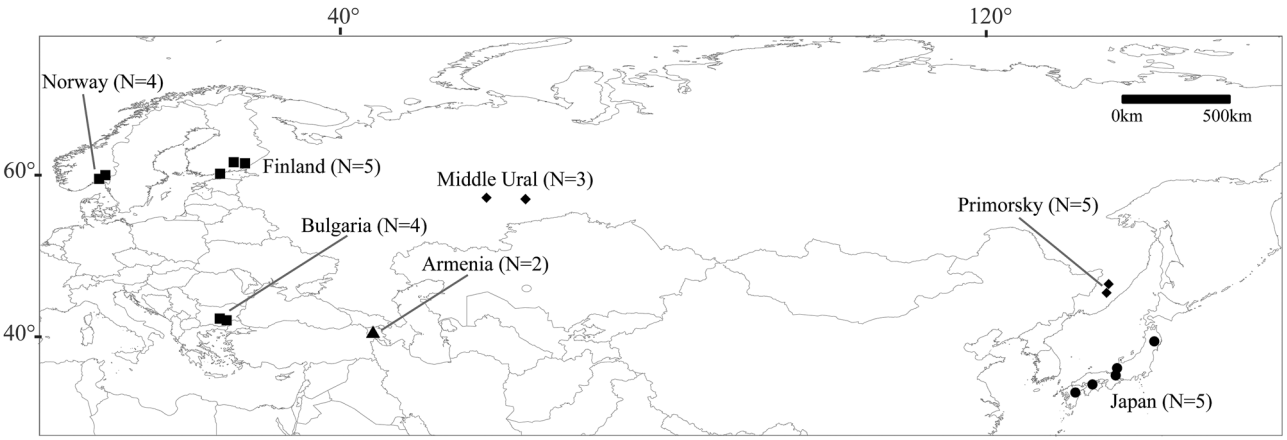

Figure 1. Map of Eurasia showing locations where *Meles* specimens used in this study were collected. Circles, rhombus, squares and triangle represent Japanese, Asian, European and Southwest Asian badgers, respectively. The profile of the samples is shown in Table 1.

Table 1. Profiles of *Meles* samples examined in this study

Species	Sample code	Tracking code	Number of clones	Sex	Location	Tissue
Meles meles	MEL-2011-68	BA	27	Female	Stara Planina, Bulgaria	Muscle
	MEL-2011-112	BB	26	Female	Sredna gora, Bulgaria	Muscle
	MEL-2011-110	BC	24	Female	Stara Planina, Bulgaria	Muscle
	MEL-2012-28	BE	27	Female	Kazanlak, Bulgaria	Muscle
	MZH-KN46943	FA	30	Female	Punkaharju, Finland	Muscle
	MZH-KN49014	FC	31	Male	Porvoo, Finland	Muscle
	MZH-KN49015	FD	31	Male	Porvoo, Finland	Muscle
	MZH-KN49016	FE	31	Female	Porvoo, Finland	Muscle
	MZH-KN49050	FF	30	Male	Porvoo, Finland	Muscle
	Nm4(204-68)	NA	24	Unknown	Akershus, Norway	Muscle
	Nm5(2547)	NB	28	Unknown	Akershus, Norway	Muscle
	Nm18(122/71)	NC	25	Unknown	Akershus, Norway	Skin
	Nm19(1839)	ND	24	Unknown	Oslo, Norway	Skin
Meles leucurus	#416829	UA	28	Female	Middle Urals, Russia	Muscle
	#452634	UB	34	Male	Middle Urals, Russia	Muscle
	#452478	UC	30	Male	Middle Urals, Russia	Muscle
	#1833	RC	24	Male	Primorsky Krai, Russia	Muscle
	#1748	RE	24	Female	Primorsky Krai, Russia	Muscle
	#1747	RF	25	Female	Primorsky Krai, Russia	Muscle
	#1746	RG	24	Female	Primorsky Krai, Russia	Muscle
	#1749	RH	23	Female	Primorsky Krai, Russia	Muscle
Meles canescens	MEL-Ar1	AR1	25	Unknown	Armenia	Muscle
	MEL-Ar2	AR2	26	Male	Armenia	Muscle
Meles anakuma	MEL-2	JA	24	Female	Gifu, Japan (Honshu)	Muscle
	MEL-MR1	JB	24	Female	Iwate, Japan (Honshu)	Liver
	MEL-H17-004	JC	25	Female	Kochi, Japan (Shikoku)	Muscle
	MEL-K10	JD	24	Male	Ohita, Japan (Kyushu)	Muscle
	HB99001	JF	24	Male	Tokyo, Japan (Honshu)	Skin

PCR AMPLIFICATION

We amplified fragments of *DRB* exon 2 from individuals of all four *Meles* species by PCR using the *Meles*-specific forward primer Meme-DRBex2F (5′-CGT CCC CAC AGG ACA TTT C; Sin *et al.*, 2012a) and the *Mustela lutreola* reverse primer (5′-CTC GCC GCT GCA CCG TGA AG; Becker *et al.*, 2009). PCR reactions were carried out in 25 µL volumes containing 1× buffer (Mg^{2+} plus), 125 µM each dNTP, 7.5 pmol each phosphorylated primer, 40–100 ng of total genomic DNA and 0.625 U of PrimeSTAR GXL DNA polymerase (Takara Bio, Kusatsu, Japan). The reaction conditions in a Takara Dice Touch thermal cycler were 2 min at 94 °C; 30 cycles for 10 s at 98 °C, 15 s at 60 °C and 30 s at 68 °C; and a final hold at 4 °C. PCR products were checked by electrophoresis on a 2% agarose gel and visualized with ethidium-bromide florescence.

CLONING AND SEQUENCING

PCR products (281 bp, including the forward and reverse primer sites) from *DRB* exon 2 were purified with a QIAquick PCR Purification Kit (Qiagen), ligated into pBluescript II SK+ (Agilent Technologies, Santa Clara, CA, USA) and transformed into *Escherichia coli* JM109 competent cells. After selection of positive clones containing target fragments by blue/white screening and colony PCR, liquid cultures were grown overnight at 37 °C, and plasmids were purified with a QIAprep Spin Miniprep Kit (Qiagen). Randomly selected positive inserts were sequenced in both directions with M13 forward and reverse primers using a BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Walthan, MA, USA) and an ABI3730 automated DNA sequencer (Thermo Fisher Scientific). From 23 to 34 plasmid clones were obtained and sequenced for each *Meles* individual. Sequences were aligned, checked and edited with MEGA 6.0. (Tamura *et al.*, 2013) and Seqman (Swindell & Plasterer, 1997) in the DNASTAR Lasergene package and were verified as MHC class II sequences by means of BLAST searches (Altschul *et al.*, 1990) of the DDBJ/EMBL/GenBank database. Sequences were identified as bona fide MHC genes, according to the criteria established for the dog MHC system (Kennedy *et al.*, 2000), briefly as follow: the forward and reverse sequence data were accordant to each other; identical sequences from at least two individuals or from two independent PCR reactions of the same individual were identified as true variants; and singular unique sequence reads were omitted, as they may have been artefacts. Verified sequences were named by using the conventions of Klein *et al.* (1990). Confirmed MHC class II *DRB* exon 2 sequences from our study have been submitted to GenBank under accession numbers LC180283–LC180339.

DATA ANALYSIS

All sequences were aligned by using Clustal W1.6 (Thompson, Higgins & Gibson, 1994) in MEGA 6.0, and distinct putative alleles were identified. As measures of the sequence diversity under *P*-distance method, the number of putative alleles in each species and the average sequence divergence among those putative alleles were recorded. Also for the same data sets DnaSP v.5.10.01 (Librado & Rozas, 2009) and MEGA 6.0 were used to estimate the ratio $\omega = d_N/d_S$ of non-synonymous (d_N) to synonymous (d_S) substitution rates by the method of Nei & Gojobori (1986); this ratio provides a measure of selective pressure at the level of individual sites (Yang & Nielsen, 2002). Values of $d_N/d_S > 1$ indicate positive selection, while $d_N/d_S = 1$ and $d_N/d_S < 1$ indicate neutral evolution and purifying selection, respectively. Values of d_N, d_S and d_N/d_S were calculated separately for antigen-binding sites (ABSs) and non-ABS codon positions (because ABSs bind directly to a peptide derived from a pathogen and are expected to be evolved under strong positive selection), as determined by Reche & Reinherz (2003). *Z*-tests for historical positive selection (Nei & Kumar, 2000) were performed in MEGA 6.0. Alternatively, four PAML models (Yang *et al.*, 2000) were tested providing evidences of selection intensities among sites: M1a (nearly neutral), M2a (positive selection), M7 (beta) and M8 (beta and ω). The likelihood ratio tests (LRTs) were performed to determine whether positive selection models provide best fit to our data, vs. null models (M2a vs. M1a and M7 vs. M8, respectively). Codons under positive selection were identified by Bayes Empirical Bayes inference (suits for small data sets and does not effected by recombination events; Yang, Wong & Nielsen, 2005) for models M2a and M8 implementing in CODEML in PAML 4 package (Yang, 2007). The mixed-effect model evolution (MEME) and fixed-effect likelihood (FEL) analyses provided by DATAMONKEY, a web server for the HyPhy Package (Murrell *et al.*, 2012), were also used to identify positively selected sites with posterior probability > 95%. The same website was used to check signatures of recombination applying the genetic algorithm recombination detection method.

To illustrate the phylogenetic relationships among the MHC alleles of the four *Meles* species in the framework of related carnivore taxa (EMBL/DDBJ/GenBank database data from species in the Mustelidae, Canidae and Felidae), a Bayesian phylogenetic analysis was conducted in MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003) running for 30 million generations, sampling every 100 generations. Data were partitioned by gene codon sites, and the best fitting models for tree construction were selected in KAKUSAN v4 (Tanabe, 2011). Parameter values sampled from the chains

were checked for convergence by using Tracer v1.6.0 (Rambaut *et al.*, 2014), and first 25% of generations were discarded in 'burn-in' stage.

RESULTS

ALLELIC DIVERSITY

In total, sequences of 742 clones were obtained from 28 individuals in the 4 *Meles* species, from an average of 26.5 clones per individual. The confirmed MHC class II *DRB* exon 2 sequences were 242 bp long without the primer regions and encoded 80 amino acids comprising 85% of the β1 domain. The numbers of distinct alleles found in any single individual ranged from three to eight. Up to eight alleles per individual were found in the European badger (Table 2), seven in the Asian badger (Table 3) and five in the Japanese badgers (Table 4). The lowest number was three for European and Japanese badgers, and Asian badgers with four alleles. In addition, five alleles were detected in each of two Southwest Asian badger individuals (Table 5). The minimum number of diploid loci in each specimen was thus from two to four in the different individuals.

The total number of distinct alleles detected per species was 19 in the European badger, of which 16 (*Meme-DRB*05–20*) were novel, that is undetected in previous studies; 19 in the Asian badger (*Mele-DRB*01–19*), 11 in the Japanese badger (*Mean-DRB*01–11*); and seven (*Meca-DRB*01–07*) in the Southwest Asian badger (Tables 2–5). The average divergence among the distinct alleles (nucleotide diversity in the allele alignment) ranged from 0.11 for the Japanese badger to 0.14 for the European badger (Table 6).

Among the 80 amino acid sites (Fig. 2) encoded by the studied *DRB* segment, 29 (36.3%) were polymorphic in

Table 2. Frequencies of MHC class II *DRB* alleles in the European badger (*Meles meles*)

Individual identity	*Meme-DRB**																					Number of verified alleles†
	01	02	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	02*	03*	
NA				+	+	+	+													+	+	4 (6)
NB				+				+	+	+										+	+	4 (6)
NC				+	+	+														+	+	3 (5)
ND				+			+	+	+	+										+	+	5 (7)
BA	+	+		+							+					+						5
BC	+										+	+									+	3 (4)
BB	+	+		+							+		+	+	+	+						8
BE	+		+	+								+			+	+						6
FA	+	+		+										+		+	+			+		6 (7)
FC		+		+														+				3
FD		+																+	+			3
FE	+	+									+	+						+		+		5 (6)
FF		+	+													+	+	+	+			6

*Presumed pseudogenes containing deletions.
†In parentheses, number of verified alleles including presumed pseudogenes.

Table 3. Frequencies of MHC class II *DRB* alleles in the Asian badger (*Meles leucurus*)

Individual identity	*Mele-DRB**																			Number of verified alleles
	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	
UA	+					+									+	+				4
UB				+										+	+		+		+	5
UC						+						+	+					+		4
RC			+			+	+	+	+	+	+									7
RE		+		+			+				+	+	+							6
RF						+	+	+	+	+	+									6
RG		+		+		+							+	+						5
RH	+	+	+	+	+		+													6

Table 4. Frequencies of MHC class II *DRB* alleles in the Japanese badger (*Meles anakuma*)

Individual identities	*Mean-DRB**													Number of verified alleles†
	01	02	03	04	05	06	07	08	09	10	11	01*	02*	
JA				+	+	+	+	+						5
JB				+	+	+	+	+						5
JF	+	+	+									+	+	3 (5)
JC					+					+	+			3
JD	+		+					+	+				+	4 (5)

*Presumed pseudogenes containing deletions.
†In parentheses, number of verified alleles including presumed pseudogenes.

Table 5. Frequencies of MHC class II *DRB* alleles in Southwest Asian badger (*Meles canescens*)

Individual identity	*Meca-DRB**							Number of verified alleles
	01	02	03	04	05	06	07	
AR1	+	+	+	+	+			5
AR2	+	+			+	+	+	5

Table 6. Mean nucleotide divergence (π) among the distinct alleles of each species, and rates of non-synonymous (d_N) and synonymous (d_S) substitutions and their ratio ($d_N/d_S \pm$ SE) for antigen-binding site (ABS) codons, non-ABS codons and all codons in *DRB* exon 2 for four *Meles* species

Substitution type		*Meles meles*	*Meles leucurus*	*Meles canescens*	*Meles anakuma*
d_N	ABS	2.20 ± 0.11	2.05 ± 0.19	0.89 ± 0.35	1.07 ± 0.16
	Non-ABS	0.38 ± 0.06	0.19 ± 0.07	0.19 ± 0.20	0.20 ± 0.08
	Overall	0.79 ± 0.03	0.69 ± 0.14	0.35 ± 0.28	0.39 ± 0.13
d_S	ABS	1.30 ± 0.13	1.75 ± 0.29	0.53 ± 0.51	1.12 ± 0.39
	Non-ABS	0.88 ± 0.11	0.86 ± 0.19	0.49 ± 0.45	0.49 ± 0.19
	Overall	0.98 ± 0.11	1.06 ± 0.22	0.49 ± 0.46	0.631 ± 0.253
d_N/d_S	ABS	1.68	1.17	1.68*	0.96
	Non-ABS	0.43*	0.221*	0.38*	0.41*
	Overall	0.81*	0.654*	0.69	0.63*
π		0.14 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01

*Statistically different from the prediction of neutral evolution, $P < 0.05$.
Putative ABS codons were those determined by Reche & Reinherz (2003).

the Southwest Asian badger, 35 (43.8%) in the Asian badger, 38 (47.5%) in the Japanese badger and 43 (53.8%) in the European badger.

Two sequences (*Mean-DRB*PS01–PS02*) in the Japanese badger and two (*Meme-DRB*PS02–PS03*) in the European badger were inferred to be pseudogenes (Tables 2 and 4; Fig. 2), due to deletion of 34 nucleotides at the beginning of *DRB* exon 2 in all four sequences that resulted in a frameshift. These presumable pseudogenes were excluded all statistical analyses but phylogenetic analysis.

Selection and recombination analysis

To evaluate selection pressure, we calculated the ratio of non-synonymous to synonymous substitution rates for positions in ABSs, non-ABS codons and all codons (Table 6), for the data sets of distinct alleles of each species. For three of these data sets, non-synonymous substitutions exceeded synonymous substitutions for predicted ABS codons ($k = 18$), implying positive selection, although ω was significantly greater than 1 only for the Southwest Asian badger. In contrast, for the non-ABS positions ($k = 62$) and overall, synonymous

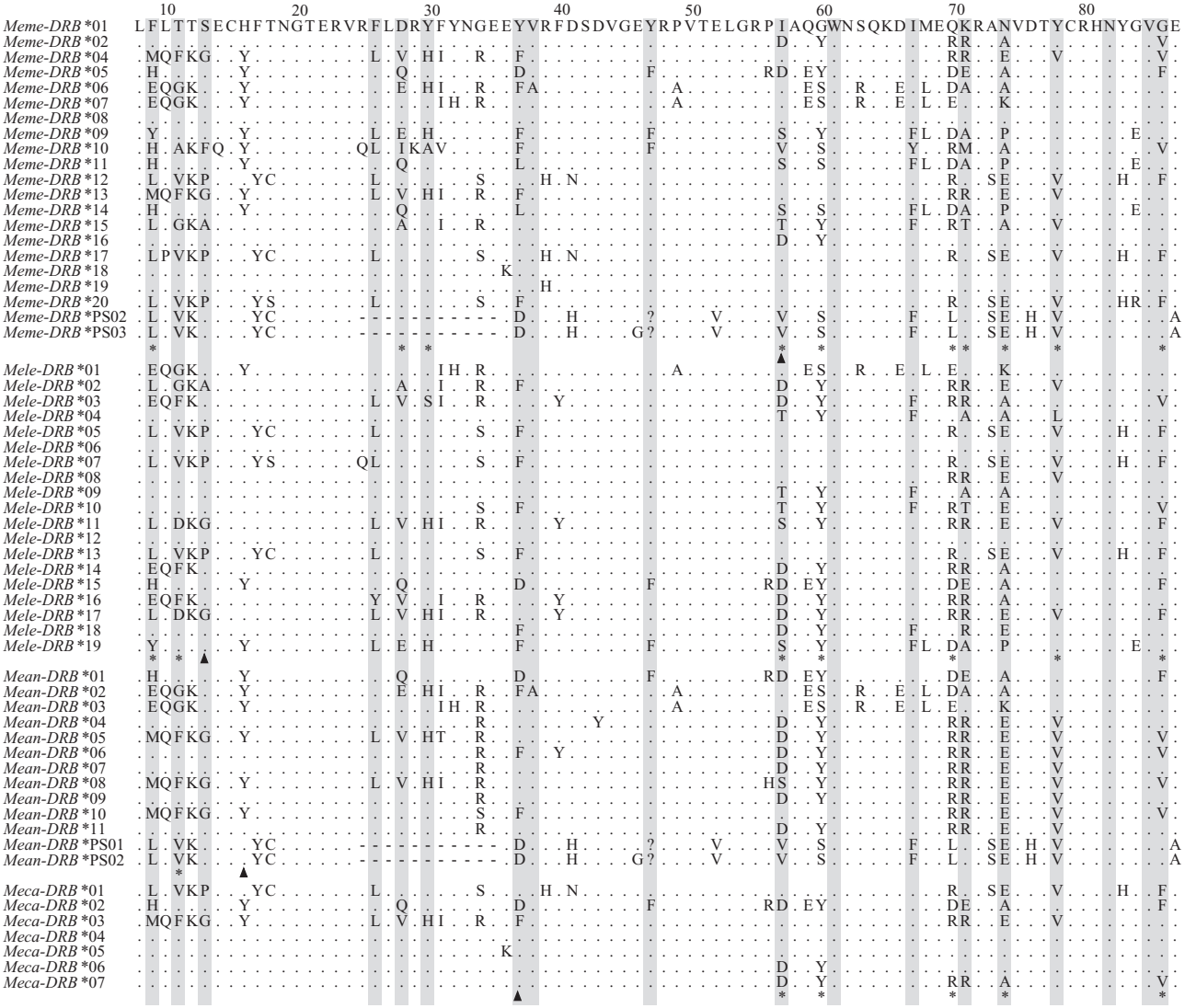

Figure 2. Deduced amino acid sequences for exon 2 of the MHC class II *DRB* gene sequenced in this study from European, Southwest Asian, Asian and Japanese badgers. The numbers at the top indicate the amino acid position in the β1 domain. Single letters represent amino acids; dots indicate identity with the first sequence in the list; dashes indicate amino acid deletions. Putative ABSs as determined by Reche & Reinherz (2003) are shaded. Asterisks indicate sites subject to positive selection as determined by the PAML, FEL and MEME analyses. Triangles are the single breakpoint (SBP) recombination sites.

substitution rates were greater (and except for the Southwest Asian badger, significantly greater) than non-synonymous substitution rates. The PAML, FEL and MEME analyses provided evidence for positive selections at the codon level in *DRB* exon 2 for all four species-wise sequence sets. More positive selection signatures were detected in M8 (beta and ω) model than in M2a (positive selection). Parameter estimates for M2a and M8 (Table 7) indicated that 3%–25% sites under positive selection with ω = 1.31 to 14.38 for these species, respectively. LRT demonstrated that positive selection models of M2a and M8 provided significantly better fit to our data, but not of the Japanese badger, than the models (M1a and M7) of neutral evolution (Table 7). These sites subjected to positive selection in PAML models were shown in Table 7. The FEL method detected only one site under positive selection in Asian and Southwest Asian badgers at 86th positions. In contrast, MEME (most badger individuals used in this study were the same as those analysed by Tashima *et al.* (2011a) and Kinoshita *et al.* (2017), where mitochondrial DNA and Y-chromosomal DNA were investigated, and no evidence of hybridization in these individuals was reported) identified three sites in the Asian badger, five in Southwest Asian and European badgers, but only one in the Japanese badger (Table 7).

Table 7. Codons under positive selection (CPS), log-likelihood (lnL) values and parameter estimates under different models of codon evolution and the values of test statistic (TS) and posterior probability (*P*) for the log-likelihood test (LRT) of MHC class II *DRB* exon 2 genes in the four Eurasian badger species

Species	Models	lnL values	Parameter estimates	CPS	LRT	TS values	*P* value
Meles anakuma	M1a	−773.17	$P_0 = 0.73, P_1 = 0.27, \omega_0 = 0.04, \omega_0 = 1.00$	Not allowed	M1a vs. M2a	0.89	0.64
	M2a	−772.73	$P_0 = 0.74, P_1 = 0.09, P_2 = 0.17, \omega_0 = 0.04, \omega_1 = 1.00, \omega_2 = 1.45$	NS			
	M7	−774.76	$P = 0.108, q = 0.292$	Not allowed	M7 vs. M8	4.06	0.13
	M8	−772.73	$P_0 = 0.75, P = 4.80, q = 99.00, P_1 = 0.25, \omega = 1.31$	9, 11^{M}, 28, 32, 37, 57, 86			
Meles leucurus	M1a	−1157.58	$P_0 = 0.77, P_1 = 0.23, \omega_0 = 0.05, \omega_0 = 1.00$	Not allowed	M1a vs. M2a	10.32	> 0.01
	M2a	−1152.42	$P_0 = 0.75, P_1 = 0.21, P_2 = 0.05, \omega_0 = 0.05, \omega_1 = 1.00, \omega_2 = 3.79$	86			
	M7	−1159.28	$P = 0.13, q = 0.38$	Not allowed	M7 vs. M8	14.46	> 0.001
	M8	−1152.05	$P_0 = 0.82, P = 0.32, q = 2.97, P_1 = 0.18, \omega = 1.77$	9, 57, (60, 70), 78, 86^{M,F}			
Meles meles	M1a	−1198.77	$P_0 = 0.77, P_1 = 0.23, \omega_0 = 0.06, \omega_0 = 1.00$	Not allowed	M1a vs. M2a	7.64	> 0.05
	M2a	−1194.95	$P_0 = 0.77, P_1 = 0.08, P_2 = 0.15, \omega_0 = 0.08, \omega_1 = 1.00, \omega_2 = 2.07$	NS			
	M7	−1203.48	$P = 0.18, q = 0.47$	Not allowed	M7 vs. M8	21.01	> 0.001
	M8	−1192.97	$P_0 = 0.82, P = 0.59, q = 4.89, P_1 = 0.19, \omega = 1.89$	9, 28, 30, 57^{M}, 60^{M}, (70), 71, (74, 78), 86			
Meles canescens	M1a	−691.71	$P_0 = 0.76, P_1 = 0.24, \omega_0 = 0.04, \omega_0 = 1.00$	Not allowed	M1a vs. M2a	11.22	> 0.01
	M2a	−686.10	$P_0 = 0.75, P_1 = 0.23, P_2 = 0.03, \omega_0 = 0.05, \omega_1 = 1.00\ \omega_2 = 14.38$	86,			
	M7	−693.18	$P = 0.12, q = 0.33$	Not allowed	M7 vs. M8	12.58	< 0.01
	M8	−686.88	$P_0 = 0.98, P = 0.13$, q = 0.38, $P_1 = 0.03, \omega = 14.04$	(57, 60, 70), 74^{M}, 86^{M,F}			

ω is ratio of d_N and d_S (d_N/d_S); *Pn* is the proportion of amino acids in ω*n* site classes; *P* and *q* are the parameters of the beta distribution, all the values of lnL, ω, *Pn*, ω*n*, *P* and *q* were calculated in CODEML of PAML 4 package. CPS were identified in models M2a and M8 by Bayes Empirical Bayes, mixed-effect model evolution (MEME) and fixed-effect likelihood (FEL) analyses with posterior probabilities > 95%, codons with > 99% were bolded; codons identified in FEL were marked with superscribed F, in MEME with M. Codons in parentheses were detected in MEME only. NS indicates not significant. TS equals twice the difference of lnL values of alternative models vs. null models. d.f. = 2 for all LRTs. P values were obtained by comparison of TS to a χ^2 distribution using CHI2 in PAML 4 package.

Most codons identified as targets to positive selection in analyses of FEL, MEME and PAML models coincided with the ABS codon positions, a few next to ABSs as shown in Figure 2.

The genetic algorithm method found 30 potential breakpoints in the Japanese badger, 35 in the Asian, 40 and 29 in the European and Southwest Asian badgers, respectively. However, only one was significant for recombination in each badger species. These breakpoints were localized at different positions: positions 16, 13, 57 and 37 in Japanese, Asian, European and Southwest Asian badgers, respectively (Fig. 2).

DRB EXON 2 GENE PHYLOGENY

In the Bayesian phylogenetic analysis, which also involved all available *DRB* sequences of related carnivores from database (Fig. 3), the *Meles* exon 2 sequences do not make a single cluster but are interspersed among sequences in other taxa. Most are still included in a clade consisting exclusively of Mustelidae, except for five sequences from three *Meles* species that group with canid *DRB*s (*Mean-DRB*02-03*, *Meme-DRB*06-07* and *Mele-DRB*01*). Within the mustelid clade, *Meme-DRB*15* from the European badger and the other three Asian badger alleles are basal, followed by other alleles forming a large stepwise phylogeny. While some small, isolated sub-clades contain *Meles* alleles from only a single species (e.g. *Mean-DRB*04-06-07-09-11* and *Mele-DRB*04-09-10*), most *Meles* clades contain alleles from two or more *Meles* species. In some cases, *Meles* alleles are embedded in clades otherwise monophyletic for other mustelid genera (e.g. *Mele-DRB*19* and *Meme-DRB*09*, *-11* and *-14* in a *Mustela* clade) or even for a genus in another family (*Mean-DRB*02-03*, *Meme-DRB*06-07* and *Mele-DRB*01* in a *Canis* clade), or comprise a sister clade to alleles from other mustelid genera (e.g. *Meles* alleles forming a sister clade to alleles from *Taxidea* and *Gulo*). Thirteen of the alleles detected in the European badger, including presumable pseudogene alleles, were shared (i.e. identical in nucleotide sequence) by one to three of the other *Meles* species (Table 8).

DISCUSSION

MHC *DRB* DIVERSITY IN *MELES* SPECIES

The MHC genes are among the best genetic markers for quantifying adaptive genetic variation in wild animal populations of concern to conservation (Sommer, 2005). In other mammal taxa, the *DRB* genes have shown the highest variation among all MHC class II genes (Kriener *et al.*, 2001; Go *et al.*, 2002; Bowen *et al.*, 2006). Our study on MHC class II *DRB* exon 2 allowed a comparison of genetic variation among four *Meles* species for the first time, and a comparison with related taxa. The *Meles* MHC *DRB* exon 2 indeed showed higher variability than other studied mustelid species so far (Becker *et al.*, 2009; Sin *et al.*, 2012a; Nishita *et al.*, 2015). In addition, comparing to the previous data of Sin *et al.* (2012a) from seven European badger individuals, our study detected more alleles, more variable amino acid sites and higher genetic diversity. This can probably be attributed to a larger sample size and a broader distribution of samples (Norway, Bulgaria and Finland; Fig. 1, Tables 1 and 2). This disparity in results related to sample size suggests that more alleles will likely be detected in the Southwest Asian badger, for which our sample size was low. Furthermore, we found three to eight putative alleles per individuals. The result is not in agreement with previously reported results by Sin *et al.* (2012a, 2015). This might be due to the copy number variation of *DRB* gene (Freeman *et al.*, 2006), which is also one of the main factors for maintenance of MHC diversity among and/or within species (Bonhomme *et al.*, 2008; Mehta, Nonaka & Nonaka, 2009), even within populations (Llaurens, McMullan & van Oosterhout, 2012). It might be, however, also owing to the different methodology used in our and previous studies: Sin *et al.* (2012a) sequenced 12–26 clones (but 6–13 clones containing PCR-amplified fragment using genomic DNA as a template) per individual for seven European badgers. The probability of finding all gene copies of five loci in an individual is > 90% if 23 colonies per individual are sequenced (Eimes *et al.*, 2011) and 48 clones for 99.99% (Lenz *et al.*, 2009); subsequently, Sin *et al.* (2015) analysed a hundred number of European badgers applying reference strand-mediated conformation analysis (RSCA). To detect all of the possible alleles, RSCA method also needs allele library for that gene (Lenz *et al.*, 2009), which only four alleles are available for the badger species. Meanwhile, few number of alleles resulted from RSCA in hundreds of individuals in the UK population also indicates that this population more likely has lower allelic diversity among all *DRB* loci (i.e. same alleles in more than one locus) compared with the populations in our study. However, we could not rule out the demerits of gold standard genotyping method: cloning and sequencing. Thus, further studies are needed for the confirmation.

DRB exon 2 encoding the β1 domain is the most important region in presenting antigenic peptides to the immune system (Hughes & Yeager, 1998). In the 80 amino acid regions studied, half of the polymorphic sites were located in the 18 ABSs. The most polymorphic sites in the *Meles DR* β-chain were positions 9, 11, 13, 28, 30, 37, 57, 70, 71 and 74 in the β1 domain

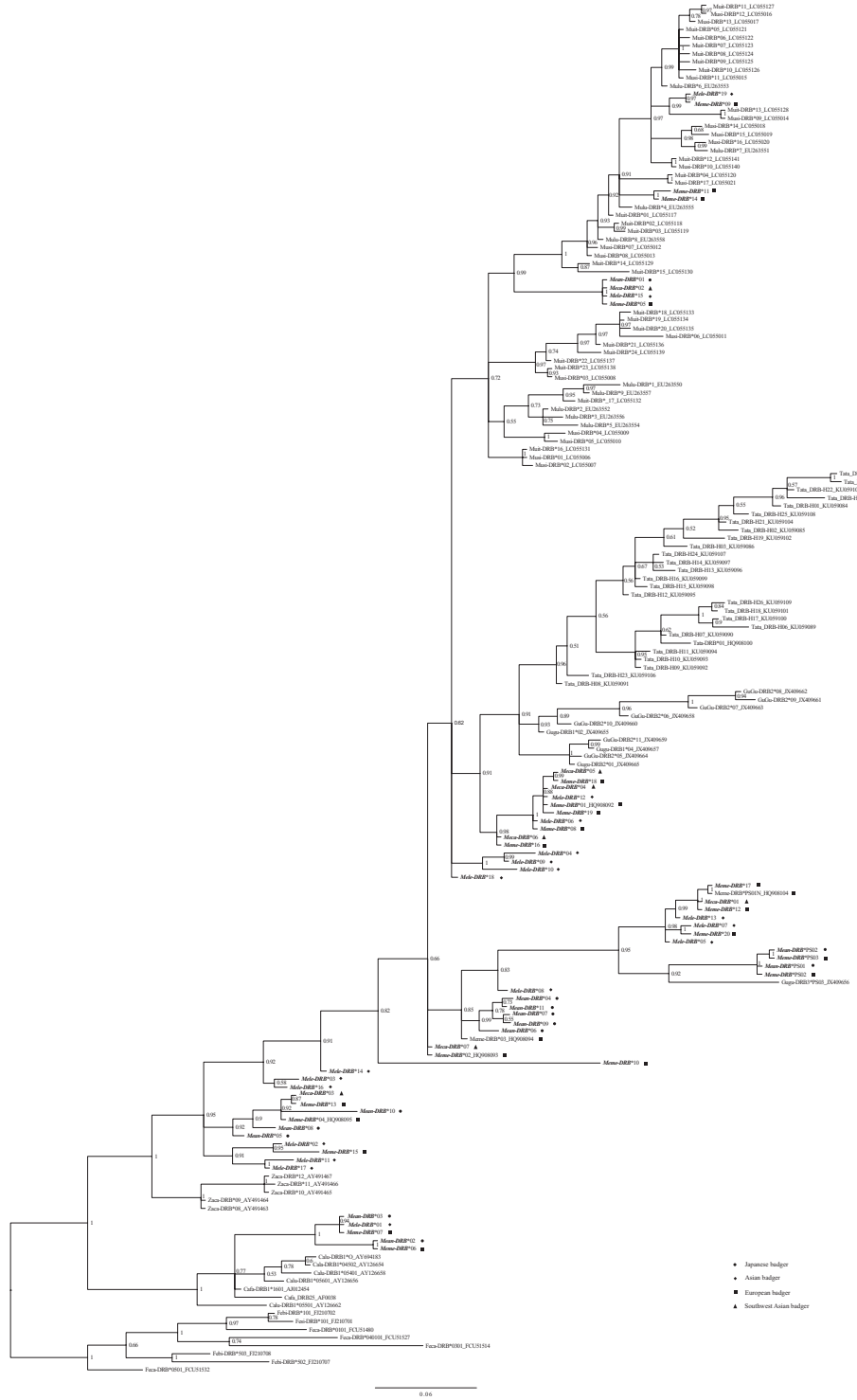

Figure 3. Bayesian phylogenetic tree of MHC class II *DRB* exon 2 from the four *Meles* species examined in this study and from other species in Canidae, Felidae and Mustelidae. The numbers near nodes are posterior probabilities. Alleles detected from European, Asian, Southwest Asian and Japanese badgers in this study are shown in bold font. For previously reported nucleotide sequences, GenBank accession numbers follow the allele names. Species abbreviations in the allele names are as follows: *Febi*, *Felis silvestris bieti*; *Feca*, *Felis catus*; *Fesi*, *Felis silvestris*; *Cafa*, *Canis familiaris*; *Cala*, *Canis latrans*; *Calu*, *Canis lupus*; *Zaca*, *Zalophus californianus*; *Gugu*, *Gulo gulo*; *Tata*, *Taxidea taxus*; *Muit*, *Mustela itatsi*; *Mulu*, *Mustela lutreola*; *Musi*, *Mustela sibirica*; *Meme*, *Meles meles*; *Mele*, *Meles leucurus*; *Meca*, *Meles canescens*; *Mean*, *Meles anakuma*.

Table 8. MHC class II *DRB* alleles shared between two or more of the *Meles* species included in this study

Meles meles	*Meles anakuma*	*Meles canescens*	*Meles leucurus*
*Meme-DRB*05*	*Mean-DRB*01*	*Meca-DRB*02*	*Mele-DRB*15*
*Meme-DRB*07*	*Mean-DRB*03*		*Mele-DRB*01*
*Meme-DRB*01*		*Meca-DRB*04*	*Mele-DRB*12*
*Meme-DRB*PS02*	*Mean-DRB*PS01*		
*Meme-DRB*PS03*	*Mean-DRB*PS02*		
*Meme-DRB*06*	*Mean-DRB*02*		
*Meme-DRB*12*		*Meca-DRB*01*	
*Meme-DRB*13*		*Meca-DRB*03*	
*Meme-DRB*18*		*Meca-DRB*05*	
*Meme-DRB*16*		*Meca-DRB*06*	
*Meme-DRB*02*		*Meca-DRB*07*	
*Meme-DRB*08*			*Mele-DRB*06*
*Meme-DRB*09*			*Mele-DRB*19*

PS, predicted pseudogenes containing deletions.

(Fig. 2). These positions are also highly polymorphic in other species (Suárez *et al.*, 2006; Becker *et al.*, 2009). The high variation in *DRB* exon 2 allows species or populations to present a large repertory of antigens, increasing their defence against parasitic infection (Hughes & Nei, 1992). Meantime, it might be basis to increase the fitness of the offspring, because individuals prefer mates that had the most dissimilar MHC genes to their own (Yamazaki *et al.*, 1976), as MHC class II-based mating preference was found in the European badgers, *M. meles*, in UK (Sin *et al.*, 2015).

Selection and recombination analysis

We estimated the d_N/d_S ratio (ω) to illustrate positive selection acting on MHC *DRB* exon 2. A high d_N/d_S ratio has been reported for ABS codons in other mustelid species (Becker *et al.*, 2009; Sin *et al.*, 2012a; Nishita *et al.*, 2015; Rico *et al.*, 2015, 2016), suggesting that positive selection acts on the *DRB* gene in these species. We likewise obtained values of $\omega > 1$ in the species-wise data sets, indicating positive selection on ABS codons. At the same time, values of $\omega < 1$ for non-ABS codons and overall indicated purifying selection (Table 6), by which non-synonymous substitutions that affect normal gene function are eliminated (Hughes & Yeager, 1998). The FEL, MEME analyses and PAML models likewise revealed some amino acids to be under positive selection (Fig. 2), which would contribute to high genetic diversity. In addition, recombination events between alleles at different loci with exons that encode ABSs could also contribute to allelic diversity in MHC genes (Bos & Waldman, 2006; Sin *et al.*, 2012b). MHC *DRB* gene variations shaped by recombination have been reported in birds (Minias *et al.*, 2016; Balasubramaniam *et al.*, 2017) and even in mustelid species of the least weasel, *Mustela nivalis* (Nishita *et al.*, 2017). In the least weasel, a large number of species/population-specific alleles were identified with one breakpoint recombination event. Likewise, we also found signs of single breakpoint recombination evens in *DRB* exon 2 for all of the four Eurasian badger species (Fig. 2). This indicates that the recombination may have increased the allelic diversity of MHC *DRB* genes in these species. Therefore, both the selection and recombination signatures in *DRB* exon 2 suggested that selection and recombination have maintained the high genetic and allelic variations observed in the four badger species (Tables 2–5).

Although selection and point mutation have been proposed as the major factor of driving and maintaining MHC polymorphism (Nei & Rooney, 2005), excessive MHC diversity in some mustelid species are generally caused by sexual selection (Sin *et al.*, 2015), diversifying (Rico *et al.*, 2015, 2016) and balancing selection (Sin *et al.*, 2012a, b; Nishita *et al.*, 2015, 2017), through competition between hosts and pathogens. Pathogen-driving selection could operate through heterozygote advantage, a rare-allele advantage and fluctuating selection (Spurgin & Richardson, 2010). The rare-allele advantages and/or fluctuating selection have been suggested as one of the selective forces in European badgers (Sin *et al.*, 2014). We found some population/region-specific alleles in European badgers (Table 2) and Asian badgers (Table 3): for instance, alleles *Meme-DRB*06-11* were observed only in Norwegian individuals, *Meme-DRB*14* and *16* only in Bulgarian individuals and *Meme-DRB*18–20* only in Finish individuals. *Meme-DRB*03*, which was found in UK individuals (Sin *et al.*, 2012a, 2014), was not found in any regions or species in this study, suggesting that it might be a UK-specific allele. The

alleles *Meme-DRB*01* and *04* observed in UK individuals (Sin *et al.*, 2012a), which were associated with pathogens pressures (Sin *et al.*, 2014), were also found in Bulgarian and Finish individuals, indicating that these individuals could be under the same pathogens pressures. Intriguingly and expectedly, *Meme-DRB*01* associated with the infection intensity of mustelid herpesvirus (MHV) that was detected all individuals examined (Sin *et al.*, 2014) was also identified in all Eurasian badgers but not in Japanese badgers (Table 8), implying that the occurrence of the MHV was probably common among Eurasian badger populations and/or species. In this regard, however, more individuals of the Japanese badger should be analysed to confirm this because we studied a very small number of samples in Japanese badgers (five individuals were obtained from three different island populations, see Table 1). Although there are no enough reports about pathogens in the Eurasian badger so far, it seems that some of pathogens hosted by these species are common among them and some are novel to populations or species (Hancox, 1980; Eisenberg *et al.*, 2013; Chiari *et al.*, 2014; Harasawa, Orusa & Giangaspero, 2014; Wodecka, Rymaszewska & Skotarczak, 2014; Moreno *et al.*, 2015; Hornok *et al.*, 2017). This is in some extent in accordance with species and population-specific alleles and with common alleles of them. However, further studies are still needed to investigate this and to find any associations between those pathogens and alleles/haplotypes in other *Meles* species or populations.

Trans-species polymorphism

The Eurasian badger belongs to the subfamily Melinae (Mustelidae, Carnivora), which contains only the genera *Meles* and *Arctonyx* (Marmi *et al.*, 2004). Paleontological and molecular data suggest that the radiation of *Meles* occurred in the Pleistocene. The divergence of the European and Asian badger lineage from the extinct *Meles thorali* lineage is estimated to have been at the Middle to Late Villafranchian boundary (1.8 Mya; Marmi *et al.*, 2006; Madurell-Malapeira *et al.*, 2011; Abramov & Puzachenko, 2013). The European, Southwest Asian, and North and East Asian badgers diverged separately since the end of Pliocene, at the onsets of glacial ages, with the Japanese badger diverging from continental Asian badgers 0.21–1.09 Mya (Marmi *et al.*, 2006) when sea levels decreased and land bridges formed, connecting the Japanese islands with the continent (Emery, Hiroshi & Beverly, 1971).

Several studies have attempted to resolve the phylogenetic relationships among Eurasian badgers, using the autosomal loci *ACTC*, *BGN*, *CFTR*, *CHRNA1*, *TS* and *TTR* (Del Cerro *et al.*, 2010); the Y-chromosome *SRY* gene (Tashima *et al.*, 2011a) and CAN-SINE (Tashima *et al.*, 2011b); and the mitochondrial control region (Marmi *et al.*, 2006; Del Cerro *et al.*, 2010; Tashima *et al.*, 2011a). Our reconstruction of the phylogenetic relationships among MHC class II *DRB* alleles in *Meles* and some other canid, felid and mustelid taxa do not help in further resolution of the interspecies relationships within *Meles*. In contrast to the other studies, sequences from the species we included from these three families did not form monophyletic groups by species (Becker *et al.*, 2009; Nishita *et al.*, 2015). Instead, most sub-clades within Mustelidae contained alleles from multiple species within genera (e.g. from various *Meles* species), alleles from species across genera (e.g. *Meles* sequences embedded within a clade otherwise containing *Mustela* alleles) or alleles from species across families (e.g. a clade containing alleles from three of the four *Meles* species embedded within a clade otherwise containing only alleles from the genus *Canis*). These are all instances of TSP, where ancestral alleles were retained across speciation events as allelic lineages, resulting in a pattern in which some alleles in one species more similar to some alleles in another species than to the other alleles in either species (Klein, 1987; Edwards & Hedrick, 1998; Klein *et al.*, 1998). Our results indicate that Canidae and Mustelidae retain MHC *DRB* alleles representing allelic lineages that contributed to MHC variability already in the common ancestor of these two families. Similarly, TSP has been reported in other mammals (Edwards & Hedrick, 1998; Goda *et al.*, 2010) and birds (Kohyama *et al.*, 2015a, b).

The ratio of non-synonymous to synonymous substitution rates at putative ABSs and positively selected sites detected in the PALM methods, the MEME and FEL analyses indicates positive selection in the *Meles DRB* genes. At the same time, TSP, such as shown by our phylogenetic analysis (Fig. 3), is generally attributed to balancing selection. In the vocabulary of MHC studies, these selective forces are inferred to have contributed to the maintenance of ancestral variation across long periods. Two things should be emphasized, however. First, as with several other animal groups (but not all), the results imply the presence of multiple copies of *DRB* genes in the *Meles* MHC complex, but so far, we have no information about the identity of particular alleles with different loci, or about the origin and maintenance of paralogous relationships of the loci through the phylogeny (did the allelic lineages at physical loci evolve independently through the species tree history). Second, the sharing of several identical alleles among several *Meles* species (13 alleles including two pseudogenes) suggest that there indeed has been little, if any, sequence evolution since the species

divergence in some 2 Mya (Marmi *et al.*, 2006); in one case, an allele was even shared by all four species. Therefore, the statistical inference of the action of the selective forces that moulded the diversity should principally refer to evolution through the pre-Pleistocene time scale > 2 Mya, and it may be unclear how that relates to the patterns of polymorphisms in more recent time. The divergence of Canidae and Mustelidae has been estimated at 123 Mya (Nyakatura & Bininda-Emonds, 2012); they shared an MHC *DRB* lineage, and examples of alleles shared between families have been reported in other mammals as well (Go *et al.*, 2002). Working out the genomic organization of *DRB* genes, and of its variability within and among species, will be essential for understanding the evolution of the intra- and trans-species polymorphisms of these genes across Carnivora.

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