Molecular Evolutionary Studies on the Major Histocompatibility Complex of Japanese and Russian Raccoon Dogs, *Nyctereutes procyonoides*

(日本およびロシアにおけるタヌキ ***Nyctereutes procyonoides*** の主要組織適合遺伝子複合体に関する分子進化学的研究)

PhD Dissertation

By

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Let all that I am praise the LORD;
may I never forget the good things he does for me.

Psalms 103:2

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Abstract

Raccoon dogs, *Nyctereutes procyonoides*, are native to East Asia, but have been introduced into western Russian and eastern Europe. They have been reported to be affected by various pathogens causing diseases, endoparasites, and viruses. Nevertheless, these pathogens have not prevented the raccoon dog's wide expansion in Far Eastern Asia and Europe. Recently, the major histocompatibility complex (MHC) genes have been widely analyzed to assess the immunological fitness and evolutionary adaptation in different populations, an essential key for conservation biology. Therefore, this study presents the allelic diversity and selection mechanism in the evolution of MHC class I and class II genes of Japanese and Russian raccoon dogs.

A total of 23 class II *DRB* alleles and a total of 48 novel MHC class I alleles were detected from 36 and 31 individuals of Japanese and Russian raccoon dogs, respectively. For both MHC class I and class II *DRB*, some alleles were found across the species' range, while others were geographically restricted. Similarly, the ratio of non-synonymous to synonymous substitution rates for codons at the predicted antigen-binding sites for both classes were greater than 1, indicating that raccoon dog's MHC class I and class II *DRB* genes have evolved under positive selection. Mixed effect model of evolution analysis and an algorithm to detect recombination showed positive selection sites at the amino acid level in both class I and class II molecules. Overall the results suggest that the diversity of MHC class I and class II *DRB*

were influenced and maintained by recombination, pathogen-driven positive selection, and geographical barriers.

For MHC class II *DRB*, the Bayesian phylogenetic tree revealed no evidence of trans-species polymorphism (TSP), but instead showed the monophyletic relationships within the Canidae clade. The lack of TSP suggests a possible influence of species-specific pathogens driven by their environments and their long historical divergence from other canids. For MHC class I genes, Bayesian phylogenetic trees showed no evidence of TSP with alleles from carnivore species in other families, but did detect TSP between raccoon dogs and the domestic dog, indicative of long term balancing selection in canids.

This study is presented into two chapters: Chapter 1 provides the results and interpretation of the MHC class II genes of Japanese and Russian raccoon dogs, which is already published in the Biological Journal of the Linnean Society last September 10, 2019 with (https://doi.org/10.1093/biolinnean/blz153). While, Chapter 2 presents the results and interpretation of the MHC class I genes of Japanese and Russian raccoon dogs, which is currently under peer review.

General Introduction

Nyctereutes procyonoides, commonly known as the raccoon dog, is a medium-sized carnivore that belongs to the family Canidae. The distribution of this species extends from northern Vietnam, through eastern China, to Far Eastern Russia and Mongolia. In Japan, the species occurs throughout the four main islands (Hokkaido, Honshu, Shikoku, Kyushu) (Kauhala & Saeki, 2016). This animal has become widespread in northern and eastern European countries as it was introduced for fur production in the middle of the 20th century. Currently, *Nyctereutes procyonoides* consist of six subspecies namely: *albus*, *koreensis*, *orestes*, *procyonoides*, *ussuriensis*, and *viverrinus* (Kauhala & Saeki, 2016). Morphological and genetic data suggests that the Japanese raccoon dogs can be classified into two more subspecies: *N. p. viverrinus* on Honshu, Shikoku, Kyushu, and many small island, and *N. p. albus* found only in Hokkaido island; and these are also speculated to be a different species from the continental populations (Ward *et al*., 1987; Kim *et al*., 2013, 2015).

The raccoon dog helps to mitigate insect pests and facilitate seed dispersal due to its omnivorous feeding behavior. It is affected by various pathogens causing diseases (Singer *et al*., 2009; Qui *et al*., 2009) and endoparasites (Al-Sabi *et al*., 2013; Laurimaa *et al*., 2016). It has also been reported to be a carrier of viral infectious diseases such as Asian tick-born meningoencephalitis virus, canine distemper, paratyphoid fever, anthrax, tuberculosis, and rabies virus. The raccoon dog is also included among the most invasive species in European

countries and is the main vector of rabies, alveolar echinococcosis, and sarcoptic mange, which are very dangerous to human health (Kauhala & Kowalczyk, 2011). In Japan, they have been reported to be affected with canine distemper virus infection in Wakayama Prefecture (Suzuki *et al*., 2015). Among the Canidae, the raccoon dog's adventurous nature, omnivorous behavior, ancestral lineage of canids, and high percentage of intracellular and extracellular pathogens makes it ideal to study selection on the major histocompatibility complex (MHC) in non-model canid species.

The MHC genes are widely studied because of their importance in the evolutionary ecology as well as their role in the immune response to pathogens (Piertney &Oliver, 2006; Sommer, 2005). The MHC is subdivided into three major subfamilies, classes I, II, and III. MHC class II proteins are expressed in antigen presenting cells which bind peptides from extracellular pathogens to helper T-cells(CD4+) (Hughes & Yeager, 1998). MHC class I proteins are expressed in all nucleated cells and essential in the defense against intracellular pathogens such as virus proteins by binding and presenting the degraded intracellular peptides from them to cytotoxic T-cells (CD8+) (Sommer, 2005). Generally, MHC is a good model in understanding the evolutionary processed of vertebrates as the genes are exposed to constant selective pressure in response to the rapidly changing immune response of a population (Hedrick, 1994).

The genetic diversity among MHC genes is influenced by various evolutionary factors, including variations in copy number (Piertney &Oliver, 2006), balancing selection (Sommer, 2005), and birth-and-death evolution (Nei *et al*., 1997). Studies on MHC gene evolution in mammals have been reported in both class I (Zhu *et al*., 2013; Sin *et al*., 2012; Abduriyim *et al*., 2019; Kuduk *et al*., 2012) and class II genes (Lukas *et al*., 2004; Wagner, 2003; Morris, 2009; Ploshnitsa *et al*., 2012; Nishita *et al*., 2015, 2018; Marshall *et al*., 2016; Abduriyim *et al*., 2017; Amaike *et al*., 2018; Saka *et al*., 2018). Their allelic diversity has been greatly

influenced by recombination and positive selection. However, in the family Canidae, only the *Canis* genus has been well studied and evolutionary studies of MHC in other non-model genera of the Canidae is scarce.

With this information, this study investigated the allelic diversity and selection mechanism of evolution of MHC genes in Japanese and Russian raccoon dogs. Specifically, in Chapter 1 of this dissertation, the variation of MHC class II *DRB* gene across different geographical populations was examined, with the goal of understanding the selection that has shaped the evolution of MHC class II *DRB* in raccoon dogs. In Chapter 2, the allelic diversity was characterized and the patterns of MHC class I evolution especially in the α1 and α2 domains, which form a peptide binding groove in raccoon dogs from Japan and Russia, are discussed.

Chapter 1

Molecular evolution of MHC class II *DRB* gene in Japanese and Russian raccoon dogs

INTRODUCTION

The raccoon dog is affected by various pathogens causing diseases (Singer *et al*., 2009; Qi *et al*., 2009) and endoparasites (Al-Sabi *et al*., 2013; Laurimaa *et al*., 2016). Nevertheless, these pathogens and parasites have not prevented the raccoon dog's wide expansion in Far Eastern Asia and Europe. To understand the phylogeographical history of raccoon dogs, population genetic patterns have been investigated using neutral gene markers (Ward *et al*., 1987; Pitra *et al*., 2010; Kim *et al*., 2013, 2015; Drygala *et al*., 2016; Paulauskas *et al*., 2016; Hong *et al*., 2018); however, the role of adaptive genetic loci has barely been explored in this species.

Genes in the MHC are the most polymorphic in vertebrates (Klein, 1987). Among the MHC genes, class II *DRB* exon 2 has been widely studied across vertebrates, due to higher levels of polymorphism than in other MHC loci (Hughes & Yeager, 1998; Sin *et al*., 2012); in terrestrial mammals, data have been reported in primates (Lukas *et al*., 2004) and other carnivores (Wagner, 2003; Morris, 2009; Ploshnitsa *et al*., 2012; Nishita *et al*., 2015, 2018;

Marshall *et al*., 2016; Abduriyim *et al*., 2017; Amaike *et al*., 2018; Saka *et al*., 2018; Hosotani *et al.*, 2020). Yachimori *et al*., (1997) reported two MHC *DRB* alleles from raccoon dogs using restriction fragment length polymorphisms (RFLPs), but they did not explain the evolution of raccoon dog MHC genes. In this chapter, the MHC class II *DRB* gene from raccoon dogs from Japan and Russia was sequenced, to examine variation in *DRB* diversity across the different geographical populations, with the goal of understanding how selection has shaped the evolution of the MHC class II *DRB* gene in raccoon dogs.

MATERIALS AND METHODS

Samples and DNA extraction

Blood and tissue samples (muscles or kidney) were obtained from a total of 36 individuals: 32 samples were from native individuals (28 from Japanese islands and four from Far Eastern Russia), and four were from an introduced population in Western Russia (Figure 1; Supplementary table 1). All samples came from road kills or legal hunting. Total DNA was extracted from all samples with a DNeasy Blood and Tissue Kit (Qiagen), and stored at 4°C.

Polymerase Chain Reaction Amplification

A 284-bp fragment of MHC class II *DRB* exon 2 was amplified by polymerase chain reaction (PCR) using forward primer NP030 (5'-ATCCTCTCTCTGCAGCACATTTC-3') and reverse primer NP032 (5'-TCGCCGCTGCACCGTGAAGCTCTC-3') (Yachimori *et al*., 1997). PCR amplifications were performed in 25-µL reaction volumes, each containing 0.625 U PrimeSTAR GXL (Takara Bio), 5 µL of 5× PrimeSTAR GXL buffer, 2 µL of Dntp MIXTURE (2.5 Mm EACH), 0.3 µL (25 pmol/µL) of each phosphorylated forward and reverse primer, and 1 µL of DNA extract. Reaction conditions in a Takara Thermal Cycler Dice Touch were 94 °C for 2 min; 30 cycles of 98 °C for 10 s, 60 °C for 15 s and 68 °C for 15 s, and a final

hold at 4 °C. PCR products were electrophoresed on a 3% agarose gel and visualized with ethidium bromide fluorescence under UV illumination.

Figure 1. Sampling locations for native raccoon dogs examined in this study. Number (*N*) in parentheses indicates sample sizes.

Cloning and Sequencing

PCR products were purified with a QIAquick PCR Purification Kit (Qiagen), ligated into pBluescriptII SK+ vector (Agilent Technologies), and transformed into *Escherichia coli* JM109 competent cells. Blue/White screening was conducted to select clones containing PCR amplicons. An average of 25 positive colonies per individual were cultured individually overnight at 37 °C in a 2 × YT medium broth, and plasmids were purified with NucleoSpin Plasmid EasyPure spin columns (Macherey-Nagel). Plasmid inserts were sequenced by using M13 forward and reverse primers (Messing, 1983). A BigDye Terminator v.3.1 Cycle Sequencing Kit (Life Technologies) and an ABI 3730 DNA automated sequencer. Sequences obtained were aligned by using MEGA 7 (Kumar *et al*., 2016).

Sequences were determined to be MHC *DRB* variants according to the criteria set by nomenclature rules for the dog major histocompatibility system (Kennedy *et al*., 2000). BLAST-N searches (Altschul *et al*., 1990) of the NCBI GenBank database were conducted to confirm that sequences were MHC class II *DRB* exon 2 or previously reported pseudogenes. All sequences identified were named with the prefix *Nypr-DRB** followed by a number according to the standard rules for MHC nomenclature in non-human species (Klein *et al*., 1990).

Data Analysis

*Nypr-DRB*s from each geographical region were analyzed by calculating Tajima's *D* (Tajima, 1989), to identify whether the DNA sequence evolved by directional or balancing selection. To determine whether antigen binding sites (ABSs), non-ABSs, and the overall amino acid sequence were under positive selection. The ratio between non-synoymous (d_N) and Synonymous (d_S) substitution rates per nucleotide sites was calculated using the Nei–Gojobori method (Nei & Gojobori, 1986) with Jukes–Cantor correction (Jukes & Cantor, 1969). The ratio d_N/d_S (ω) was tested for significant deviations from neutrality by using the codon-

based Z-test in MEGA 7 (Kumar *et al*., 2016). ABS positions were identified based on ABSs in the human HLA-DR molecule (Bondinas *et al*., 2007). In addition, mixed effects model evolution (MEME) and genetic algorithm for recombination detection (GARD) implemented in DATAMONKEY (Murrell *et al*., 2012) were used to detect amino acid positions that have undergone positive selection or contain recombination breakpoints, respectively.

A Bayesian phylogenetic analysis was conducted to determine the relationships among *DRB* allelic sequences obtained in our study, and from GenBank for selected felids, other canids, and humans. A phylogenetic tree was reconstructed by using MrBayes v.3.2.6 (Ronquist & Huelsenbeck, 2003) with 120 million generations sampled every 100 generations and with the first 25% of generations discarded as burn-in. The optimal models of nucleotide substitution selected by KAKUSAN4 (Tanabe, 2007) under Bayesian Information Criterion 4 (sample size = 116) were HKY85_Gamma (First codon position) and GTR_Gamma (second and third codon positions). Parameter values sampled from the chains were examined for convergence using Tracer v.1.6 (Rambaut *et al*., 2004).

RESULTS

Allelic Diversity

In all, 829 clones of MHC class II *DRB* exon 2 from the 36 raccoon dogs (average, 25 clones per individual) were sequenced. The obtained sequences were 237 bp long without the primer sequences, encoding 79 amino acids and comprising around 83% of the β1 domain (285 bp). In all, 31 nucleotide sites (13%) in the 237-bp region of *DRB* exon 2 were polymorphic. Within the deduced 79 amino acid sequence, 11 out of 20 polymorphic sites were located in the 19 ABSs, and amino acid position 11 was the most polymorphic site in the β1 domain (Figure 2).

Of the 23 *DRB* alleles identified (Fig. 2), 22 (*Nypr-DRB*03–*24*) were novel. The number of alleles found for each individual ranged from one to four (Table 1), which suggests that the Eurasian raccoon dog has at least two MHC class II *DRB* diploid loci.

Table 1. Distribution of MHC class II *DRB* alleles among geographical populations of raccoon dogs in Russia and Japan

Region	Sample name	*Nypr-DRBs*																						Number of identified alleles
		**01*	**03*	**04*	**05*	**06*	**07*	**08*	**09*	**10*	**11*	**12*	**13*	**14*	**15*	**16*	**17*	**18*	**19*	**20*	**21*	**22*	**23*	**24*	
Hokkaido	HKD_1	+																							1
	HKD_2	+																							1
	HKD_3	+	+	+	+																				4
	HKD_4	+																							1
	HKD_5	+		+																					2
Northern Honshu	NHS_1	+	+																						2
	NHS_2	+			+																				2
	NHS_3		+			+																			2
	NHS_4	+					+																		2
	NHS_5		+		+																			+	2
Central Honshu	CHS_1	+												+											2
	CHS_2						+	+								+									3
	CHS_3																	+							1
	CHS_4	+						+																	2
	CHS_5				+			+												+					3
Southern Honshu	SHS_1	+												+											2
	SHS_2	+					+																		2
	SHS_3													+											1
	SHS_4													+					+						2
	SHS_5	+																							1
Shikoku	SHKK_1				+			+	+											+					4
	SHKK_2									+	+														2
	SHKK_3											+	+												2
	SHKK_4							+		+															2
Kyushu	KYSH_1														+			+							2
	KYSH_2	+													+										1
	KYSH_3	+			+											+									2
	KYSH_4							+							+										2
Far Eastern Russia (Native)	RSSN_1			+																				+	2
	RSSN_2		+	+	+																+				4
	RSSN_3			+	+																		+		3
	RSSN_4				+																		+		2
Western Russia (Introduced)	RSSI_1			+																	+	+			3
	RSSI_2			+																					1
	RSSI_3	+		+																					2
	RSSI_4			+				+																	2

No alleles were shared by all regions. Except for Hokkaido, each region showed one or more alleles restricted to that region. Allele *Nypr-DRB*06* was found in northern Honshu, *Nypr-DRB*17* only in central Honshu, *Nypr-DRB*19* only in southern Honshu, *Nypr-DRB*14* and **15* only in Kyushu, *Nypr-DRB*24* only in Far Eastern Russia, and *Nypr-DRB*22* only in western Russia. The highest number of region-specific *Nypr-DRB* alleles was observed on

Shikoku, with five unique alleles (*Nypr-DRB*09–*13*) not detected elsewhere. Allele *Nypr-DRB*01* was the most frequently shared between Japanese and Russian populations, and was detected in all five individuals sampled from Hokkaido. *Nypr-DRB*04* was shared only by the populations in Hokkaido and Russia. Similarly, *Nypr-DRB*07* was detected only in the three regions (northern, central, and southern) of Honshu.

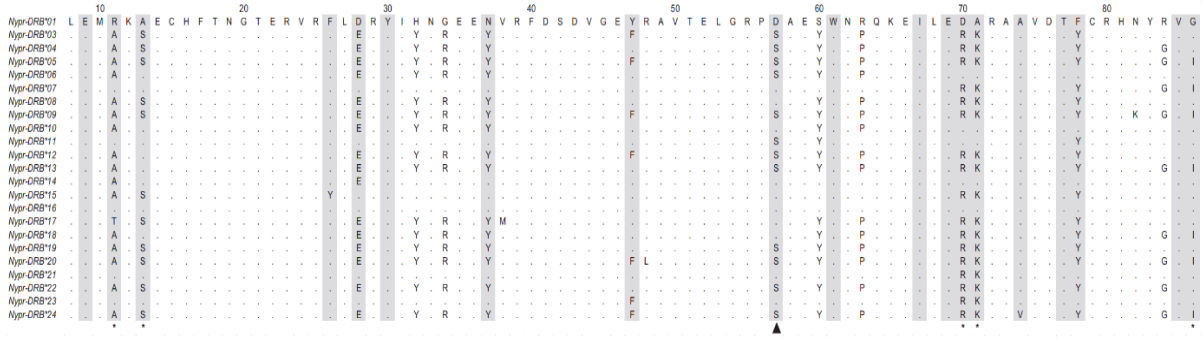

Figure 2. Amino acid sequences deduced from partial sequences of MHC class II *DRB* exon 2 from raccoon dogs. Identity with the sequence from *Nypr-DRB*01* is indicated by dots. Numbers above the sequences indicate the amino acid position in the β1 domain. Grey shading indicates antigen binding sites under positive selection, as calculated by the genetic algorithm for recombination detection (GARD).

Selection on MHC class II *DRB* exon 2

Tajima's *D* value for each region and the values for Japan and Russia overall were significantly positive ($P < 0.05$) (Table 2), indicating that balancing selection has acted on *Nypr-DRB* loci in both the native and the introduced raccoon dog populations. Tajima's *D* values were highest for Japan overall ($D = 4.003$), northern Honshu ($D = 4.238$) and for all native populations overall ($D = 4.134$). The value for the introduced population in western Russia was also significantly positive ($D = 1.780$, $P < 0.05$).

Table 2. Sequence diversity of MHC class II *DRB* exon 2 in raccoon dogs by sampling region

Region	*m*	*S*	*Ps*	θ	π	Tajima's *D*	*P*	Number of distinct alleles
Overall Japan (Native)	629	30	0.127	0.018	0.046	4.003	0.019	
Hokkaido	113	24	0.101	0.02	0.049	1.083	0.014	0
Northern Honshu	109	25	0.105	0.02	0.049	4.238	0.023	1
Central Honshu	94	26	0.11	0.021	0.039	2.512	0.031	1
Southern Honshu	121	23	0.097	0.018	0.032	2.235	0.017	2
Shikoku	98	25	0.105	0.02	0.037	2.394	0.02	5
Kyushu	94	26	0.11	0.021	0.04	2.584	0.024	2
Overall Russia	200	26	0.11	0.019	0.033	2.107	0.015	
Far Eastern Russia (Native)	93	20	0.084	0.017	0.035	3.24	0.024	1
Western Russia (Introduced)	107	21	0.089	0.017	0.027	1.78	0.02	1
Overall (without introduced)	722	30	0.127	0.018	0.046	4.134	0.017	

m, number of sequences; *S*, number of segregating sites; *Ps*, number of segregating sites divided by the total number of sites; θ, 4Nμ for an autosomal gene for a diploid organism; π, nucleotide diversity; *D*, Tajima's test statistics; *P*, significance value for Tajima's *D*.

Values of d_N for ABSs, non-ABSs, and all codons in *Nypr-DRB* alleles from native and introduced populations were greater than d_S, with $\omega > 1$ (Table 3), indicating that these alleles evolved under positive selection. Especially noteworthy, ω values were more than 2 at ABSs for both native and introduced populations. A MEME analysis provided evidence of positive selection at the amino acid level. Five amino acid positions (asterisks, Figure 2) showed evidence of positive selection, and all of these positions coincided with codons contributing to ABSs inferred from the human HLA-DR molecule. Finally, recombination analysis using GARD showed a single recombination breakpoint (triangle, Figure 2) located at the amino acid positions 57 in the β1 domain.

Table 3. Ratio (ω) of non-synonymous (d_N) to synonymous (d_S) substitution rates for antigen binding sites (ABSs), non-ABSs and all amino acids in MHC class II *DRB* alleles from native and introduced populations of raccoon dogs

Population	Position	Number of codons	d_N	(±SD)	d_S	(±SD)	$\omega = (d_N/d_S)$
Native	ABS	17	0.231	(±0.076)	0.096	(±0.064)	2.406
	Non-ABS	62	0.019	(±0.008)	0.013	(±0.011)	1.462
	Overall	79	0.059	(±0.014)	0.03	(±0.014)	1.967
Introduced	ABS	17	0.258	(±0.092)	0.095	(±0.062)	2.716
	Non-ABS	62	0.025	(±0.010)	0.023	(±0.016)	1.087
	Overall	79	0.067	(±0.018)	0.037	(±0.018)	1.811

Trans-species Polymorphism

The Bayesian phylogenetic tree, including *Nypr-DRB*s and representative sequences from felids, other canids and humans, showed all *Nypr-DRB* alleles we identified formed a monophyletic group within a larger canid clade (Figure 3). The *Nypr-DRB* alleles identified thus show no evidence of trans-species polymorphism. The *Nypr-DRB* clade in turn formed an unresolved polytomy with individual sequences and small clades from the red fox (*Vuvu-DRB*s) and arctic fox (*Vula-DRB*s).

DISCUSSION

DRB allelic diversity in raccoon dogs

Overall, the MHC class II *DRB* alleles showed high variation. The high genetic variation in *DRB* exon 2 is beneficial because it provides a broader spectrum of pathogens that an organism can be familiar with and defend itself against (Jeffery & Bangham, 2000). A minimum of one and a maximum of four putative alleles for each individual are found, indicating that raccoon dogs have at least two functional *DRB* loci. The result is not in agreement with its closest relative, the red fox (*Vulpes vulpes*), in which only one *DRB* locus has been reported (Ploshnitsa *et al*., 2012). Variation in copy number among and/or within species has also been reported across other organisms, such as in mustelids (Nishita *et al*., 2015; Abdurayim *et al*., 2017) and birds (Minias *et al*., 2018). According to Nei *et al*., (1997), gene duplication is thought to produce variation in the copy number of loci, and it is believed to be a driving force in obtaining high allelic diversity in the MHC.

Among the 23 *Nypr-DRB* alleles we identified, 22 were novel. From RFLP data, Yachimori *et al*., (1997) reported only two *DRB* alleles and only one of these, *Nypr-DRB*01*, was detected in our study. The higher number of alleles we obtained could have been due to

Figure 3. Bayesian phylogenetic tree for MHC class II *DRB* exon 2 from raccoon dogs and other representatives from Felidae and Canidae. Numbers near nodes are posterior probability values. The DDBJ/EMBL/GenBank accession number of each nucleotide sequence is shown followed by the allele name. Abbreviations for species names: Nypr, *Nyctereutes procyonoides*; Vuvu, *Vulpes vulpes*; Vula, *Vulpres lagopus*; Cafa, *Canis lupus familiaris*; DRB1, DLA-DRB and DLA-DRB1, *Canis* sp.; Urli, *Urocryon littoralis*; Prbe, *Prionailurus bengalensis*; FLA-DRB1, *Acinonym jubatus*; Lyru, *Lynx rufus*; Lyly, *Lynx lynx*; Lepa, *Leopardus pardalis*, Leti, *Leopardus tigrinus*; Pale, *Panthera leo*; Pati, *Panthera tigris*; HLA, human.

higher sample sizes, more sampling localities or methodology (using PCR and cloning rather than RFLP). Moreover, each geographical population except for Hokkaido had at least one unique *DRB* allele, with the Shikoku population in Japan having the highest number of regionally restricted alleles. Locally restricted alleles could have been maintained by native pathogen-driven balancing selection (Hughes & Yeager, 1998). On the other hand, alleles shared among some regions could be affected by widespread pathogens. Furthermore, among the nine *Nypr-DRB*s found in Russia, two alleles, *Nypr-DRB*04* and *Nypr-DRB*21*, were shared between Far Eastern and Western Russia. *Nypr-DRB*04* was high in frequency among individuals in both populations, reflecting the origin of the Western population from the Far Eastern population. Individuals of Hokkaido shared three alleles (*Nypr-DRB*03–*05*) with Far Eastern Russia, which could have been facilitated by the formation of land bridges between these regions during the last glacial maximum (LGM) (McKay, 2012).

DRB selection mechanism in raccoon dogs

Tajima's *D* values were significantly greater than zero in all represented populations, suggesting that balancing selection has influenced the evolution of *DRB* alleles in all these groups. For both native and introduced populations (Table 3), ω was greater than 1; values were nearly 2–3 times as high for codons involved in ABSs than for other sites (non-ABSs), but were close to a value of 2 overall for both types of population. Strong positive selection on ABSs has also been observed in species closely related to raccoon dogs, including the red fox (ω = 2.72, Amaike *et al*., 2018; ω = 2.7, Marshall *et al*., 2016), Arctic fox (*Vulpes lagopus*) (ω = 3.3, Ploshnitsa *et al*., 2012), and domestic dog (*Canis lupus familiaris*) and wolf (*Canis lupus*) (ω = 2.8 and 2.6, respectively, Seddon & Ellengren, 2002). The values of ω = 2.4 and 2.7 for ABSs in native and introduced populations, respectively, were similar to the values in the previous studies. The results from the MEME and GARD analyses revealed five codons

under positive selection and one recombination breakpoint, respectively. Positive selection at the amino acid level could have contributed to maintaining the high allelic diversity of MHC *DRB* exon 2 in raccoon dogs.

Trans-species polymorphism in raccoon dogs

Trans-species polymorphism (TSP) among MHC genes is a common pattern reported among Canidae and Felidae, having been observed in studies on the Arctic fox (Ploshnitsa *et al*., 2012), red fox (Marshall *et al*., 2016; Amaike *et al*., 2018), wolf (Hedrick *et al*., 2002), domestic dog (Wagner, 2003), bobcat (*Lynx rufus*; Marmesat *et al*., 2017) and lynx (*Felis lynx*; Marmesat *et al*., 2017). In contrast, this study observed no trans-species polymorphism for the *Nypr-DRB*s that were analyzed.

The absence of TSP in raccoon dog *DRB*s indicates that this species diverged from other canids long enough ago that no ancestral alleles have been retained. The genus *Nyctereutes* appeared about 9 Mya (Kauhala & Saeki, 2004), and most species in the genus have gone extinct (Kurten, 1968; Tedford & Zhangxiang, 1991; Wang & Tedford, 2008). As the only surviving species in the genus, *N. procyonoides* is phylogenetically distant from other canid species, which may account for the lack of trans-species polymorphism. Osborne *et al*. (2013) reported a similar case in the New Zealand sea lion (*Phocarctos hookeri*), which shows high *DRB* variation but for which there is no evidence of trans-species polymorphism. Osborne *et al*., (2013) also discussed that the absence of trans-species polymorphism was perhaps due to an epizootic event that led to the selection of few ancestral alleles from which the current diversity arose. Phylogenetic analyses of MHC *DRB* alleles from extinct *Nyctereutes* species, amplified from fossil material, should be conducted to seek earlier traces of trans-species polymorphism in *Nyctereutes DRB*s.

TSP has been noted for MHC alleles across considerable phylogenetic distance (Klein *et al*., 1998) – across genera of Felidae, across Mustelidae and Ursidae, and across Mustelidae and Canidae – and phylogenetic distance alone may not explain a failure to detect it in the raccoon dog. This species might have experienced distinct pathogens over a long periods of time, compared to other canids, as its environment and ecological niche differ from those other canids. Although the phylogenetic tree showed the *Nypr-DRB* alleles in the raccoon dog to be most closely related to alleles from the Arctic fox and red fox, raccoon dogs do not significantly compete for food or habitat with these foxes (Kauhala & Kowalczyk, 2011), and may well come into contact with different pathogens. Finally, the alleles detected may have been limited by the PCR primer set used, and other primers should be explored to confirm the lack of TSP in *Nypr-DRB*s.

The presence of a single *Nypr-DRB* clade suggests that these identified *Nypr-DRB* alleles came from a single ancestral sequence. Two lineages were observed in the *Nypr-DRB* clade, with *Nypr-DRB*10* and *Nypr-DRB*14* as basal alleles. Neither of the basal alleles is the most frequent, and they are restricted to Shikoku, and central and southern Honshu, respectively. In this study, the central Honshu and Shikoku populations had the greatest allelic variation among the regions, with a total of eight alleles. These data suggest that raccoon dogs have inhabited these areas for a long time, and may have expanded their range from here to the northern regions. Dobson (1994) concluded that *N. procyonoides* could have originated in Hondo (Honshu, Shikoku, and Honshu) based on records of Middle Pleistocene fossils of *N. procyonoides* in Hondo (Kawamura, 1991), and its absence from the extensive Early and Middle Pleistocene mammal assemblages in China (Han & Xu, 1965). However, Dobson & Kawamura (1998) later reasoned that *N. procyonoides* may have arisen from *N. sinensis*, a Pliocene to Middle Pliocene fossil species from China that invaded Hondo via land bridges that connected Hondo and mainland China. Because this study is limited to the MHC *DRB* gene

from Japanese and Russian raccoon dogs, further investigation using different genetic markers, analysis of samples from other East Asian regions and more archaeological evidence should be explored to fully understand the evolutionary history of *N. procyonoides* in Far East Asia.

Chapter 2

Molecular evolution of MHC class I genes in Japanese and Russian raccoon dogs

INTRODUCTION

In recent years, the molecular evolution of MHC class I and class II has been studied extensively; however, genes in MHC class I genes are more difficult to genotype than those in class II (Venkataraman *et al*., 2007). The class I protein consists of a single amino acid chain (α chain) encoded by seven exons. Among the seven exons, the most polymorphic are exon 2 and 3, which encode amino acids involved in forming peptide-binding grooves in the extracellular α1 and α2 domains, respectively (Bjorkman *et al*., 1987).

The genetic diversity among MHC genes is influenced by various evolutionary factors, including variations in copy number (Piertney & Oliver, 2006), balancing selection (Sommer, 2005), and birth-and-death evolution (Nei *et al*., 1997). Studies on MHC I evolution in mammals, including the giant panda (Zhu *et al*., 2013), four badger species (Sin *et al*., 2012; Abduriyim *et al*., 2019), and the brown bear (Kuduk *et al*., 2012) indicate that their allelic diversity has been greatly influenced by recombination and positive selection. Furthermore, in the badger, the α1 and α2 domains appear to have undergone different evolutionary histories

(Abduriyim *et al.*, 2019; Sin *et al.*, 2012). In the family Canidae, only the genus *Canis* has been well studied and studies of MHC class I evolution for non-model genera are scarce.

The raccoon dog (*Nyctereutes procyonoides*; Canidae), carries infectious diseases such as Asian tick-borne meningoencephalitis, canine distemper, paratyphoid fever, anthrax, tuberculosis, and rabies. This species is among the most invasive mammals in European countries, where it is the main vector for rabies, alveolar echinococcosis, and sarcoptic mange, which are dangerous to human health (Kauhala & Kowalczyck, 2011). In Japan, raccoon dogs infected with canine distemper virus have been reported from Wakayama prefecture (Suzuki *et al*., 2015). The raccoon dog's omnivorous mode of feeding, and high prevalence of intracellular pathogens makes it ideal for studying selection on MHC class I in a non-model canid species. The aim of this chapter was to characterize the allelic diversity of the MHC class I α1 and α2 domains and their patterns of evolution in raccoon dogs from Japan and Russia.

MATERIALS AND METHODS

Samples and DNA extraction

In total, 31 blood or tissue samples from raccoon dogs were used (Supplementary Table 2), 23 from the Japanese Islands, four from Far Eastern Russia, and four from Western Russia (Figure 4). Some samples were common to those in the MHC class II (Chapter 1) study by Bartocillo *et al.* (2020). DNA from all samples was extracted by using a DNeasy Blood and Tissue Kit (Qiagen) and then stored at 4 °C until use.

Figure 4. Sampling location for native raccoon dogs examined in this study. Number (*N*) in parentheses indicates sample sizes.

Polymerase Chain Reaction (PCR)

MHC class I DLA 88 exon 2 N forward primer 5'-TCTCACCCGTCGGCTCCGCAG-3' and MHC class I DLA 88 exon 3 reverse primer 5'-AGGCGAGATCGGGGAGGC-3' (Wagner *et al*., 2000) were used for PCR amplification. Reactions were performed in 25-µL

reaction volumes, each comprising of 0.625 U PrimeSTAR GXL (Takara Bio), 5 µL of 5× PrimeSTAR GXL buffer, 2 µL (25 pmol/µL) of each phosphorylated forward and reverse primers, and 1 µL of DNA extract (60 – 100 ng of genomic DNA). Reaction conditions in the Takara Thermal Cycler Dice Touch were 98 °C for 2 minutes; 30 – 35 cycles of 98 °C for 10 s and 68 °C for 45 s; with a final extension of 68 °C for 10 minutes, with a final hold of 4 °C. PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide florescence under UV illumination, and then purified by using QIAquick PCR Purification Kit (Qiagen).

Cloning and Sequencing

Cloning and sequencing were done as described by Bartocillo *et al*. (2020) (Chapter 1). On average, 27 positive clones per individual were sequenced using M13 forward and reverse primers. Sequences were aligned and checked using MUSCLE in MEGA 7 (Kumar *et al*., 2016). DnaSP v.5 (Librado & Rozas, 2009) was used to determine identical sequences among all sequences obtained. Sequences were then identified as bona-fide variants based on the criteria associated with the nomenclature rules for dog DLA (Kennedy *et al*., 2000). Accepted bona-fide sequence in this study was defined as having an identical sequences derived from at least two raccoon dog individuals or from two separate PCR reactions, while unique single sequences were rejected as chimeras. BLAST-N searches (Altschul *et al*., 1990) of the NCBI GenBank were conducted to identify identical or homologous sequences. Bona-fide true alleles were named according to the rules for MHC nomenclature in non-human species (Klein *et al*., 1990) in descending order of frequency of occurrence. Nucleotide sequences obtained in this study were deposited in the DNA Databank of Japan (DDBJ) under accession numbers LC554227-LC554274.

Data Analysis

Putative functional *Nypr-MHC1* alleles were analyzed by calculating Tajima's *D* (Tajima, 1989) to determine whether the DNA sequences evolved by directional or balancing selection. To determine whether the antigen-binding sites (ABSs), non-ABSs, and the overall amino acid sequences of the α1 and α2 domains were under positive selection, ratios between non-synonymous (d_N) and synonymous (d_S) substitution rates per nucleotide sites were calculated by using the Nei-Gojobori method (Nei & Gojobori, 1986) with the Jukes-Cantor correction (Jukes and Cantor, 1969). ABS positions were inferred from the ABSs in human MHC proteins (Bjorkman *et al*., 1987). The mixed effect model of evolution (MEME) and genetic algorithm for recombination detection (GARD) in DATAMONKEY (Murrell *et al*., 2012) were used to identify amino acid sites that have undergone positive selection or represent recombination breakpoints, respectively. MrBayes v.3.2.6 (Ronquist & Huelsenbeck, 2003) was used to obtain a Bayesian phylogenetic tree for allelic sequences from *Nypr-MHC* representative species in Felidae, Canidae, Mustelidae, Ursidae; and human. KAKUSAN 4 (Tanabe, 2007) was used to select optimal models of nucleotide substitution. Phylogenetic analyses were conducted separately for four data partitions — the partial open reading frame (ORF; exon 2 and 3 concatenated), exon 2, exon 3, and intron 2. All phylogenetic trees were reconstructed from two Markov Chain Monte Carlo (MCMC) runs of 10 million generations for exon 3 and intron 2, 15 million generations for exon 2, and 20 million generations for the concatenated exon 2 and 3. All the chains were sampled every 100 generations, with the first 25% samples discarded as burn-in.

RESULTS

Allelic diversity of MHC class I gene in raccoon dogs

MHC class I fragments of 708–810 bp long were sequenced from 31 raccoon dog individuals from Japan and Russia, with an average of 27 clones sequenced per individual. Each sequence consisted of exon 2 (267 bp, 89 amino acids), exon 3 (273 bp, 92 amino acids), and the intervening intron 2 (168–270 bp). In all, 48 novel *Nypr-MHC1* alleles were identified (Table 4). One to six putative functional alleles were detected per individual indicating that there are at least three MHC class I loci in the raccoon dog. Exon 2 was somewhat higher in nucleotide diversity than exon 3 (Table 5), which means that there were more polymorphic amino acid sites in the α2 domain than in the α1 domain.

Table 5. Sequence polymorphism in MHC class I exon 2, exon 3, and intron 2 of raccoon dogs from Japan and Russia.

Fragments	Exon 2	Exon 3	Intron 2
Variable sites	22	28	31
Parsim informative sites	17	25	30
Mutations	26	33	31
Number of amino acids	89	92	-
Polymorphic amino acid sites	12	16	-
Nucleotide diversity	0.02777	0.02286	0.07282

Dashes indicate no values calculated, because the intron is non-coding.

The distribution of MHC class I alleles among the geographical populations (Table 4) shows that although no alleles were shared among all regions, *Nypr-MHC1*01* and **02* were the most broadly distributed; however, each detected in six of the eight regions; neither was detected in Hokkaido or Far Eastern Russia. *Nypr-MHC1*02* was the allele most frequently detected among the individuals analyzed. *Nypr-MHC1*08* was shared by Hokkaido, Honshu, and Shikoku Islands, and *Nypr-MHC1*20* was shared only by Northern, Central, and Southern Honshu. The native populations in Far Eastern Russia shared three alleles (*Nypr-MHC1*04, *06, *09*) with the introduced population in Western Russia. The degree of geographical restriction varied considerably, ranging from no alleles specific to a region (central Honshu) to 10 region-specific alleles (Hokkaido).

Table 4. Distribution of MHC class I alleles among geographical populations of raccoon dogs from Japan and Russia

Region	Sample name	*Nypr - MHC1*																																																Number of identified alleles
		**01*	**02*	**03*	**04*	**05*	**06*	**07*	**08*	**09*	**10*	**11*	**12*	**13*	**14*	**15*	**16*	**17*	**18*	**19*	**20*	**21*	**22*	**23*	**24*	**25*	**26*	**27*	**28*	**29*	**30*	**31*	**32*	**33*	**34*	**35*	**36*	**37*	**38*	**39*	**40*	**41*	**42*	**43*	**44*	**45*	**46*	**47*	**48*	
Hokkaido	HKD_1								+		+													+							+	+									+									6
	HKD_2								+		+																	+			+										+									5
	HKD_3								+		+																		+													+								4
	HKD_5															+		+																									+	+						4
Northern Honshu	NHS_1		+												+					+										+																				4
	NHS_2		+																		+					+																			+	+				5
	NHS_3	+							+								+																																	3
	NHS_4		+					+																									+																	3
	NHS_5														+		+			+																		+									+			5
Central Honshu	CHS_1	+				+														+																														3
	CHS_2	+																																																1
	CHS_3		+					+													+																													3
	CHS_4		+					+	+																+									+																5
Southern Honshu	SHS_2	+						+	+												+																		+											5
	SHS_3			+																																														1
	SHS_4		+			+																+																										+		4
	SHS_5	+		+																																														2
Shikoku	SHKK_2		+			+						+																																						3
	SHKK_3		+			+						+																																						3
	SHKK_4	+							+									+				+			+							+																		6
Kyushu	KYSH_2	+	+										+																																					3
	KYSH_3																		+								+																							2
	KYSH_4							+																										+	+					+										4
Far Eastern Russia (Native)	RSSN_1				+		+			+																																								3
	RSSN_2				+		+																																											2
	RSSN_3				+		+																																											2
	RSSN_4									+													+													+														3
Western Russia (Introduced)	RSSI_1		+							+																																								2
	RSSI_2	+											+																																					2
	RSSI_3									+				+																																				2
	RSSI_4				+		+							+																							+												+	5

Selection mechanism of MHC class I gene in raccoon dogs

Selection pressure on functional domains in the raccoon dog MHC class I protein was analyzed to determine possible evolutionary differences between α1 (encoded by exon 2) and α2 (exon 3). Tajima's *D* values for both domains were significantly greater than 1 (Table 6) indicating that balancing selection has influenced the allelic diversity in both exons. The ω

Table 6. Sequence diversity of MHC class I exons 2 and 3 in raccoon dogs

Gene region	*m*	*S*	*Ps*	*θ*	*π*	Tajima's *D*	*P*
Exon 2	658	22	0.0824	0.0117	0.0269	3.1924	$P < 0.01$
Exon 3	658	28	0.1026	0.0145	0.0364	3.8264	$P < 0.01$

m, number of sequences; *S*, number of segregating sites; *Ps*, number of segregating sites divided by the total number of sites; *θ*, 4Nμ for an autosomal gene for a diploid organism; *π*, nucleotide diversity; *D*, Tajima's test statistics; *P*, significance value for Tajima's *D*.

(d_N/d_S) value was markedly greater than 1 for the ABSs in each exon (Table 7), indicating that positive selection on codons encoding ABSs in both the α1 and α2 domains has influenced allelic diversity. The MEME analysis provided evidence of positive selection at the amino acid level for both domains [asterisks in Supplementary Figures S1 (α1 domain) and Supplementary Figures S2 (α2 domain)], but the α2 domain displayed more sites (three) under positive selection than the α1 domain (one). Three out of the four sites under positive selection coincided with ABS codons inferred from the human HLA-A2 locus (Bjorkman, 1987). The GARD analysis detected one recombination breakpoint in exon 3, but none for exon 2. The results indicate that in the α1 domain, positive selection was the main factor leading to allelic diversity in the raccoon dog, whereas in the α2 domain, both positive selection and recombination could have influenced the allelic diversity.

Table 7. Ratio (ω) of non-synonymous (d_N) to synonymous (d_S) substitution rates for antigen binding sites (ABSs), non ABSs, and all amino acids in MHC class I exons 2 and 3 in raccoon dogs. *SD*, standard deviation

Population	Position	Number of codons	d_N	(± *SD*)	d_S	(± *SD*)	$\omega = (d_N / d_S)$
Exon 2	ABS	16	0.145	(± 0.060)	0.049	(± 0.044)	2.9592
	Non-ABS	73	0.009	(± 0.005)	0.018	(± 0.012)	0.5
	Overall	89	0.03	(± 0.011)	0.024	(± 0.011)	1.25
Exon 3	ABS	15	0.252	(± 0.088)	0.111	(± 0.078)	2.2702
	Non-ABS	77	0.013	(± 0.006)	0.007	(± 0.004)	1.8571
	Overall	92	0.047	(± 0.013)	0.019	(± 0.008)	2.4737

Phylogenetic Analysis of MHC class I in raccoon dogs

Among the Bayesian phylogenetic analyses of separate genetic regions, exon 2 and 3 sequences from the raccoon dog MHC class I (*Nypr-MHC1*) and those from other carnivores showed instances of trans-species polymorphism (TSP); For exon 2 (Figure 5), two large clades contained both dog and raccoon dog sequences—in other words, in two cases, raccoon dog sequences were more closely related to a group of dog sequences than other raccoon dog sequences. The same situation was evident from exon 3 (Figure 6), although the groupings of dog and raccoon dog sequences were different than exon 2. However, phylogenetic analysis of concatenated exon 2 and 3 sequences (partial ORF) (Figure 7) showed ambiguous evidence for TSP; one clade of dog DLA alleles formed the sister group to a monophyletic subclade containing all the *Nypr-MHC1* alleles, with other dog DLA alleles outside these two subclades, although the node linking the clades was weakly supported (posterior probability, 0.89). In addition, intron 2 sequences (Supplementary Figure S3), mostly formed clades by species in the phylogenetic analysis (Figure 8), with the exception that one group of raccoon dog sequences was closely related to the dog sequences (DLA) than to other raccoon dog sequences. No specific patterns in the geographic distribution of alleles were detected in any phylogenetic trees.

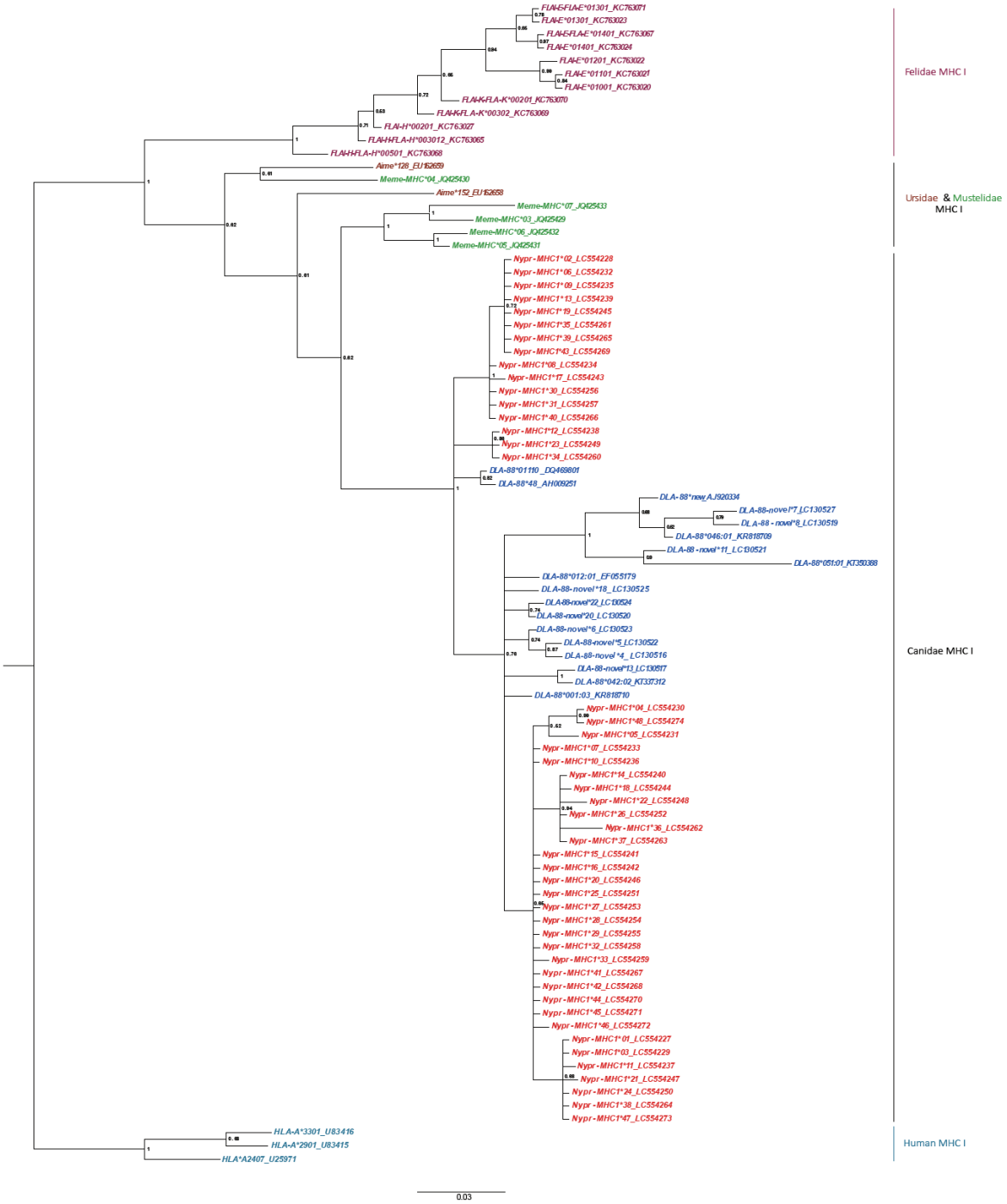

Figure 5. Bayesian phylogenetic tree for exon 2 of MHC class I alleles from raccoon dog; representative species of canids, felids, mustelids, and ursids; and human. Alleles of different species are shown in different colors. For other information, see the caption to Fig. 2.

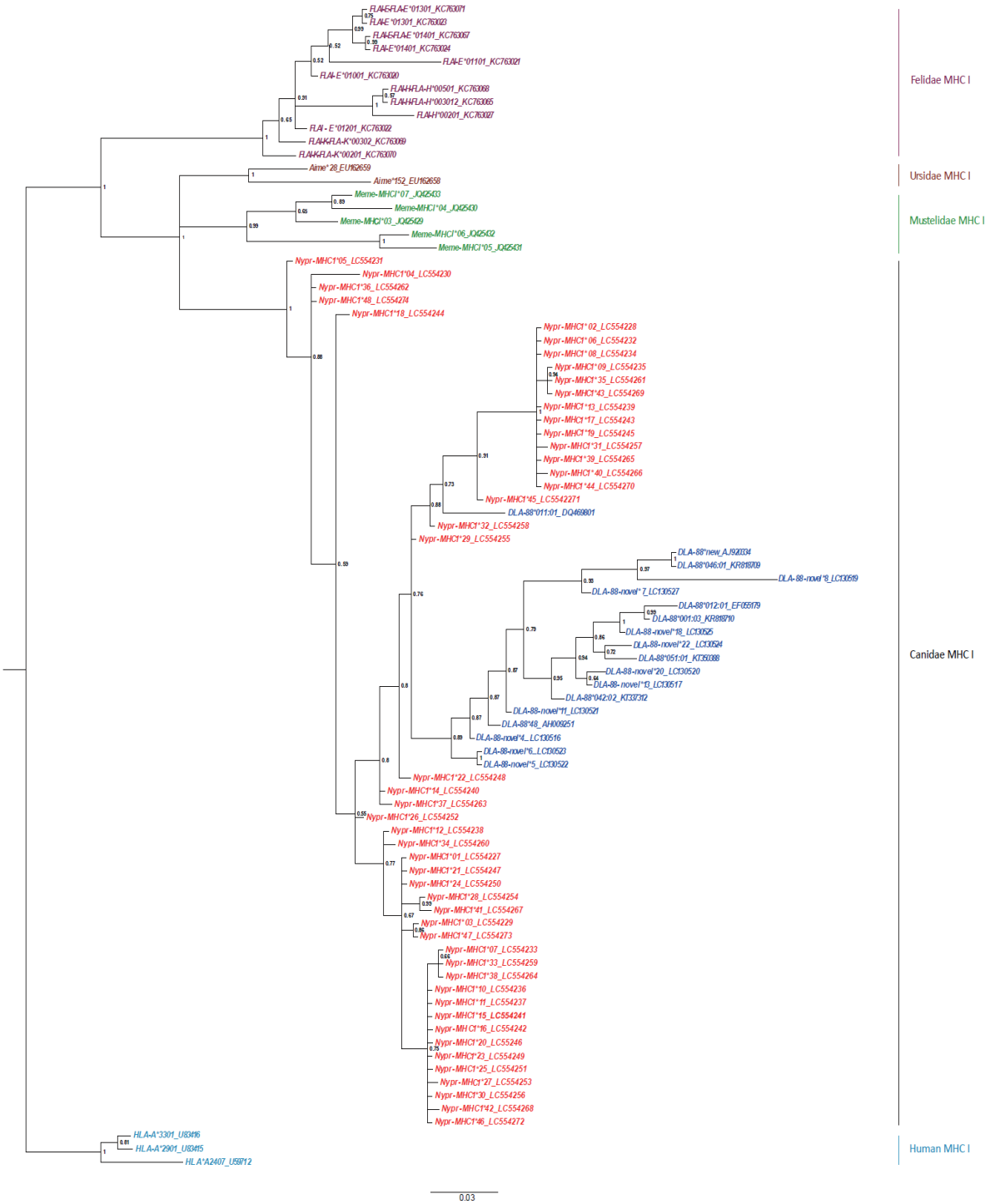

Figure 6. Bayesian phylogenetic tree for exon 3 of MHC class I alleles from raccoon dogs; representative species of canids, felids, mustelids, and ursids; and humans. Alleles of different species are shown in different colors. For other information, see the caption to Fig. 2.

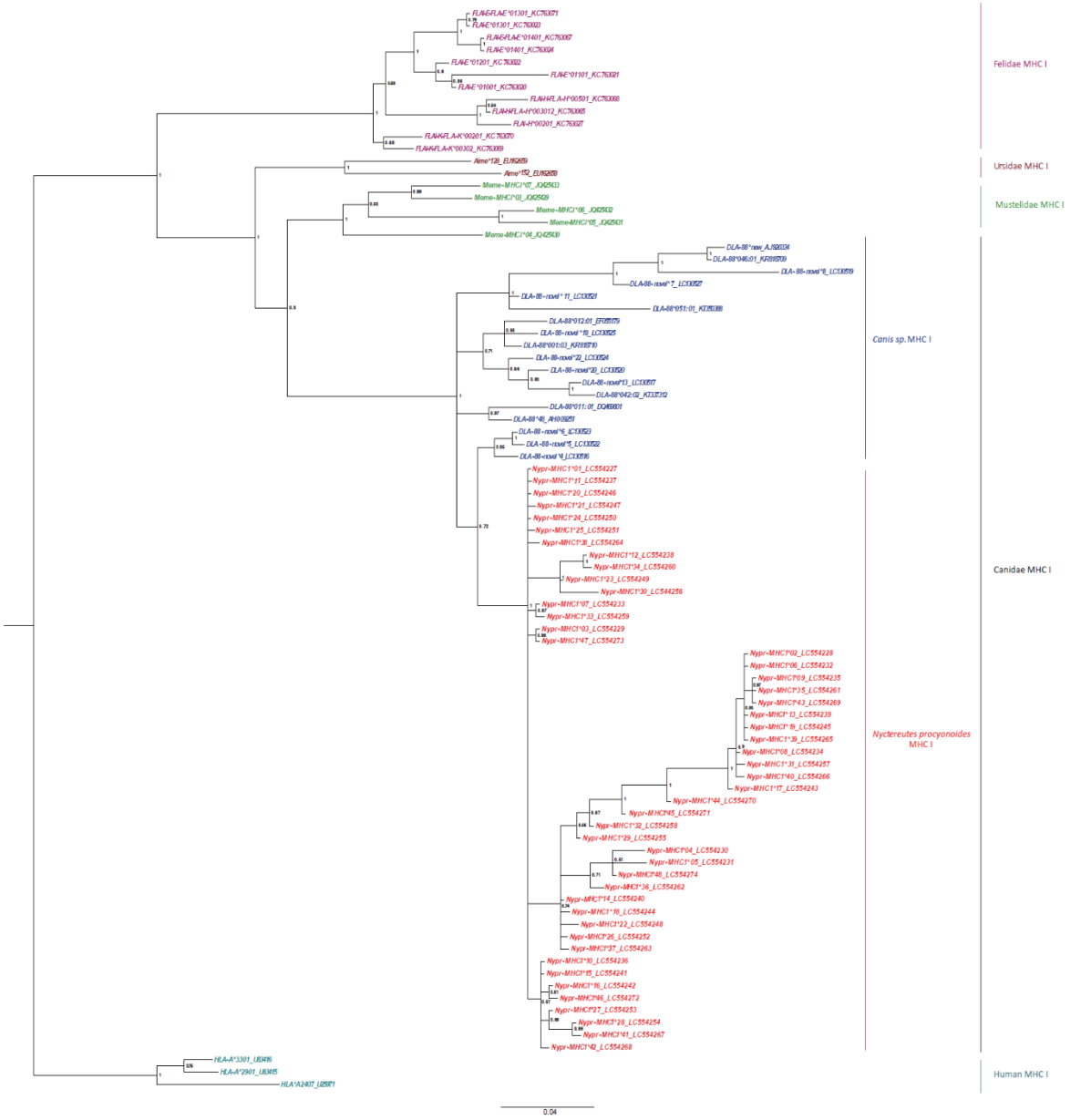

Figure 7. Bayesian phylogenetic tree for the partial open reading frame (concatenate exon 2 and exon 3 sequences) of MHC class I alleles from raccoon dogs (indicated by red characters); representative species of canids (blue), felids (purple), mustelids (green), and ursids (brown); and human (light blue). Numbers near the nodes are posterior probability values. DDBJ/EMBL/GenBank accession numbers for nucleotide sequences are included after allele names for sequences retrieved from GenBank. Abbreviations within alleles names indicating different species: *Nypr*, raccoon dog; *DLA-88*, domestic dog; *FLA*, domestic cat; *Meme*, European badger; *Aime*, giant panda; HLA, human.

Figure 8. Bayesian phylogenetic tree for intron 2 of MHC class I alleles from raccoon dogs; representative species of canids, felids, mustelids, and ursids; and human. Alleles of different species are shown in different colors. For other information, see the caption to Fig. 2.

DISCUSSION

Allelic diversity of MHC class I genes in raccoon dogs

This is the first study to characterized MHC class I genes in the raccoon dog. MHC class I genes are generally divided into two subgroups, classical (class Ia), and non-classical (class Ib), according to the properties of the encoded protein (Tanoka & Kasahara, 1998; Halenius *et al.*, 2015). Class Ia is highly polymorphic at the population level. Classical MHC class I proteins present peptide ligands from intracellular proteins (pathogens) on infected or

cancerous cells to CD8+ T cells. On the other hand, class Ib comprises many types of genes encoding proteins is mediating inhibitory or activating signals in natural killer (NK) cells (Tanoka and Kasahara, 1988; Halenius *et al*., 2015).

It is confirmed that all nucleotide sequences obtained in this study were classical class Ia sequences based on the standard structure of MHC class I (Kaufman *et al*., 1994) and polymorphism at deduced ABSs in both the α1 and α2 domains. One to six alleles per individual were found, indicating that raccoon dogs have at least three functional classical MHC class I gene loci. Overall, this study identified 48 novel *Nypr-MHC1* alleles, the level of allelic variation was higher than in most other mammalian species: 16 alleles in the giant panda (Zhu *et al*., 2012); 21 in the chimpanzee (Maibach *et al*., 2017); 17 alleles in the wolf (Liu *et al*., 2017); three in the domestic cat (Yuhki *et al*., 2008); 42 in the bonobo (Maibach *et al*., 2017); 26 in the sable (Zhao *et al.*, 2020); and 37 in the brown bear (Kuduk *et al*., 2012). The domestic dog is an apparent exception among canids, showing higher allelic variation, with 73 known alleles (Wagner *et al*., 2002; Ross *et al*., 2012; Miyamae *et al.*, 2017; Venkataraman *et al*., 2017). This might be the result of higher sampling effort in this well-studied model species. By the same token, this study was limited to the Japanese and Russian raccoon dog populations, and additional alleles might be detected if raccoon dogs from other areas had been included.

Exposure over a long period of time to a wide variety of intracellular pathogens like the canine distemper virus (Suzuki *et al*., 2015), SARS (IASR, 2005), rabies (Kurosawa *et al*., 2017), *Trichinella* sp. (Pozio, 2000), and six bacterial and five viral pathogens (Sutor *et al*., 2014) may have been the driving force for this high variation in *Nyrp-MHC1* in raccoon dogs. The high allelic MHC class I variation in raccoon dogs, along with their high dispersal capability, adaptation to a wide range of climatic and environmental conditions (Caut *et al*., 2008), omnivory, and a high reproductive rate (Kauhala & Kowalczyck, 2011) could explained

their success in expansion in Europe after being introduced there from their native range in East Asia.

Bartocillo *et al.* (2020) (Chapter 1) reported similar high variation of MHC class II *DRB* alleles in raccoon dogs, with both locally restricted and widespread alleles. Locally restricted alleles could have resulted from balancing selection with the presence of some native pathogens (Hughes & Yeager, 1998). Interestingly, the highest number of locally restricted alleles (10) was found in Hokkaido and the seven most frequent alleles identified in this study were not found in this island. The geographical isolation of Hokkaido from other parts of Japan by the Tsugaru straits during the last glacial maximum (Ohshima, 1991) could have prevented the gene flow that would have promoted genetic divergence of MHC I among the raccoon dogs of Japan. On the other hand, widespread pathogens might explain alleles shared among regions. Kurosawa *et al.*, (2017) reported that rabies originated in Japan locally and then spread throughout the country. Not only the distribution but also the pattern of spread of infectious disease might have had an effect on the allelic variation of MHC I genes in raccoon dogs.

Evolution of MHC class I genes in raccoon dogs

Evidence of historical positive selection was found on both the α1 and α2 domains of MHC class I proteins in Japanese and Russian raccoon dogs based on the ratio of the non-synonymous and synonymous substitution rates. The results were congruent with a study on wolf MHC class I genes (Liu *et al*., 2017), which showed strong positive selection. Similarly, Bartocillo *et al.* (2020) detected strong positive selection in MHC class II *DRB* exon 2 in raccoon dogs. Separate analysis of the two exons indicated stronger positive selection on exon 2 (encoding the α1 domain) than on exon 3 (encoding α2 domain). Specifically, for exon 2, only ABS codons showed evidence of strong positive selection, which was also observed in studies of badgers that examined both exons 2 and 3 in MHC class I genes (Sin *et al*., 2012;

Abduriyim *et al*., 2019). In contrast, for exon 3, not only ABS codons but also non-ABS codons (that is, for all codons) showed evidence of strong positive selection. In addition, there were fewer polymorphic amino acid sites in both domains in the raccoon dog compared to other carnivore species reported by Sin *et al.*, (2012) and Abduriyim *et al.*, (2019). Nevertheless, even with a low number of polymorphic amino acid sites and ABS-restricted polymorphic sites, the high allelic variation in raccoon dogs was found. This indicates that positive selection could have contributed to maintaining the high allelic variation of MHC class I in raccoon dogs. Positive selection on ABSs is favorable to a population, as it gives a wider range of antigen peptides for recognition of a wider variety of pathogens, and increases the chance of survival from pathogenic infections (Hughes and Nei, 1992; Hughes and Yeager, 1998; Jeffery & Bangham, 2000).

TSP is a phylogenetic pattern that results from the retention of ancestral allelic lineages across divergence events, including speciation (Penn & Potts, 1999), and a typical phenomenon showing the contribution of balancing selection on a gene (Klein *et al.*, 1998). Whereas a phylogenetic analysis of MHC class II *DRB*, Bartocillo *et al.* (2020) (Chapter 1) found no pattern of TSP; instead, all raccoon dog sequences formed a clade within Canidae. Our analyses provided some evidence for TSP in MHC class I between the raccoon dog and domestic dog, but not between the raccoon dog and representatives of other carnivore families (Felidae, Mustelidae, Ursidae). Analysis of the partial ORF of exon 2 and 3 showed only weak or no evidence of TSP. However, a separate analysis of exon 2 showed a more strongly supported pattern of TSP, in which two clades emerged that each included both raccoon dog and domestic dog sequences; one of these clades showed relatively high (posterior probability = 0.98) branch support.

Indications of TSP for exons 2 and 3 (and especially exon 2) were congruent with the Tajima's *D* values (D = 3.1924 for exon 2; D = 3.8264 for exon 3), which indicate that these

sequences have evolved under balancing selection. However, no clear evidence of TSP was observed even though the Tajima's *D* value is sufficiently high, perhaps, it could be due to the raccoon dogs lack of closely related species. Data on MHC class I genes in canids are scarce, and studies on other species should be conducted to further understand the extent of TSP in canids.

Intron 2 sequences in the raccoon dog and domestic dog showed remarkably low variation compared to their exon sequences, and relatively few variations compared to intron 2 from representatives of other canid families. The phylogenetic analysis of intron sequences was quite interesting, as it showed two distinct clades of raccoon dog sequences, one of which was highly supported (posterior probability = 1), and the other of which was the sister group to domestic dog intron sequences. The results were similar to the study of intron between HLA-A, -B, -C by Cereb *et al.* (1996), whereby introns 1, 2, 3 were relatively conserved than their neighboring exon 2 and 3. Introns between HLA-A, -B, -C genes in humans have been reported to be conserved and locus-specific, a byproduct of interallelic recombination and subsequent genetic drift which then leads to the homogenization of introns over evolutionary time (Cereb *et al.*, 1997). It has been established before that introns have no apparent purpose but studies provide evidence that introns may provide a vital role in the long-term survival of genes by preventing degenerative interlocus recombination (Kricker *et al.*, 1992). Overall, the presence of two highly conserved clades of introns in the raccoon dog is still a mystery and studies on MHC I introns in the Canidae should be explored.

The results from positive selection and recombination analyses revealed one codon (amino acid site) in exon 2 and three codons in exon 3 that are under positive selection and a recombination breakpoint in exon 3 only. Positive selection at the amino acid level could have contributed to maintain the high allelic diversity of exon 2, whereas for exon 3, both positive selection and recombination could have contributed to allelic diversity. Overall, the differences

in the intensity of positive selection between the two exons, the different phylogenetic relationships evident in their trees, and the presence of recombination in only exon 3 suggest that exon 2 and 3 encoding α1 and α2 domains, respectively, of MHC class I genes in the raccoon dog have different evolutionary histories. This agrees with the conclusion of Sin *et al.* (2012) and Abduriyim *et al.* (2019) that separate evolutionary analyses are necessary for the two MHC class I domains.

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General Conclusion

This dissertation presents the first study to characterized the allelic diversity and selection mechanism that influenced the evolution of the MHC genes of raccoon dogs, *Nyctereutes procyonoides* in Japan and Russia. Specifically, in Chapter 1 extensive diversity was shown in the *DRB* gene of the Japanese and Russian raccoon dogs, which provides a broader spectrum of pathogens that an organism can be familiar with and defend against. These basic findings are consistent with the research showing that the copy number variation in the MHC *DRB* gene is one of the driving force in obtaining high allelic diversity. Overall, genetic evidence was provided that the variation in the *DRB* gene may have been influenced by pathogen-driven selection and geographical barriers based on the presence of locally restricted and shared alleles among different geographical areas.

Furthermore, the present findings in Chapter 1 confirmed that strong positive selection and recombination at the amino acid level have contributed in maintaining the high allelic diversity of MHC *DRB* exon 2 in raccoon dogs. The absence of TSP in raccoon dog *DRB*s indicates that this species diverged from other canids long enough ago that no ancestral alleles have been retained and that the raccoon dog might have experienced distinct pathogens over a long period of time compared to other canids, as its environment and ecological niche differ from those other canids. In addition, these findings provide additional information about the

claim that *N. procyonoides* have originated in Honshu, Shikoku, and Honshu islands through the presence of two lineages observed in the *Nypr-DRB* clade.

Chapter 2 provides the first report to the high diversity of MHC class I alleles in raccoon dogs. This high diversity in MHC class I alleles in raccoon dogs have been influenced by copy number variation and exposure to wide variety of intracellular pathogens in a very long period of time. Collectively, the results appear consistent with MHC class II DRB whereby variations in the MHC class I have been influenced by pathogen-driven selection and geographical barriers based on the presence of locally restricted and shared alleles among different geographical areas.

Additionally, findings in Chapter 2 confirmed that historical positive selection and recombination were the main force that have acted in the evolution of the MHC class I genes. Separate analysis of the two exons indicated stronger positive selection on exon 2 and 3. The analyses provided some evidence for TSP in MHC class I between raccoon dogs and domestic dogs. A separate analysis of exon 2 showed a more strongly supported pattern of TSP whereas exon 3 showed only weak evidence of TSP. The presence of recombination was evident in exon 2, but absent in exon 3. Overall, the different results based on separate analysis of exon 2 and exon 3 suggests that α1 and α2 domains of MHC class I underwent different evolutionary histories, which agreed with Sin *et al.,* (2012) and Abduriyim *et al.,* (2019), whereby separate evolutionary analysis of each MHC class I domain is necessary to understand.

Taken together, this study provides new knowledge about the evolutionary diversity and mechanism of MHC genes in raccoon dogs. This offer a powerful tool in assessing the adaptive immune system capability, and susceptibility to infectious and autoimmune diseases of raccoon dog, an essential information in conservation biology. This work shed more light on the molecular evolutionary adaptation of non-model species, and free-ranging animal

populations in mammals, especially in the Canidae. The results represent an excellent initial step toward understanding the selection mechanism of MHC genes among non-model species of canids. The findings add to the growing body of literature that may guide policy makers in assessing conservation actions among raccoon dogs in Japan and Russia and/or help in the policy making of introducing raccoon dogs to non-native geographical areas for fur production.

Because this study is limited to the allelic diversity and evolutionary selection mechanism of MHC class I and class II genes of raccoon dogs from Japan and Russia, future research using different genetic markers, additional sampling size, analysis of samples from other East Asian regions and European regions, and archeological samples should be explored to fully grasps the extent of the evolutionary history and allelic diversity of MHC genes in *N. procyonoides*. In canids, MHC data is mostly limited to the genus *Canis* and future studies on other non-model canids could fruitfully explore this issue further. Future studies could also investigate the correlation between parasitic infections and MHC diversity of raccoon dogs in Japan and Russia. Despite of these, this work could be a framework for more exploration in the evolutionary and population genetic studies, and conservation actions for *Nyctereutes procyonoides*.

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Supplementary Tables

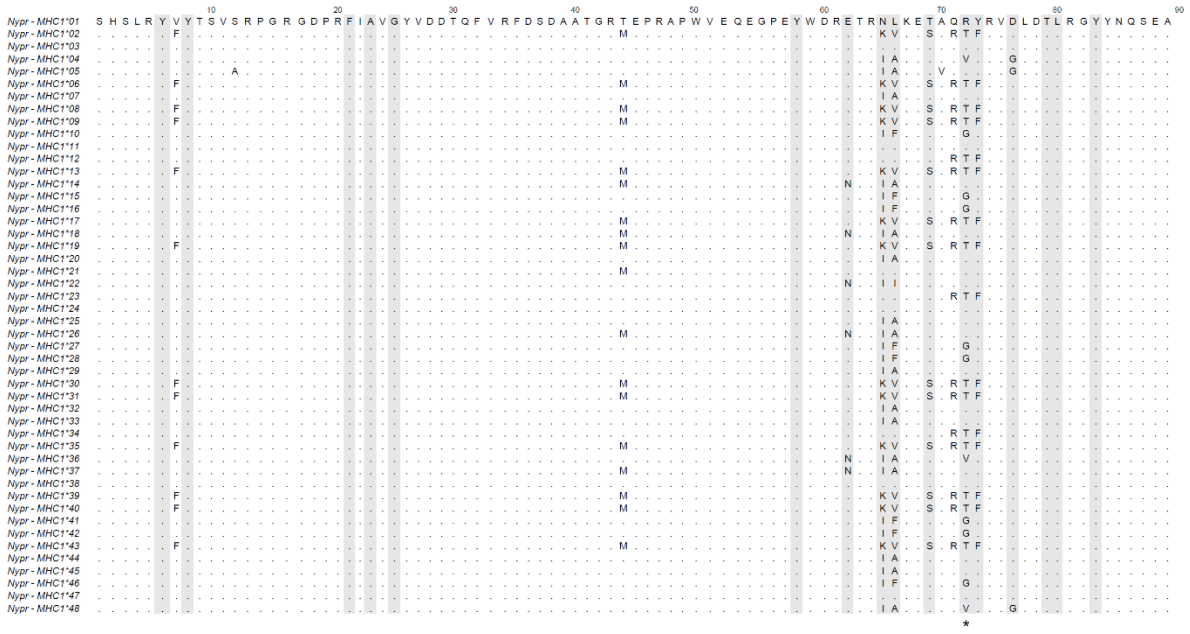
Supplementary Table 1. Sample information for raccoon dogs examined for MHC *DRB* class II gene

Sampling code	Sample name	Number of plasmid clones	Sex	Tissue	Region	Location	Supplier
OB1	HKD_1	20	Male	Blood	Hokkaido	Kamishihoro Town	S. Fujimoto (Obihiro Zoo)
E1	HKD_2	23	Male	Blood	Hokkaido	Numata Town	G. Bando (Asahiyama Zoo)
CH2	HKD_3	26	Male	Muscle	Hokkaido	Urahoro Town	H. Yanagawa (Obihiro University of Agriculture and Veterinary Medicine)
M1	HKD_4	24	Male	Blood	Hokkaido	Sapporo City	Y. Nishine (Sapporo Maruyama Zoo)
M2	HKD_5	24	Male	Blood	Hokkaido	Sapporo City	Y. Nishine (Sapporo Maruyama Zoo)
YA1	NHS_1	20	Female	Blood	Northern Honshu	Sendai City, Miyagi Prefecture	H. Kato (Yagiyama Zoological Park)
YA2	NHS_2	23	Male	Blood	Northern Honshu	Sendai City, Miyagi Prefecture	H. Kato (Yagiyama Zoological Park)
YA3	NHS_3	22	Female	Blood	Northern Honshu	Sendai City, Miyagi Prefecture	H. Kato (Yagiyama Zoological Park)
YA4	NHS_4	24	Female	Blood	Northern Honshu	Kawasaki Town, Miyagi Prefecture	H. Kato (Yagiyama Zoological Park)
AK1	NHS_5	28	Female	Muscle	Northern Honshu	Shizukuishi Town, Iwate Prefecture	K. Umezu (Akita Prefectural Museum)
G1	CHS_1	22	Female	Muscle	Central Honshu	Takayama City, Kiryu, Gifu Prefecture	S. Dakemoto (Takayama City)
G2	CHS_2	25	Male	Muscle	Central Honshu	Takayama City, Gifu Prefecture	S. Dakemoto (Takayama City)
G3	CHS_3	20	Female	Muscle	Central Honshu	Takayama City, Gifu Prefecture	S. Dakemoto (Takayama City)
G4	CHS_4	25	Male	Muscle	Central Honshu	Takayama City, Gifu Prefecture	S. Dakemoto (Takayama City)
G5	CHS_5	22	Male	Muscle	Central Honshu	Hida - gun, Gifu Prefecture	S. Dakemoto (Takayama City)
O5	SHS_1	29	Male	Blood	Southern Honshu	Kobe City, Hyogo Prefecture	Kobe Oji Zoo
AS1	SHS_2	27	Female	Blood	Southern Honshu	Hiroshima City, Hiroshima Prefecture	Y. Fukumorto (Hiroshima Asa Zoological Park)
AS2	SHS_3	23	Female	Blood	Southern Honshu	Takata District, Hiroshima Prefecture	Y. Fukumorto (Hiroshima Asa Zoological Park)
AS3	SHS_4	22	Female	Blood	Southern Honshu	Hiroshima City, Hiroshima Prefecture	Y. Fukumorto (Hiroshima Asa Zoological Park)
AS4	SHS_5	32	Female	Blood	Southern Honshu	Hiroshima City, Hiroshima Prefecture	Y. Fukumorto (Hiroshima Asa Zoological Park)
TK1	SHKK_1	30	Male	Blood	Shikoku	Awa City, Tokushima Prefecture	K. Iguchi (Tokushima Zoo)
TK2	SHKK_2	24	Male	Blood	Shikoku	Kamikatsu Town, Tokushima Prefecture	K. Iguchi (Tokushima Zoo)
TK3	SHKK_3	24	Unknown	Blood	Shikoku	Kamikatsu Town, Tokushima Prefecture	K. Iguchi (Tokushima Zoo)
N1	SHKK_4	24	Female	Kidney	Shikoku	Kami District, Kouchi Prefecture	K. Iguchi (Tokushima Zoo)
K14	KYSH_1	26	Female	Muscle	Kyushu	Oita City, Oita Prefecture	M. Baba (Kitakyushu Museum of Natural History and Human History)
KGS2	KYSH_2	32	Male	Muscle	Kyushu	Kirishima City, Kagoshima Prefecture	M. Akuzawa (Kagoshima University)
KGS3	KYSH_3	22	Female	Muscle	Kyushu	Kagoshima City, Kagoshima Prefecture	M. Akuzawa (Kagoshima University)
K11	KYSH_4	26	Male	Muscle	Kyushu	Kitakyushu City, Fukuoka Prefecture	M. Baba (Kitakyushu Museum of Natural History and Human History)
SPB13	RSSN_1	21	Female	muscle	Russia (Native)	Chuguevo District, Primorsky Territory	
SPB14	RSSN_2	26	Male	muscle	Russia (Native)	Spassk District, Primorsky Territory	
SPB16	RSSN_3	26	Male	muscle	Russia (Native)	Krasnoarmeisk District, Primorsky Territory	
SPB17	RSSN_4	27	Female	muscle	Russia (Native)	Krasnoarmeisk District, Primorsky Territory	
KIR81	RSSI_1	30	Female	muscle	Russia (Introduced	Kirov Province	
KIR82	RSSI_2	24	Unknown	muscle	Russia (Introduced	Kirov Province	
SPB21	RSSI_3	27	Unknown	muscle	Russia (Introduced	Novgorod Province	
SPB22	RSSI_4	30	Unknown	muscle	Russia (Introduced	Leningrad Province	

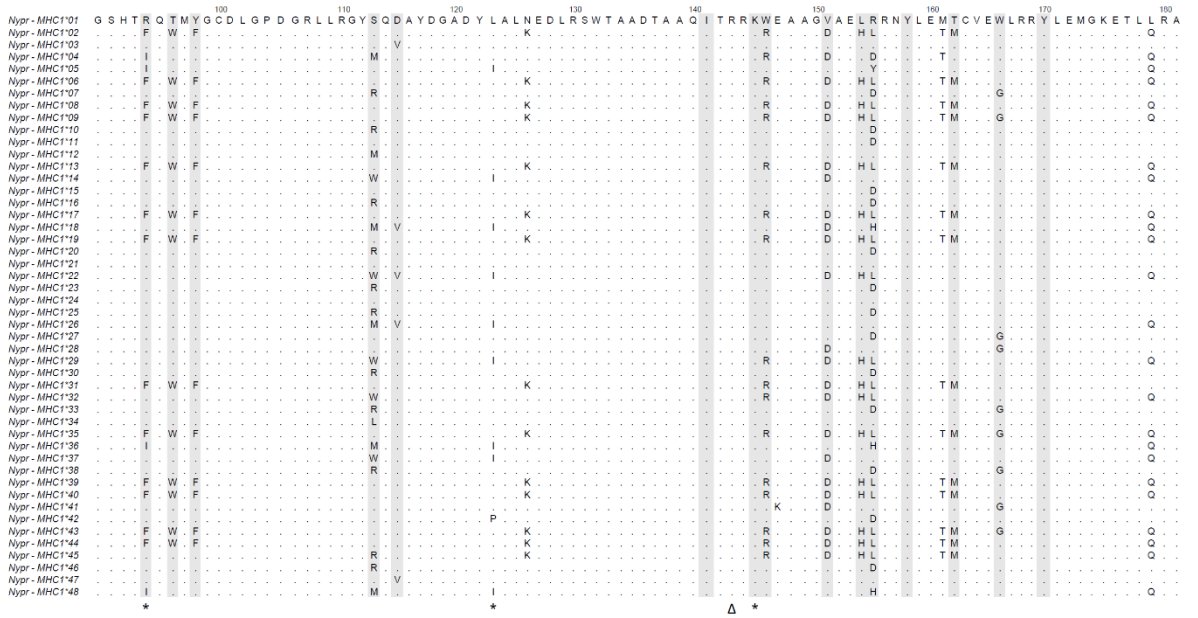
Supplementary Table 2. Sample information for raccoon dogs examined for MHC class I genes

Sampling code	Sample name	No. of plasmid clones	Sex	Tissue	Region	Location	Supplier
OB1	HKD_1	32	Male	Blood	Hokkaido	Kamishihoro Town	S. Fujimoto (Obihiro Zoo)
E1	HKD_2	26	Male	Blood	Hokkaido	Numata Town	G. Bando (Asahiyama Zoo)
CH2	HKD_3	27	Male	Muscle	Hokkaido	Urahoro Town	H. Yanagawa (Obihiro University of Agriculture and Veterinary Medicine)
M2	HKD_5	32	Male	Blood	Hokkaido	Sapporo City	Y. Nishine (Sapporo Maruyama Zoo)
YA1	NHS_1	28	Female	Blood	Northern Honshu	Sendai City, Miyagi Prefecture	H. Kato (Yagiyama Zoological Park)
YA2	NHS_2	20	Male	Blood	Northern Honshu	Sendai City, Miyagi Prefecture	H. Kato (Yagiyama Zoological Park)
YA3	NHS_3	32	Female	Blood	Northern Honshu	Sendai City, Miyagi Prefecture	H. Kato (Yagiyama Zoological Park)
YA4	NHS_4	28	Female	Blood	Northern Honshu	Kawasaki Town, Miyagi Prefecture	H. Kato (Yagiyama Zoological Park)
AK1	NHS_5	20	Female	Muscle	Northern Honshu	Shizukuishi Town, Iwate Prefecture	K. Umezu (Akita Prefectural Museum)
G1	CHS_1	23	Female	Muscle	Central Honshu	Takayama City, Kiryu, Gifu Prefecture	S. Dakemoto (Takayama City)
G2	CHS_2	29	Male	Muscle	Central Honshu	Takayama City, Gifu Prefecture	S. Dakemoto (Takayama City)
G3	CHS_3	42	Female	Muscle	Central Honshu	Takayama City, Gifu Prefecture	S. Dakemoto (Takayama City)
G4	CHS_4	24	Male	Muscle	Central Honshu	Takayama City, Gifu Prefecture	S. Dakemoto (Takayama City)
AS1	SHS_2	28	Female	Blood	Southern Honshu	Hiroshima City, Hiroshima Prefecture	Y. Fukumorto (Hiroshima City Asa Zoological Park)
AS2	SHS_3	27	Female	Blood	Southern Honshu	Takata District, Hiroshima Prefecture	Y. Fukumorto (Hiroshima City Asa Zoological Park)
AS3	SHS_4	26	Female	Blood	Southern Honshu	Hiroshima City, Hiroshima Prefecture	Y. Fukumorto (Hiroshima City Asa Zoological Park)
AS4	SHS_5	35	Female	Blood	Southern Honshu	Hiroshima City, Hiroshima Prefecture	Y. Fukumorto (Hiroshima City Asa Zoological Park)
TK2	SHKK_2	26	Male	Blood	Shikoku	Kamikatsu Town, Tokushima Prefecture	K. Iguchi (Tokushima Zoo)
TK3	SHKK_3	24	Unknown	Blood	Shikoku	Katsuura Town, Tokushima Prefecture	K. Iguchi (Tokushima Zoo)
N1	SHKK_4	29	Female	Kidney	Shikoku	Konan City, Kochi Prefecture	Noichi Zoological Prak
KGS2	KYSH_2	31	Male	Muscle	Kyushu	Kirishima City, Kagoshima Prefecture	M. Akuzawa (Kagoshima University)
KGS3	KYSH_3	28	Female	Muscle	Kyushu	Kagoshima City, Kagoshima Prefecture	M. Akuzawa (Kagoshima University)
K11	KYSH_4	23	Male	Muscle	Kyushu	Kitakyushu City, Fukuoka Prefecture	M. Baba (Kitakyushu Museum of Natural History and Human History)
SPB13	RSSN_1	31	Female	Muscle	Russia (Native)	Chuguevo District, Primorsky Territory	
SPB14	RSSN_2	22	Male	Muscle	Russia (Native)	Spassk District, Primorsky Territory	
SPB16	RSSN_3	25	Male	Muscle	Russia (Native)	Krasnoarmeisk District, Primorsky Territory	
SPB17	RSSN_4	24	Female	Muscle	Russia (Native)	Krasnoarmeisk District, Primorsky Territory	
KIR81	RSSI_1	23	Female	Muscle	Russia (Introduced)	Kirov Province	
KIR82	RSSI_2	24	Unknown	Muscle	Russia (Introduced)	Kirov Province	
SPB21	RSSI_3	28	Unknown	Muscle	Russia (Introduced)	Novgorod Province	
SPB22	RSSI_4	31	Unknown	Muscle	Russia (Introduced)	Leningrad Province	

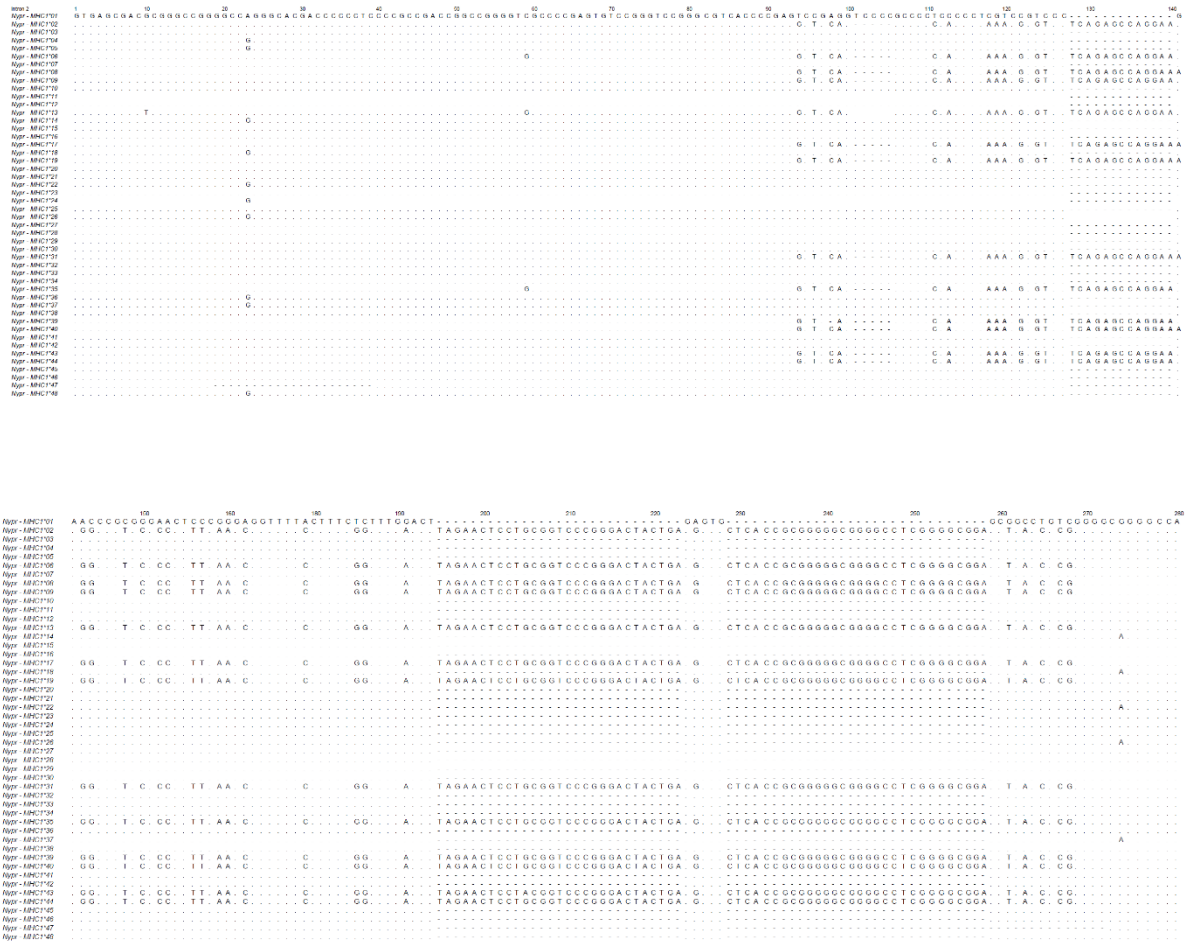
Supplementary Figures



Supplementary Figure S1. Alignment of amino acid sequences deduced from partial sequences of MHC class I exon 2 from raccoon dogs (*Nypr-MHC1*). Dots indicate the identity with the *Nypr-MHC1*01* sequence. Number at the top indicate amino acid positions in the α1 domain. Grey shading indicates antigen binding sites (ABSs) predicted from human MHC class I (Bjorkman, 1987). The asterisk indicates a recombination breakpoint, determined by a GARD (genetic algorithm for recombination detection) analysis.



Supplementary Figure S2. Alignment of amino acid sequences deduced from partial sequences of MHC class I exon 3 from raccoon dog (*Nypr-MHC1*). For other information, see the caption to Fig. S1.



Supplementary Figure S3. Alignment of intron 2 nucleotide sequences for MHC class I alleles (*Nypr-MHC1*) in raccoon dogs.