Studies of dog leukocyte antigens genotyping based on canine visceral leishmaniasis

（イヌ内臓リーシュマニア症を起点とした

イヌ主要組織適合性複合体遺伝子多型に関する研究）

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Contents

Abbreviations

APC	Allophycocyanin
AVL	Anthroponotic visceral leishmaniasis
CCS	Circular consensus sequencing
CpG	Cytosine-phosphate-guanine
CVL	Canine visceral leishmaniasis
DLA	Dog leukocyte antigen
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HE	Hematoxylin and eosin
HLA	Human leukocyte antigen
kDNA	Kinetoplast minicircle DNA
MHC	Major histocompatibility complex
NGS	Next-generation sequencing
PBS	Peptide binding site
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
SMRT	Single-Molecule Real-Time
SNV	Single-nucleotide variant
Tregs	Regulatory T cells
VL	Visceral leishmaniasis

Notes

Contents of this thesis have been published as follows:

1. Konno H, Yokoyama N, Tamura Y, Aoshima K, Nakao R, Takiguchi M, Katakura K. An experimental challenge model for *Leishmania donovani* in beagle dogs, showing a similar pattern of parasite burden in the peripheral blood and liver. Parasitol Res 121, 3569-3579, 2022.

2. Konno H, Miyamae J, Kataoka H, Akai M, Miida H, Tsuchiya Y. Dog leukocyte antigen genotyping across class I and class II genes in beagle dogs as laboratory animals. Immunogenetics 76, 261-270, 2024.

3. Konno H, Miyamae J, Kajitani R, Kugou K, Kataoka H, Akai M, Ishizaka T, Chiba K. PacBio long-read amplicon sequencing of full length of dog leukocyte antigen genes. Exp Anim, in press.

General introduction

Visceral leishmaniasis (VL) in humans is primarily attributed to two closely related *Leishmania* species: *Leishmania donovani* and *Leishmania infantum* (syn. *Leishmania chagasi*). The former is predominantly responsible for anthroponotic VL, particularly in the Indian subcontinent and regions of East Africa. In contrast, *L. infantum* is the causative agent of zoonotic VL, with dogs serving as the principal reservoir for the parasite across the Mediterranean, North Africa, the Middle East, West Asia, China, and Latin America [10,11,19,64]. While *L. infantum* inflicts canine VL (CVL), presenting a wide range of clinical manifestations from subclinical forms to fatal outcomes [7,11,21,24], research into the pathogenesis of *L. donovani* in naturally infected dogs remains limited and largely overlooked.

In previous epidemiological studies, *L. donovani* DNA or anti-*Leishmania* antibodies in the canine blood were detected[4,5,28,46,49,97,98], suggesting that dogs may play a role in the *L. donovani* transmission cycle as a reservoir in VL endemic areas. Therefore, experimental assessment is needed to understand the parasitological and immunological aspects in dogs with *L. donovani* infection. Beagle dogs are widely used in elucidating the pathogenesis of various immune-related diseases and infections including *L. infantum*[61,65,94,105].

The major histocompatibility complex (MHC) is integral to the immune response, facilitating the presentation of antigenic peptides to T cells. MHC molecules are categorized into two main classes (class I and class II), each serving distinct roles in immune function. The MHC genes, known as human leukocyte antigen (HLA) genes in humans, are among the most polymorphic in vertebrate genomes, with HLA gene polymorphisms playing recognized roles in the risk, susceptibility, and severity of diverse immune-related conditions, including transplant rejection [6], autoimmune diseases [27], infectious diseases [73], and adverse drug reactions [48]. HLA class I and class II gene polymorphisms are also associated with susceptibility to several parasitic infections such as leishmaniasis [93], trypanosomiasis [92], and malaria [66].

In dogs, the MHC is referred to as the dog leukocyte antigen (DLA). Epidemiological evidence indicated that the risk of CVL infection varies among different dog breeds, suggesting a genetic component to susceptibility [95]. This variation in susceptibility may be reflected in the distinct DLA profiles observed across various breeds

[74]. In the CVL studies, previous research has demonstrated significant associations between specific DLA class II alleles and the course of *L. infantum* infection in a group of naturally infected dogs [86]. In addition, the study using beagle dogs experimentally infected with *L. infantum* have reported associations between specific DLA class II alleles and increased anti-*Leishmania* antibody levels [105]. Since the surface proteins of *L. donovani* have shown the potential to bind DLA class I molecules [60], DLA class I alleles may also be important as a risk factor for CVL. Therefore, the information on DLA class I and class II gene polymorphisms is crucial to understand their role in the immune or clinical responses to CVL in dogs. However, only a few investigations have simultaneously analyzed both class I and class II genes in individual dogs [74]. The accumulation of polymorphism data for both DLA class I and class II genes represents a significant approach for investigating the link between canine MHC gene polymorphisms and individual immune response to CVL.

HLA variants located outside of the peptide binding site (PBS) have also been associated with susceptibility to parasitic infections in humans [29,42,91]. Therefore, the method using long-read sequencing technologies has been developed for HLA genotyping to resolve the full-length sequences [67]. Similar to the advancements in humans, full-length DLA sequencing is required to identify risk mutations for CVL in dogs. In recent research on DLA genotyping, a method using next-generation sequencing (NGS) has intensified investigations [37,85]. However, polymerase chain reaction (PCR)-sequence-based typing (SBT) has conventionally been the gold standard in DLA genotyping. To the best of our knowledge, no reports on DLA genotyping utilizing long-read sequencing technology have been published.

In Chapter 1, an experimental challenge model was established by inoculating beagle dogs with *L. donovani* promastigotes to investigate the parasitological and clinical progression of the infection. In Chapter 2, the genetic background of beagle dogs as an experimental animal was characterized by analyzing both DLA class I and class II genes using conventional PCR- SBT, providing a baseline for understanding MHC diversity. Finally, in Chapter 3, a novel DLA genotyping assay was developed to overcome the limitations of conventional methods. This assay utilized long-range PCR amplification of the entire gene lengths followed by Pacific Biosciences (PacBio) Single-Molecule Real-Time (SMRT) sequencing, enabling the comprehensive identification of extended alleles and regulatory polymorphisms at full length sequences of DLA genes.

Chapter 1.

An experimental challenge model for *Leishmania donovani* in beagle dogs

1. Introduction

To assess the clinical, parasitological, and immunological responses in dogs against *L. infantum*, the construction of experimental canine models for *L. infantum* was carried out by considering many factors, including the breed of dogs, stage of the parasite, number of parasites, route of inoculation, and use of vectors [1,17,63,77,82]. However, studies on the experimental infection of dogs with *L. donovani* have never been reported except for one report in two separate studies in which hematological, biochemical, and pathological changes were assessed in German shepherd dogs [52,53]. The lack of experimental studies of CVL by *L. donovani* may be attributed to the concept of anthroponotic transmission of *L. donovani* in humans [64].

It is worth noting that the detection of *L. donovani* DNA by PCR assay or anti-*Leishmania* antibodies by rk39 immunochromatographic test in peripheral blood collected from stray dogs has been reported in India [98], Bangladesh [4,5], Sudan [28,46,97], and Ethiopia [49]. Furthermore, *Phlebotomus argentipes* and *Phlebotomus orientalis*, the known vectors for *L. donovani* in the Indian subcontinent and Ethiopia, respectively, have been reported to take blood meals from domestic animals, including dogs [3,16]. The presence of *Leishmania* DNA in canine blood suggests that dogs play a role in the reservoir of the *L. donovani* transmission cycle in VL-endemic areas, although parasite isolation was rarely successful. Thus, an experimental assessment is needed to understand the parasitological and immunological aspects of *L. donovani* infection in dogs.

In Chapter 1, beagle dogs were inoculated with *L. donovani* promastigotes to construct an experimental challenge model for *L. donovani* in dogs. Challenged dogs were examined for clinical signs and hematological and biochemical parameters for up to 8 months after inoculation. T lymphocyte subsets, including $CD4^+$, $CD8^+$, and $CD4^+CD25^+Foxp3^+$ T cells in the peripheral blood were examined by flow cytometry as the role of regulatory T cells (Tregs) has been reported in the persistence of the same *L. donovani* parasites in the liver of immunodeficient murine VL model [109]. The detection of parasite DNA in the peripheral blood, liver biopsy materials, and spleen autopsy materials was conducted using real-time PCR. Furthermore, the detection of parasite antigens in the liver was performed by immunohistochemistry.

2. Materials and methods

2.1 Animals and ethics statement

A total of three 1-year-old male beagle dogs (approximately 10 kg) were purchased from Hokudo Co., Ltd. (Sapporo, Japan). The dogs were born to different parents in a breeding company. None of the negative control dogs were used due to the 3R principle in animal experiments, in which the number of animals per experiment is reduced to the absolute minimum. All dogs received physical examination and vaccines against leptospirosis, rabies, hepatitis, adenovirus, coronaviruses, distemper, parainfluenza, and parvovirus before the initiation of the experiment. Dogs (A, B, and C) were housed in an animal biosafety level 2 facility in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of Graduate School of Veterinary Medicine, Hokkaido University. Dogs were kept in separate cages and given a standard dog diet and tap water. This study was conducted following the guidance of the Institute for Laboratory Animal Research, which was based on Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. This experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Hokkaido University (Permit Number: 13-0149), following the guidelines of the American Association for Accreditation of Laboratory Animal Care International.

2.2 Parasites and experimental infection

Because of the unavailability of *L. donovani* strains isolated from dogs, we used the *L. donovani* Sudan strain (HOM/SU/62/2S-25M-C2), which was originally isolated from humans and has been characterized by *in vitro* experiments as well as mouse models in previous studies [20,50,51,109]. Promastigotes of *L. donovani* of the frozen stabilized parasites were cultured at 25°C in M199 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 50 µg/ml gentamycin. Dogs were inoculated with 5×10^8 stationary phase promastigotes/kg body weight in 2 ml saline intravenously via the jugular vein. Dogs were observed for a period of up to 8 months after parasite inoculation. At the end of this study, dogs were euthanized by intravenous injection of 50 mg/kg sodium pentobarbital (Kyoritsu

Seiyaku Co., Tokyo, Japan) and 1.0 g/kg potassium chloride. The liver and spleen tissues of euthanized dogs were subjected to DNA extraction and paraffin sections after fixation in a 10% neutral-buffered formalin solution.

2.3. Clinical assessment

Clinical manifestations of challenged dogs were checked by a facility staff veterinarian every day. At the time of blood and liver biopsy sampling, dogs were extensively examined for symptoms of VL, such as skin lesions, weight loss, enlarged lymph nodes, and hepatosplenomegaly. Rectal temperature was not monitored because of the avoidance of direct contact with challenged dogs without anesthesia as much as possible.

2.4. Hematological and biochemical analysis of peripheral blood

Dogs were sedated by intramuscular injection with 0.1 mg/kg midazolam (Midazolam, Teva Pharma Japan Inc., Nagoya, Japan) and 0.2 mg/kg medetomidine hydrochloride (Domitor, Zenoaq Nippon Zenyaku Kogyo, Fukushima, Japan). Anesthesia was reversed by intramuscular injection with atipamezole hydrochloride (Antisedan, Zenoaq Nippon Zenyaku Kogyo). A total of 10 ml of peripheral blood samples was collected from the jugular vein into an ethylenediaminetetraacetic acid (EDTA)-coated tube before parasite inoculation and after parasite inoculation at a monthly interval. Approximately 2, 3, and 5 ml of the samples were used for hematological examination, biochemical examination, and flow cytometry, respectively. In addition, plasma samples were collected after centrifugation of EDTA-blood samples at 1,100 × g at 25°C for 10 min.

Clinical laboratory tests were outsourced to a reference laboratory (Hokudo Co., Ltd.). Hematological parameters included the number of erythrocytes, white blood cells, and platelets, and the value of hematocrit, hemoglobin level, mean cell hemoglobin level, mean cell volume, and mean cell hemoglobin concentration. Differential white blood cells were also determined using an automated counter and hemoglobinometer. In addition, biochemical parameters, such as albumin, albumin/globulin ratio, creatinine, urea, alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, and γ-glutamyl transpeptidase were determined using an autoanalyzer in the company (The Daiichi Kishimoto Clinical Laboratories, Sapporo, Japan).

2.5. rk39 immunochromatographic test

A rapid immunochromatographic strip test, rk39 dipstick (Kalazar Detect™, In Bios. International, Inc., Seattle, WA, USA), was conducted on each canine plasma sample (50 µl) for the qualitative detection of antibodies to visceralizing *Leishmania* species. According to the manufacturer's guideline, when two pink lines (control and test) appeared, even in faint, the test was determined as positive.

2.6. Liver biopsy

Dogs were sedated as described above, and a liver biopsy was performed under ultrasound guidance using a portable ultrasound imaging system (SeeMore™, Medico's Horata Inc., Tokyo, Japan). A 14-gauge biopsy needle (Fine-Core, Toray Medical Co. Ltd., Tokyo, Japan) was percutaneously inserted and maintained in the left and lateral lobes to avoid the gallbladder during penetration of the liver. Two liver tissue samples (20–30 mg each) were taken from each dog for real-time PCR and histopathological examination. The liver biopsy samples were collected before and at 1, 3, 5, and 7 months after parasite inoculation.

2.7. Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were separated using a Percoll reagent (Percoll™ Plus, GE Healthcare Japan, Tokyo, Japan) adjusted to a density of 1.077 g/ml in phosphate buffered saline. Buffy coat samples were collected after centrifugation at 400 × g at 25°C for 30 min and treated with NH_4Cl solution (HLB, Immuno-Biological Laboratories Co. Ltd., Takasaki, Japan) to lyse red blood cells. PBMCs were adjusted to 1×10^7 cells/ml in phosphate buffered saline containing 1.7 mM EDTA supplemented with 10% heat-inactivated FBS. To evaluate the number of canine $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$, and $Foxp3^+$ cells by flow cytometry, the following antibodies were used: fluorescein isothiocyanate (FITC) labeled mouse anti-dog CD3, FITC labeled rat anti-dog CD4, phycoerythrin (PE) labeled rat anti-dog CD4, Alexa Fluor®647 labeled rat anti-dog CD8 (AbD Serotec®, Bio-Rad, Kidlington, UK), PE labeled mouse anti-dog CD25 (clone P4A10, eBioscience, San Diego, CA, USA), and allophycocyanin (APC) labeled rat anti-mouse Foxp3 (clone FJK-16s, eBioscience). The clone FJK-16s is cross-reactive with canine Foxp3 [14], and clone P4A10 can be used to select canine Tregs [2]. Isotype-matched control antibodies, including PE-mouse IgG1κ, FITC-rat IgG2aκ, and APC-rat IgG2aκ (eBioscience) were also used.

$CD4^+$ and $CD8^+$ T cell subsets were identified by a combination of canine anti-CD3 with anti-CD4 or anti-CD8 antibodies. Foxp3 staining was carried out using a

fixation/permeabilization solution following the manufacturer's instructions. A number of 1×10^5 cells were analyzed for each sample by a BD FACSVerse™ flow cytometer and BD FACSuite™ Software (Becton, Dickinson and Company, Sparks, MD, USA).

2.8. Parasite culture

A small piece of liver and spleen sample was aseptically taken from challenged dogs at necropsy and homogenized in phosphate buffered saline. The homogenate was 10-fold serially diluted with Medium 199 (5 µg to 50 mg tissue samples/ml) and cultured in duplicate on 24-well plates in Medium 199 supplemented with 15% heat-inactivated FBS, 25 mM HEPES, and 50 µg/ml gentamycin at 25°C for 4 weeks [50].

2.9. DNA extraction and real-time PCR

Total DNA was extracted from 100 µl of whole blood as well as from 20-30 mg of liver biopsy, liver necropsy, and spleen necropsy samples using a Dneasy Blood & Tissue kit (QIAGEN, Valencia, CA, USA). Genomic DNA was also extracted from cultured *L. donovani* promastigotes using a Dneasy Blood & Tissue kit. DNA concentration was measured by a NanoDrop 2000 Spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA).

Real-time PCR was performed on the LightCycler® Nano System (Roche Diagnostic Japan, Tokyo, Japan) using primers specific for *Leishmania* mitochondrial kinetoplast minicircle DNA (kDNA), RV1 (5'-CTTTTCTGGTCCTCCGGGTAGG-3') and RV2 (5'-CCACCCGGCCCTATTTTACACCAA-3') [71]. A typical 20-µl reaction mixture contained 1× FastStart Essential DNA Green Master (Roche Diagnostic Japan), 0.25 µM of each primer, and 100 ng DNA templates from the blood, liver, and spleen. The reaction was carried out with the initial denaturation at 95°C for 5 min, followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. A standard curve was created using *L. donovani* DNA ranging from 0.005 to 500 pg. Amplification plot, melting temperature I, and threshold cycle of amplification values were generated according to the software provided with the instrument. The amount of parasite kDNA (ng/100 ng DNA of blood and tissue) was determined from three different assays in duplicate or triplicate and is expressed as the average ± SD. Based on the concentration of parasite DNA (0.4 pg/cell) determined in this study, the number of parasites per ml blood was estimated by the following formula:

No. parasites/ml blood = $A/B \times 1/0.4 \times 10^{-3}$

A = amount of kDNA in 100 ng blood DNA (pg)

B = blood volume equivalent to100 ng blood DNA (µl)

2.10. Histopathology and immunohistochemistry

Liver biopsy, liver necropsy, and spleen necropsy specimens were prepared to 2–4- μm-thick serial paraffin sections, and at least one section was stained with hematoxylin and eosin (HE). As a positive control, paraffin-embedded liver sections were prepared from a mouse inoculated with the same *L. donovani* strain in this study [109] were included. For immunohistochemistry, paraffin sections were deparaffinized using xylene and rehydrated with 70–100% ethanol. Sections were then boiled in 0.1 M citrate buffer (pH 6.0) for 20 min to retrieve the epitopes and incubated in 0.3% hydrogen peroxide in methanol at 25°C for 15 min to block endogenous peroxidase activity. After blocking nonspecific immunoglobulin binding by incubation in 10% nonfat milk at 25°C for 10 min, sections were reacted with a mouse monoclonal antibody against *Leishmania* peroxiredoxin (kindly supplied by Dr. Y. Matsumoto, University of Tokyo) diluted 1:500 in phosphate buffered saline-Tween 20 containing 10% nonfat milk at 25°C for 1 h. Sections were washed with phosphate buffered saline and incubated with horseradish peroxidase-conjugated anti-mouse IgG (N-Histofine® Simple Stain MAX-PO (M), Nichirei Biosciences Inc., Tokyo, Japan) at 25°C for 1 h. After washing sections with phosphate buffered saline, immunoreactivity was visualized by using a 3, 3′ -diaminobenzidine (Dojindo, Tokyo, Japan) solution. Hematoxylin was used for a nuclear counterstain.

3. Results

3.1 Clinical assessment and blood analysis

All three dogs inoculated with *L. donovani* promastigotes showed no obvious clinical signs, skin abnormality, nail growth, bleeding, or weight loss throughout the experimental period (8 months) (data not shown). No remarkable changes in hematological (Table 1-1) and biochemical (Table 1-2) parameters were detected, revealing none of the indications of anemia or liver dysfunction in these dogs.

Table 1-1. Hematological parameters in dogs inoculated with *L. donovani* promastigotes.

Dog	Parameter	Post inoculation (Month)							
		0	1	2	3	4	5	6	7
A	WBC (/μL)	11,380	8,080	7,260	9,060	8,220	7,790	7,990	10,670
	RBC ($\times 10^4$/uL)	756	751	747	740	699	680	687	711
	Hb (g/dL)	16.5	16.3	16.1	15.8	14.9	14.7	14.8	15.4
	Ht (%)	52	50.1	49.1	50.1	45.8	45.3	44.2	46.8
	MCV (fL)	69	67	66	68	66	67	64	66
	MCH (pg)	21.8	21.7	21.6	21.4	21.3	21.6	21.5	21.7
	MCHC (%)	31.7	32.5	32.8	31.5	32.5	32.5	33.5	32.9
	Platelet ($\times 10^4$/μL)	42.3	40.9	38.6	34.7	35.8	40.5	34.4	38.1
	Basophil (%)	0	0	0	1	0	0	0	0
	Eosinophil (%)	0	1.4	1	2	0	0	1	4
	Neutrophil Stabkernige (%)	2	0	2	1	3	2	2	4
	Neutrophil Segmented (%)	68	64.2	79	62	60	68	69	71
	Lymphocyte (%)	21	30.1	15	26	33	25	22	19
	Monocyte (%)	9	4.3	3	8	4	5	6	2
B	WBC (/μL)	ND	8,050	6,790	9,750	9,040	9,920	9,360	8,460
	RBC ($\times 10^4$/uL)	ND	869	760	743	752	762	764	813
	Hb (g/dL)	ND	19.1	16.2	16	15.9	16.4	16.5	17.4
	Ht (%)	ND	58.5	50	50.9	50.3	51.4	49.5	53.4
	MCV (fL)	ND	67	66	69	67	67	65	66
	MCH (pg)	ND	22	21.3	21.5	21.1	21.5	21.6	21.4
	MCHC (%)	ND	32.6	32.4	31.4	31.6	31.9	33.3	32.6
	Platelet ($\times 10^4$/μL)	ND	17.8	23.6	24.5	28.4	27.7	25.2	25.1
	Basophil (%)	ND	0	0	1	0	0	0	0
	Eosinophil (%)	ND	3.7	7	5	2	0	3	4
	Neutrophil Stabkernige (%)	ND	1	2	2	3	3	1	3
	Neutrophil Segmented (%)	ND	68.4	70	77	75	70	71	69
	Lymphocyte (%)	ND	22	18	10	17	25	19	18
	Monocyte (%)	ND	4.7	3	5	3	2	6	6
C	WBC (/μL)	ND	6,140	8,330	7,440	9,560	10,690	11,680	10,850
	RBC ($\times 10^4$/uL)	ND	780	821	723	828	827	825	837
	Hb (g/dL)	ND	16.9	17.9	15.8	19.3	18	18	18.6
	Ht (%)	ND	53	55.4	49.7	56.9	56.2	54.5	56.1

MCV (fL)	ND	68	67	69	69	68	66	67
MCH (pg)	ND	21.7	21.8	21.9	23.3	21.8	21.8	22.2
MCHC (%)	ND	31.9	32.3	31.8	33.9	32	33	33.2
Platelet ($\times 10^4/\mu L$)	ND	26.4	18.1	0.4	16.6	13.5	15.8	16.4
Basophil (%)	ND	0	0	0	0	0	0	0
Eosinophil (%)	ND	2.1	2	1	0	1	1	3
Neutrophil Stabkernige (%)	ND	0.2	2	3	4	5	1	5
Neutrophil Segmented (%)	ND	65.8	86	72	72	55	80	66
Lymphocyte (%)	ND	28.9	9	20	21	37	15	25
Monocyte (%)	ND	3	1	4	3	2	3	1

ND: no data, RBC: red blood cell, WBC: white blood cell, Hb: hemoglobin, Ht: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration

Table 1-2. Biochemical parameters in dogs inoculated with *L. donovani* promastigotes.

Dog	Parameter	Post inoculation (Month)							
		0	1	2	3	4	5	6	7
A	A/G	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
	Alb (g/dL)	2.7	2.6	2.5	2.6	2.5	2.5	2.6	2.6
	AST [GOT] (U/L)	34	21	21	23	23	20	20	22
	ALT [GPT] (U/L)	34	31	30	29	32	45	29	34
	γ-GT(γ-GTP) (U/L)	4	4	4	4	4	4	4	5
	ALP (U/L)	0	-	-	1	<2	<2	<2	<2
	UN (mg/dL)	21.8	11.1	13.5	14.9	12.8	12.8	9.8	11.7
	Creatinine (mg/dL)	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.5
B	A/G	0.7	0.7	0.8	0.7	0.6	0.6	0.6	0.8
	Alb (g/dL)	2.6	2.5	2.6	2.6	2.5	2.4	2.4	2.7
	AST [GOT] (U/L)	31	22	27	32	27	26	31	26
	ALT [GPT] (U/L)	29	26	34	29	33	37	30	30
	γ-GT(γ-GTP) (U/L)	3	3	5	3	4	3	3	4
	ALP (U/L)	-	-	-	1	<2	<2	<2	<2
	UN (mg/dL)	10.9	10.6	10.2	12.1	14.2	9.9	8.2	11.3
	Creatinine (mg/dL)	0.6	0.5	0.6	0.6	0.6	0.6	0.6	0.6
C	A/G	0.9	0.8	0.7	0.9	0.7	0.8	0.7	0.8
	Alb (g/dL)	2.5	2.6	2.6	2.8	2.6	2.6	2.7	2.7
	AST [GOT] (U/L)	22	20	33	41	26	30	31	30
	ALT [GPT] (U/L)	34	34	66	44	43	238	48	57
	γ-GT(γ-GTP) (U/L)	3	3	3	3	4	3	3	4
	ALP (U/L)	-	-	-	1	<2	<2	<2	<2
	UN (mg/dL)	13.9	14.2	16.6	21.3	17.3	18.4	17.2	14.5
	Creatinine (mg/dL)	0.5	0.5	0.6	0.6	0.7	0.7	0.7	0.6

-: not determined, A/G: albumin/globulin ratio, Alb: albumin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GT: γ-glutamyl transpeptidase, ALP: alkaline phosphatase, UN: urea

3.2. Detection of anti-Leishmania *antibodies by rk39 test*

Plasma samples collected before inoculation were negative, while very faint positive bands were detected in all three canine samples at 1 and 3 months after parasite inoculation. As the test lines were very faint and difficult in appearance in a photograph (Figure 1-1a), the pictures of the test bands were enhanced for visualization using a photo editing software (Figure 1-1b). However, plasma samples obtained at 7 months of inoculation showed negative reactions in all dogs (Figure 1-1b).

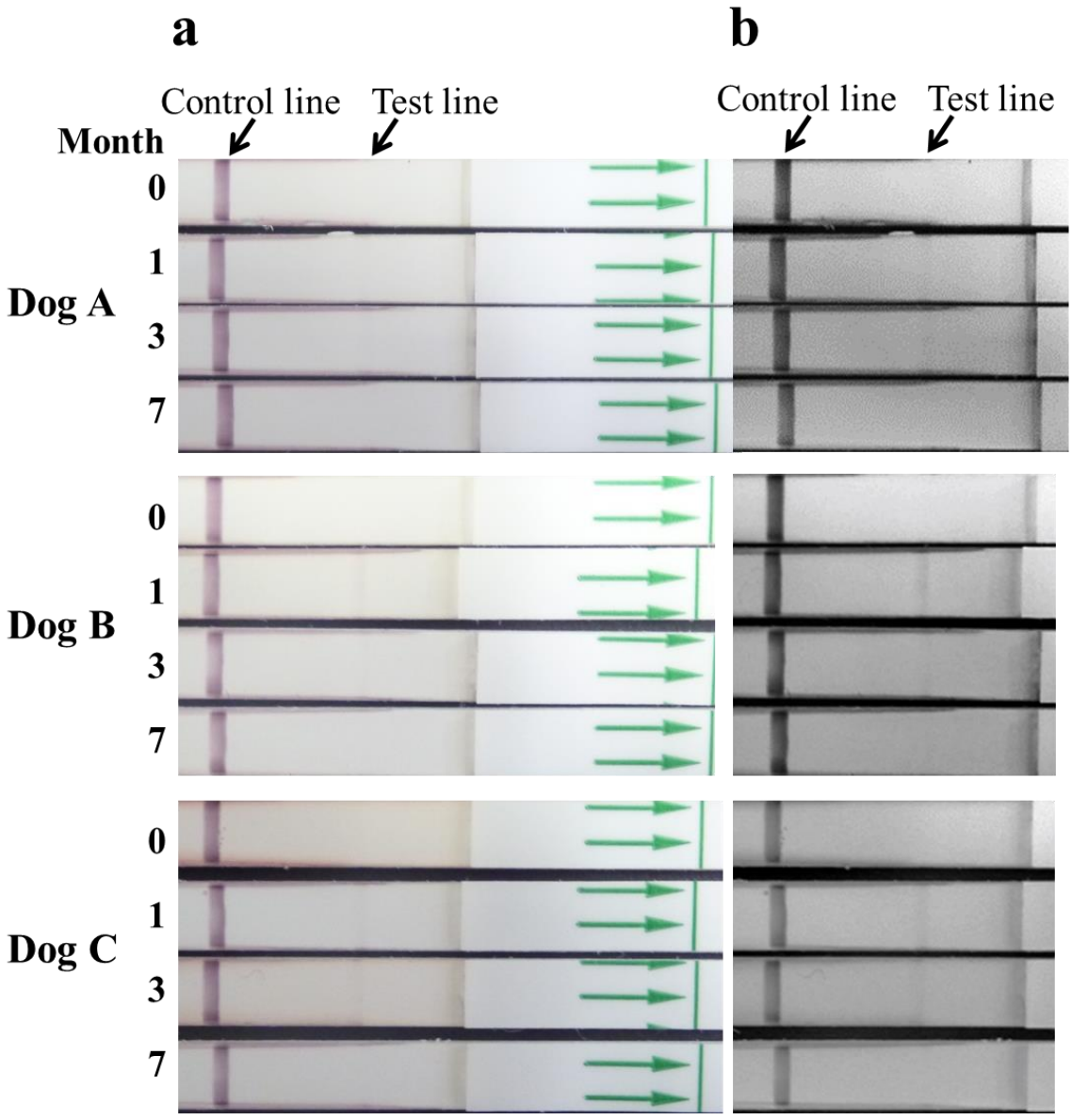

Figure 1-1. Detection of antibodies against *Leishmania* parasites in dogs inoculated with *L. donovani* promastigotes.

The reactions in rk39-dipsticks (a) were digitally enhanced using a photo editing software (b).

3.3. Flow cytometry

In an immunodeficient mouse model, FoxP3+ Tregs are involved in the persistence of *L. donovani* infection in the liver [109]. To address whether Tregs are also involved in the parasite load in the present canine model of *L. donovani* infection, the kinetics of CD4+FoxP3+ and CD4+CD25+FoxP3+ cells in the peripheral blood were examined. The light-scatter characteristics of lymphocytes were used to gate CD3+ and CD4+ T cells (Figure 1-2a and 1-2b) for further analysis of CD3+CD4+ and CD3+CD8+ T cell subpopulations (Figure 1-2c). CD25+Foxp3+ T cells were gated from CD4+ T cells (Figure 1-2d). The ratio of CD25+FoxP3+ to CD4+ T cells varied in three dogs and slightly increased at 6 months of parasite inoculation, but the percentages appeared to be consistent and no conclusive evidence was obtained (Figures 1-3).

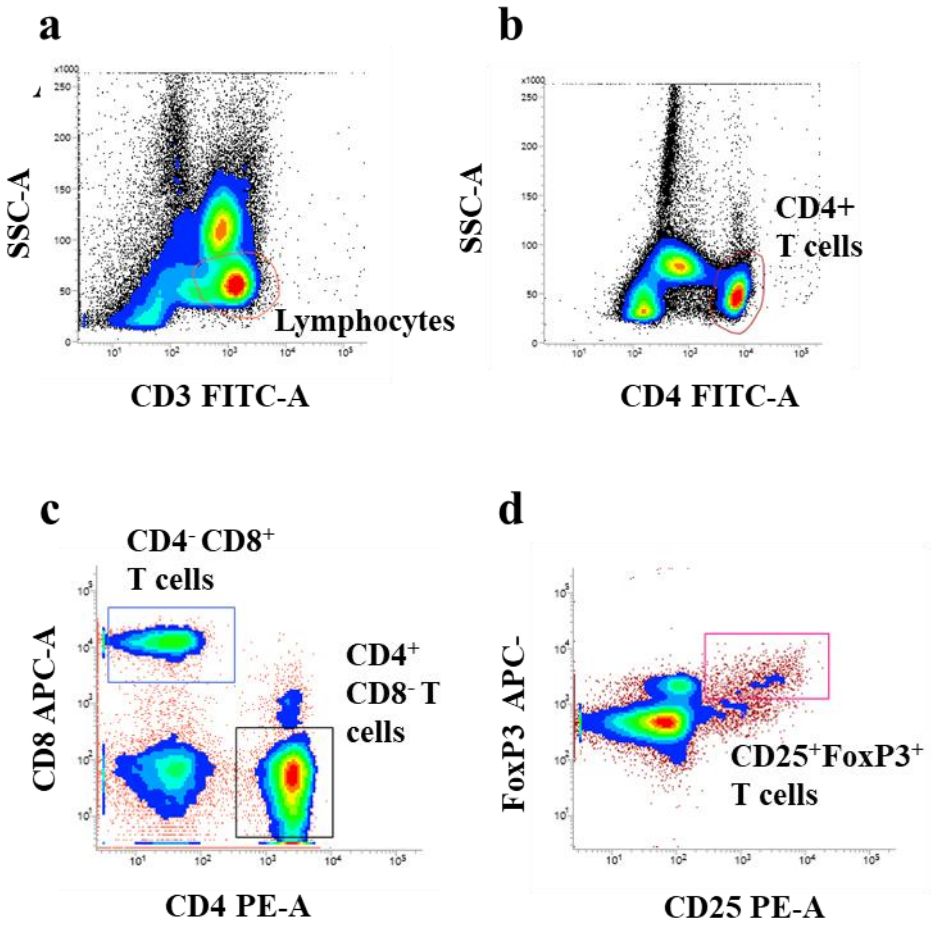

Figure 1-2. A typical flow cytometry analysis, showing $CD3^+$ (a), $CD4^+$ (b), $CD4^+$ $CD8^-$ (c), and $CD4^+CD25^+FoxP3^+$ (d) T cell subpopulations in the PBMCs from dog A collected before inoculation with *L. donovani*.

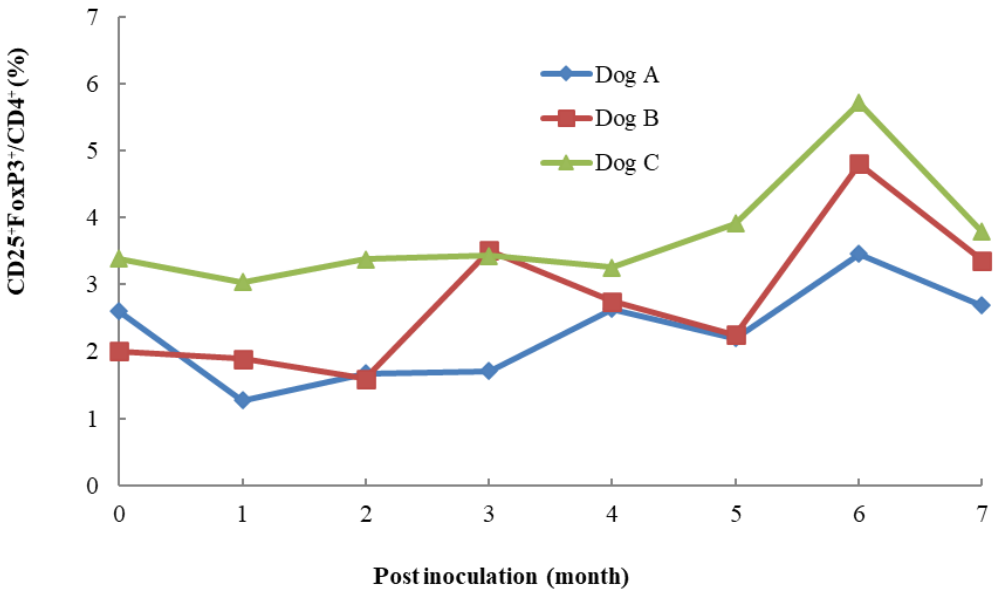

Figure 1-3. Kinetics of $CD25^{+}FoxP3^{+}/CD4^{+}$ T cell subpopulations in the peripheral mononuclear cells from dogs inoculated with *L. donovani*.

3.4. Isolation of the parasites by culture

Necropsy liver and spleen samples from the three challenged dogs were cultured in the medium for up to 4 weeks. However, no parasites were detected in the culture medium.

3.5. Real-time PCR

To assess the presence of *Leishmania* kDNA in the canine peripheral blood, real-time PCR was performed on blood samples at 1, 3, 4, 5, 6, and 7 months after inoculation. In a standardization for real-time PCR assay, an amplification (Figure 1-4a) and a standard curve ($r^2 = 0.9936$, E = 1.813, Error = 0.521) (Figure 1-4b) were clearly created in the range of 0.005 pg (5 fg) and 500 pg of *Leishmania* DNA. The melting temperature ranged from 83.19°C to 83.36°C, indicating the specific amplification (Figure 1-4c). As the DNA concentration of the parasites used in this study was 0.4 pg/cell, the detection limit in the real-time PCR assay using SYBR Green reagents was 5 fg, equivalent to a 1.25×10^{-5} parasite. A considerable amount of *Leishmania* kDNA was detected from all three canine blood DNA samples collected at 4, 5, 6, and 7 months after inoculation (Figure 1-5a). The highest amount of kDNA was calculated as 0.81 ± 0.11, 2.35 ± 2.22, and 1.10 ± 1.31pg/100 ng of blood DNA at 5 months after inoculation for dogs A, B, and C, respectively (Figure 1-5a). The DNA amount was equivalent to $0.77–2.27 \times 10^{-4}$ parasites per ml of the original peripheral blood.

Similarly, a considerable amount of parasite kDNA was also detected from the liver biopsy DNA samples in all three dogs at 5 and 7 months after inoculation (Figure 1-5b). A similar pattern of kDNA amount was observed in the peripheral blood and liver, in which the peak parasite load was detected at 5 months of parasite inoculation (Figure 1-5a and 1-5b). Further, parasite kDNA was detected from the spleen necropsy samples at 8 months after inoculation, although the amount was less compared to the level in the liver (Figure 1-6).

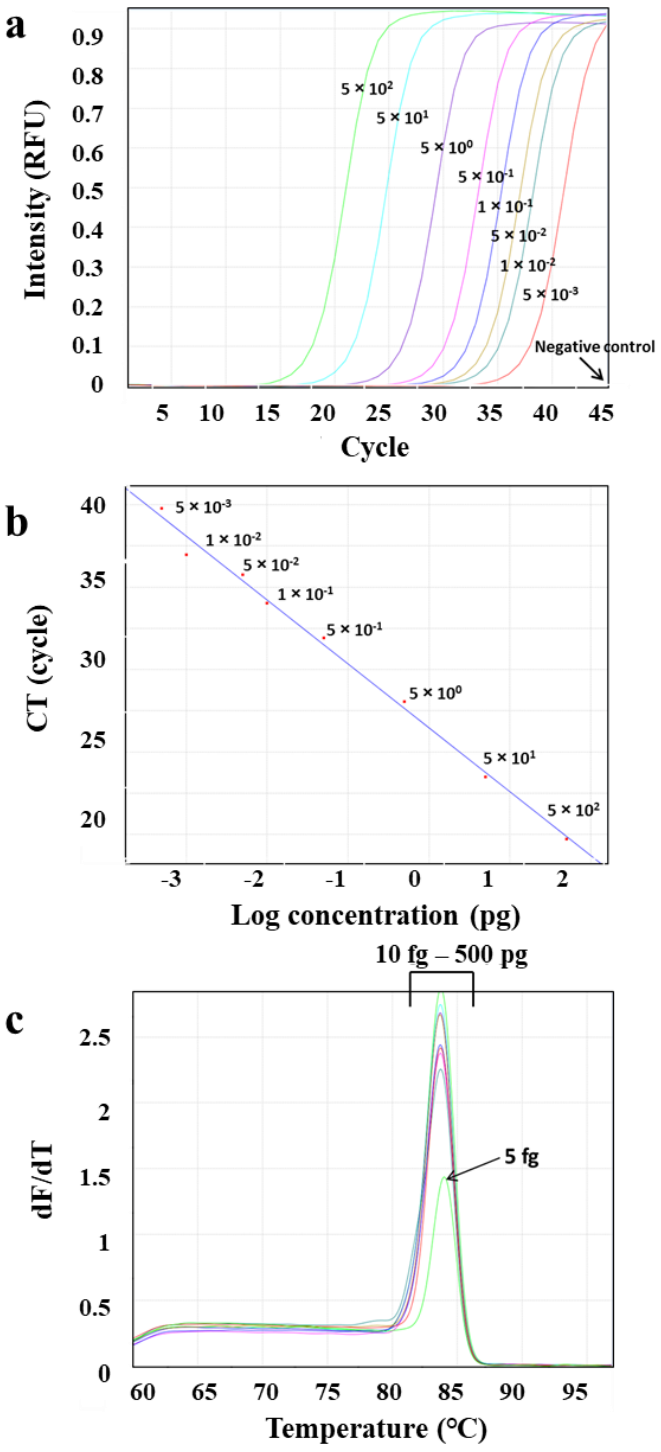

Figure 1-4. A typical amplification (a), standard curve (b) and melting curve (c) of *Leishmania* kDNA created by real-time PCR using total *Leishmania* DNA ranging from 0.005 to 500 pg.

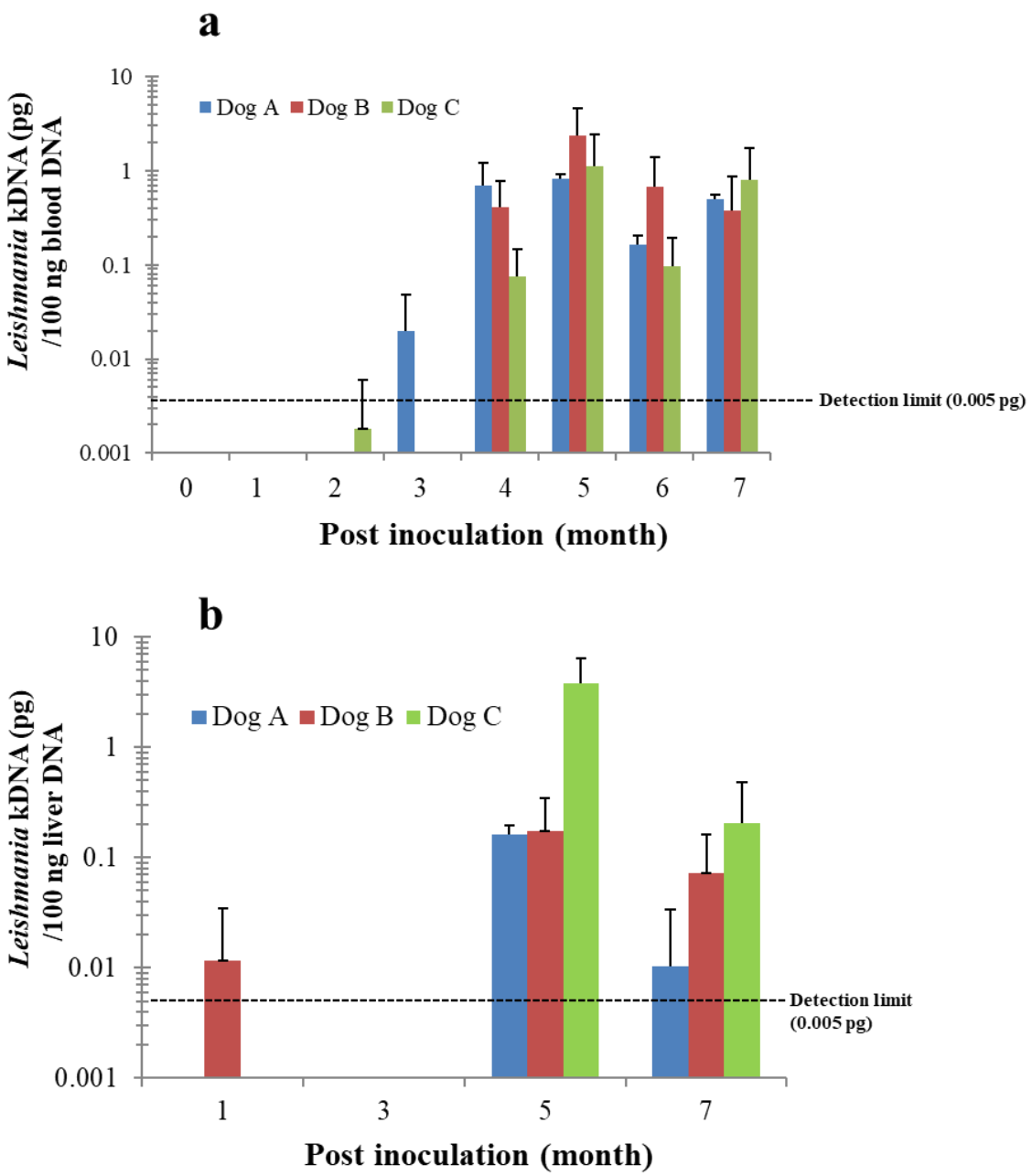

Figure 1-5. Detection of *Leishmania* kDNA using real-time PCR from the peripheral blood (a) and liver biopsy samples (b) of dogs after inoculation with *L. donovani*.

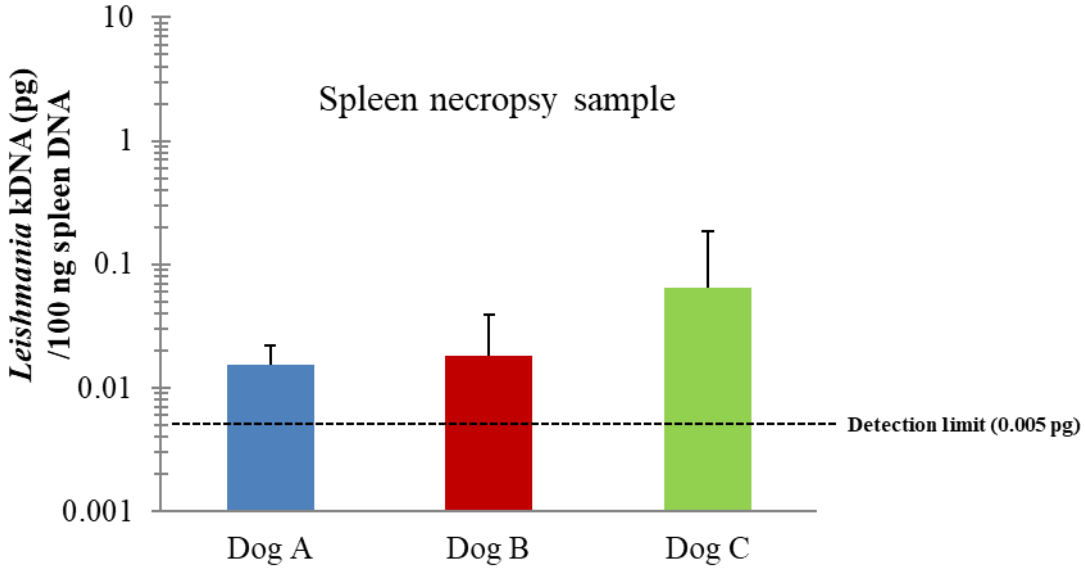

Figure 1-6. Detection of *Leishmania* kDNA using real-time PCR from the spleen necropsy samples of dogs at 8 months after inoculation with *L. donovani*.

3.6. Histopathology and immunohistochemistry

Although careful microscopic examination of HE-stained sections was conducted, there were no remarkable inflammatory responses or granuloma formation in the canine liver and spleen. Based on the results of parasite burden by real-time PCR assay, the liver biopsy sections at 5 and 7 months after parasite inoculation were further examined by immunohistochemical staining using a monoclonal antibody against *Leishmania* peroxiredoxin. The immunohistochemistry showed many reacted areas in a positive control murine liver section (Figure 1-7a). Similar staining portions were rarely observed in the liver biopsy sections, but a typical immunochemical reaction was detected in the liver biopsy section at 5 months in dog B (Figure 1-7b). However, HE-counterstaining revealed no remarkable inflammatory response around the parasite-antigen positive area (Figure 1-7c)

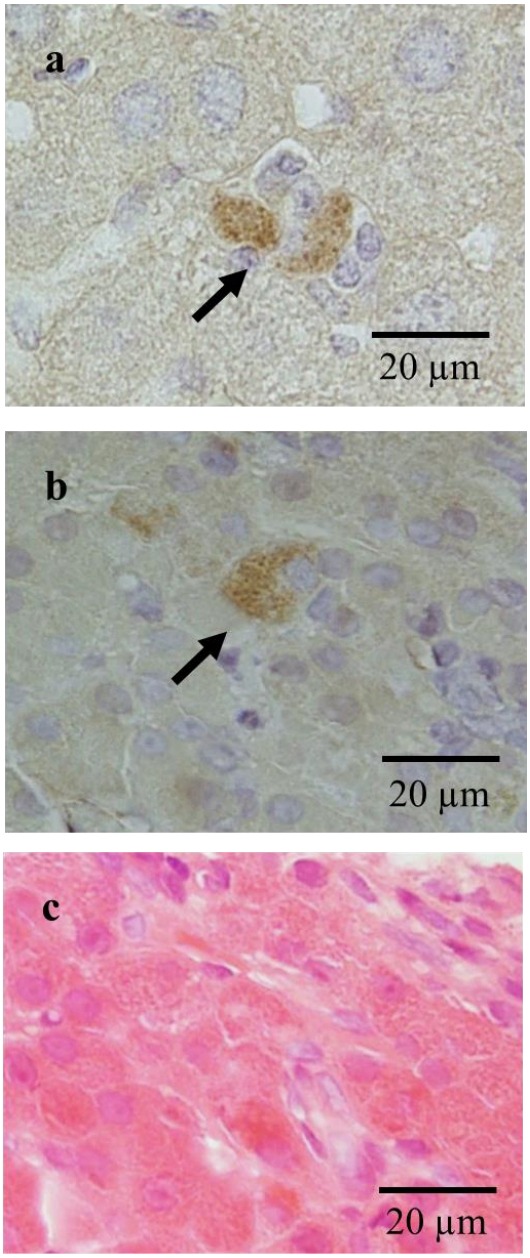

Figure 1-7. Immunohistochemistry for detection of parasite antigens.

Monoclonal antibody against *Leishmania* peroxiredoxin was reacted with a murine liver section prepared from a mouse infected with *Leishmania donovani* in the previous study as a positive control (a). A canine liver biopsy section from dog B after 5 months of inoculation with the same *Leishmania donovani* strain in this study was reacted with the monoclonal antibody (b). One of the canine liver consequent sections was stained with HE (c). Arrows in a and b indicate cells expressing *Leishmania* peroxiredoxin.

4. Discussion

In Chapter1, an experimental canine challenge model for *L. donovani* promastigotes was constructed using beagle dogs. Although this model showed an asymptomatic CVL with a very low parasite burden, a considerable amount of parasite kDNA was detected from the peripheral blood, liver, and spleen of challenged dogs. Liver biopsy sampling enabled us for the first time to compare the amount of parasite DNA in the peripheral blood and liver of the same animal. It is worth noting that the amount of parasite DNA during the experimental period showed a similar pattern with a peak at 5 months in the circulating blood and liver tissue. The blood parasite DNA may be attributed to the proliferation and dissemination of parasites or the destruction of parasites in the liver at the late phase of this experimental infection.

The isolation of the parasite from the necropsy samples was unsuccessful. This may be due to the reduction of the number of parasites after the peak parasite burden at 5 months in the liver and to the lower parasite burden in the spleen, even though parasite DNAs remained in the organs. Nevertheless, the detection of parasite DNA in the blood in this study may support the concept that dogs play a role in maintaining *L. donovani* parasites, as suggested by several reports on VL-endemic areas [4,28,46,49,98].

Only one experimental infection of dogs (German Shepherd) with *L. donovani* (Khartoum strain, probably human isolate) was reported in two separate papers [52,53]. The dogs received intravenous inoculation with the amastigotes. The dogs developed CVL symptoms, including moderate splenomegaly and lymphadenomegaly. Parasites were detected in cultures from the spleen, lymph node, liver, and bone marrow. Visceralizing species of *Leishmania*, *L. donovani* and *L. infantum*, establish long-term infection within different organs, including the liver, spleen, and bone marrow. Organ-specific immune responses have been associated with VL in murine models infected with *L. donovani*, revealing self-limitation in hepatic infection and persistent infection in the spleen, accompanied by profound structural alterations [33,34]. From the CVL model in this study, no conclusive evidence showed an increase or decrease in the percentage of $CD4^+$, $CD8^+$, $CD4^+FoxP3^+$, or $CD4^+CD25^+FoxP3^+$ T cell populations in the blood, although $FoxP3^+$ Treg subset was involved in hepatic parasite persistence in an immunodeficient mouse model [109]. A significant increase in $CD3^+CD8^+$ T cells and a reduced percentage of $CD3^+CD4^+FoxP3^+$ T cells in the peripheral blood have been reported in dogs naturally

infected with *L. infantum* [22]. A decrease of $CD4^+FoxP3^+$ T cells in the peripheral blood and spleen was reported in dogs naturally infected with *L. chagasi* [101]. Therefore, the immune response in the present asymptomatic CVL model by *L. donovani* inoculation in this study suggests that it may differ from other *Leishmania* diseases.

When considering this difference, it is important to consider that immune responses can vary due to species-specific *Leishmania* surface proteins and the host's genetic background. A comparative whole-genome analysis of major *Leishmania* species revealed that 55 out of 8,032 protein-coding genes were specific to *L. donovani*, while 60 out of 8,241 were specific to *L. infantum* [90]. Regarding the genetic factors, HLA gene polymorphisms have been reported as risk factors for VL infection in humans[25,36]. *Leishmania donovani* surface proteins[30,60] also suggest a binding affinity for specific HLA allele molecules. In dogs, the *L. donovani* surface proteins have also been analyzed to have a binding affinity to DLA class I molecules[60]. Additionally, associations between DLA gene polymorphisms and serum antibody levels have been noted in dogs during infection experiments with *L. infantum*[105]. Therefore, studying DLA polymorphisms is important not only for understanding asymptomatic infection but also for improving and standardizing the canine challenge model. Analyzing how individual immune profiles relate to infection through detailed DLA studies is essential for making the canine model more reliable and useful for future research on CVL and other canine immune-mediated diseases.

5. Summary

Leishmania donovani and *L. infantum* are closely related species. However, the former is considered the causative agent for anthroponotic VL (AVL), while the latter is known to be responsible for zoonotic VL, with dogs serving as the primary reservoir host. Although molecular detection of *L. donovani* from naturally infected dogs has been reported in AVL-endemic areas, experimental infection of dogs with this species is very limited. Here, we constructed an experimental CVL model with *L. donovani* infection using beagle dogs. During an 8-month observation period after parasite inoculation, few clinical symptoms were observed in the three inoculated dogs. The overall hematological and biochemical data of the dogs showed normal levels, and there were no remarkable changes in the peripheral $CD4^+$, $CD8^+$, $CD25^+$, or $FoxP3^+$ T cell populations. Liver biopsy sampling was conducted to monitor the parasite burden in the liver. A similar pattern of the amount of mitochondrial kinetoplast DNA was observed in peripheral blood and liver by real-time PCR analysis. In addition, parasite antigens were detected from the liver biopsy sections by immunohistochemical analysis, further supporting the existence of parasites in the liver. These results demonstrated a subclinical CVL model for *L. donovani* in beagle dogs characterized by a similar kinetics of parasite burden in the peripheral blood and liver.

Chapter 2.

Dog leukocyte antigen genotyping across class I and class II genes in beagle dogs as laboratory animals

1. Introduction

The MHC plays a crucial role in the immune response by presenting antigen peptides to T cells. There are two main classes of MHC molecules, class I and class II, which have distinct roles in immunity. MHC class I molecules are expressed on the surface of most cells [41], while MHC class II molecules are expressed on selected cells. In humans, they are constitutively expressed on antigen-presenting cells, including dendritic cells, B cells, macrophages, and thymic epithelial cells [88]. Generally, class I molecules predominantly present peptides derived from the cytoplasm and nucleus, whereas class II molecules present peptides from intracellular vesicles and organelles, which receive antigens from the extracellular space [23]. MHC genes are among the most polymorphic genes in vertebrate genomes and are known as HLA genes in humans. HLA gene polymorphisms are well documented to be associated with the risk, susceptibility, and severity of various immune-related phenomena, including transplant rejection [6], autoimmune diseases [27], infectious diseases [73], and adverse drug reactions [48]. For instance, specific MHC alleles, such as *HLA-B*57:01* in class I and *HLA-DRB1*04:01* in class II, have been linked to drug-induced hypersensitivity reactions [79] and autoimmune diseases like rheumatoid arthritis [40], respectively. Furthermore, the combination of class I and class II alleles has been associated with the risk of autoimmune diseases [102]. Therefore, genotyping both class I and class II MHC genes is crucial for understanding immune responses and their inter-individual differences.

DLA genes are similar to those in humans in terms of the basic organization of the MHC region but have differences. For example, the MHC genes are distributed on different chromosomes. Specifically, *DLA-79* is located on chromosome 18, although the other three DLA class I genes (*DLA-88*, *DLA-12*, and *DLA-64*) and four class II genes (*DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) are on chromosome 12. In addition, a previous study indicated that the *DLA-88L* gene, another DLA class I locus, was generated by a gene conversion event between *DLA-88* and *DLA-12* and located in the *DLA-12* genetic region [75]. Unlike HLA, *DLA-DP* genes have been reported to be pseudogenes [26]. Alleles of DLA are registered in the IPD-MHC database (https://www.ebi.ac.uk/ipd/mhc/) [69,70], which currently contains over 400 DLA alleles (139 *DLA-88*, 17 *DLA-12*, 9 *DLA-64*, 8 *DLA-79*, 161 *DLA-DRB1*, 30 *DLA-DQA1*, and 79 *DLA-DQB1* alleles) (release 3.11.0.0; accessed on

2nd June, 2025). Of the 139 alleles named *DLA-88*, 13 were reclassified as the *DLA-88L* gene [74]. To date, no genetic polymorphisms have been identified in the *DLA-DRA* gene, and no alleles have been registered in the IPD-MHC database.

DLA gene polymorphisms have also been shown to be associated with various pathological conditions [55,58,81]. Focusing on the CVL study, the relationship between DLA class II alleles and the course of *L. infantum* infection in a group of naturally infected dogs [86]. In addition, a study using experimentally infected beagle dogs reported associations between DLA class II alleles and increased anti-*Leishmania* antibody levels [105]. Since the surface proteins of *L. donovani* have shown potential to bind the *DLA-88* molecule [60], DLA class I alleles may also be important as a risk factor for CVL. Therefore, the information on DLA class I and class II gene polymorphisms is crucial to confirm their associations with the immune or clinical responses to CVL in dogs. However, only a few investigations have simultaneously analyzed both class I and class II genes in individual dogs [74]. One reason for this is the lack of established genotyping methods due to the structural complexity of class I genes, particularly *DLA-88*, *DLA-88L*, and *DLA-12*. Recently, Miyamae et al. [74] developed a locus-specific genotyping method for these genes, enabling the identification of multiple class I and class II alleles in the same individual using simple PCR-based amplicon sequencing [54,89,112]. The accumulation of data on both DLA class I and class II gene polymorphisms using this method is a valuable approach for analyzing the relationship between DLA polymorphisms and individual differences in immune responses.

Two strains of beagle dogs (TOYO and Marshall Beagles) have been widely used for research in Japan. Differences in body weight and sexual maturation between strains of beagle dogs have been reported, which is probably due to the genetic background [31]. However, to the best of our knowledge, no DLA gene analyses have been reported for either of these strains, nor has any comparison of DLA gene polymorphisms between them. Moreover, research on individual differences in immune-related responses has not been reported. There is thus a need to evaluate and organize the genetic diversity of DLA in laboratory beagle dogs in order to utilize these individuals for research aimed at elucidating differences in susceptibility and further pathogenic mechanisms. In the present study, we genotyped all class I and class II genes, four DLA class I and four class II genes, in 93 beagle dogs derived from the two different strains. The obtained data should facilitate the use of

laboratory beagle dogs to investigate disease processes in various immune-related responses associated with a specific DLA type.

2. Materials and methods

2.1. Animals

Ninety-three beagle dogs were used in this study. A total of 47 TOYO Beagles were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan), while 46 Marshall Beagles were obtained from Marshall Bioresources Japan (Tsukuba, Japan). The animals were returned to general housing upon the completion of this study. This study was conducted in accordance with the "Law Concerning the Protection and Control of Animals" (Japanese Law No. 105; October 1, 1973) and the "Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Organizations under the Jurisdiction of the Ministry of Health, Labour and Welfare" (Notification No. 0601001; issued by the Japanese Ministry of Health, Labour and Welfare; June 1, 2006). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd., and all animal procedures were performed following the guidelines of the Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

2.2. Genomic DNA extraction

Approximately 0.5 ml of blood was collected from the jugular vein of each animal using a disposable syringe (Terumo Corporation, Tokyo, Japan). Blood samples were placed in blood sampling tubes containing EDTA dipotassium salt (Microtainer; Nippon Becton Dickinson Company, Ltd., Tokyo, Japan). Genomic DNA was extracted from each blood sample using a QIAamp Blood Mini Kit (QIAGEN), following the manufacturer's protocol. In addition, the amount of gDNA in each sample was confirmed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. PCR of DLA genes

Table 2-1 shows the specific primer sets used for amplifying *DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1* genes. PCR amplification of *DLA-12/88L* genes followed the method previously described by Miyamae et al. [74]. The first PCR was intended to amplify the 5.6 kb genomic region of the DLA-12/88L genes. The PCR mixture, with a total volume of 50 µl, contained 20 ng of DNA, 1 unit of KOD FX polymerase (TOYOBO, Osaka, Japan), 25 µl of 2×PCR buffer, 10 µl of

2 mM deoxynucleotide triphosphate (dNTP), and 15 μM of each primer. The thermal cycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 33 cycles of 98 °C for 10 s, 58 °C for 30 s, and 68 °C for 36 s. The second PCR, using specific primer combinations for *DLA-12* and *DLA-88L* alleles, was performed to amplify the 1.6 kb region of each gene. The composition of the second PCR mixture for amplifying *DLA-12* or *DLA-88L* was the same as in the first PCR, and the thermal cycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 33 cycles of 98 °C for 10 s, 63 °C for 30 s, and 68 °C for 96 s.

For the other genes, PCR was conducted to amplify the 4.0 kb, 1.6 kb, 1.1 kb, 313 bp, 303 bp, 345 bp, and 300 bp genomic regions for *DLA-88*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*, respectively. The PCR mixture, with a total volume of 50 μl, contained 20 ng of DNA, 1 unit of KOD FX polymerase, 25 μl of 2×PCR buffer, 10 μl of 2 mM dNTP, and 15 μM of each primer. The thermal cycling conditions for these genes were as follows: initial denaturation at 94 °C for 1 min, followed by 33 cycles of 98 °C for 10 s, 63 °C for 30 s, and 68 °C for 4 min for *DLA-88*; 98 °C for 10 s, 63 °C for 30 s, and 68 °C for 90 s for *DLA-64* and *DLA-79*; 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 30 s for *DLA-DQA1*; and 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 30 s for *DLA-DRA*, *DLA-DRB1*, and *DLA-DQB1*.

Table 2-1. A list of locus-specific primers used for PCR

DLA locus	Forward/reverse	Primer sequence (5' ’o 3')	Reference
DLA-88	F	AGGGGACAATGGGACAGGAACTTGGA	Ross et al. (2012)
	R	AGGACTCAGGGAGACAGTGCGACAAGA	Miyamae et al. (2018)
DLA-12/88L (first PCR)	F	CATTTCTGTTGGGATATGTGGTAA	Ross et al. (2012)
	R	CATCAAGGGATAAGGTGAAAGA	Miyamae et al. (2018)
DLA-12 (second PCR)	F	CGACCCTAAAGGTCTGGGCTA	Ross et al. (2012)
	R	GGTGGCGGGTCACACG	Miyamae et al. (2018)
DLA-88L (second PCR)	F	CGGAGATGGAGGTGGTGA	Ross et al. (2012)
	R	GGTGGCGGGTCACACG	Miyamae et al. (2018)
DLA-64	F	CGGAGATGGAGGTGGTGA	Ross et al. (2012)
	R	GGGTGGCGGGTCAGGTAG	Miyamae et al. (2018)
DLA-79	F	CAGGGCTGACAGTGTCTCCTCTGC	Venkataramann et al. (2013)
	R	CACTGAGTCCACCCTCCCTTCC	Venkataramann et al. (2013)
DLA-DRA	F	TAATACGACTCACTATGAGGCTGGTTTCCACCCTAACTCTC	This study*
	R	GATCCCCATGTCTAGGAGTG	This study*
DLA-DRB1	F1	GTAAAACGACGGCCAGTGGATCCCCCCGTCCCCACAG	Kennedy et al. (2006)
	F2	GTAAAACGACGGCCAGTGCCGTCCCCACAGCACATTTC	Wargner et al. (1996)
	R	AGCGGATAACAATTTCACACGCCCGCTGCGCTCA	Kennedy et al. (2006)
DLA-DQA1	F	GTAAAACGACGGCCAGTGTAAGGTTCTTTTCTCCCTCTG	Kennedy et al. (2006)
	R	GGACAGATTCAGTGAAGAGAG	Kennedy et al. (2006)
DLA-DQB1	F	GTAAAACGACGGCCAGTGCTCACTGGCCCGGCTGTCTC	Kennedy et al. (2006)
	R	CACCTCGCCGCTGCAACGTG	Kennedy et al. (2006)

*: newly designed for this study

Underline indicates the sequences of universal regions assigned to specific primers.

2.4. Genotyping and haplotyping

After PCR products were purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK) for class I genes and ExoSAP-IT (Thermo Fisher Scientific) for class II genes, they were directly sequenced by Sanger sequencing (Azenta, Tokyo, Japan). The sequencing primers used for *DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1* genes are listed in Table 2-2. To verify that no missing alleles existed in *DLA-DRB1*, a double-check was performed using another forward primer (DRB1-F2, 5'-CCGTCCCCACAGCACATTTC-3' [114]) under the same PCR conditions, considering the reported possibility of allele dropout by the DRB1-F1 primers of Table 2-1 [57]. The nucleotide sequences of the PCR products for *DLA-88*, *DLA-12*, and *DLA-88L* were identified using the sequencing primers i1F-T and i3R-T. In cases where the *DLA-88* allele sequences were difficult to identify due to sequence offsets caused by nucleotide insertions and deletions in intron 2 and/or exon 3 of these alleles, additional sequencing was performed using a different primer, i2F2. For *DLA-12* and *DLA-88L*, only the PCR products with visible bands on the electrophoresis gel were subjected to sequencing. Allele assignment for each individual was performed with GENETYX-SV/RC Ver.13.1.1 (Genetyx Corporation, Tokyo, Japan) by comparing the determined nucleotide sequences with all known DLA allele sequences published in the IPD-MHC database and the National Center for Biotechnology Information (NCBI) database. We assigned a provisional allele name to the new allele. Some alleles that had not yet been assigned official names were named in accordance with a previous report [74]. To ascertain the haplotypes of *DLA-88*, *DLA-12/88L*, *DLA-DRB1*, *DLA-DQA1*, and/or *DLA-DQB1* genes located on chromosome 12, the haplotypes of homozygous dogs were initially identified, after which the haplotypes of heterozygous dogs were estimated manually based on the homozygous dogs. The program PHASE v2.1.1 [106] was used to confirm the haplotypes detected in this study. To statistically compare the frequencies of alleles and haplotypes between strains, Fisher's exact test was performed using EXSUS software version 10.1.8 (EPS Corporation, Tokyo, Japan). Bonferroni adjustments for multiple comparisons were not performed, as recommended in similar studies [84,107].

Table 2-2. A list of sequencing primers

DLA locus	Primer name	Primer sequence (5' to 3')	Reference
DLA-88	i1F-T	AGGGGGTCGGGCGGGGT	Miyamae et al. (2018)
	i3R-T	GAGTCCATATTCCCTTCCTGG	Miyamae et al. (2018)
	i2F2	GGTTTTACTTTCTCTTTGGACTG	This study*
DLA-12, 88L	i1F-T	AGGGGGTCGGGCGGGGT	Miyamae et al. (2018)
	i3R-T	GAGTCCATATTCCCTTCCTGG	Miyamae et al. (2018)
DLA-64	i1F-T	AGGGGGTCGGGCGGGGT	Miyamae et al. (2018)
	DLA-64_i3R	CCAGCGGGAGGGGAGATC	This study*
DLA-79	DLA79-i1F	CCACCGACCAGCCCCTAAGC	This study*
	DLA79-i3R	CACTGAGTCCACCCTCCCTTCC	Venkataramann et al. (2013)
DLA-DRA	T7	TAATACGACTCACTATGAGGC	Universal
DLA-DRB1	M13-20	GTAAAACGACGGCCAGTG	Universal
	M13rev	AGCGGATAACAATTTCACA	Universal
DLA-DQB1, DLA-DQA1	M13-20	GTAAAACGACGGCCAGTG	Universal
DLA-DQB1	DQB1-S-R	CACCTCGCCGCTGCAACGTG	Kennedy et al. (2006)

*: newly designed for this study

Underline indicates the sequences of universal regions assigned to specific primers.

2.5. Subcloning to resolve genotyping ambiguity

To resolve ambiguity in DLA genotyping, PCR products that exhibited ambiguity were subcloned into the pTA2 cloning vector using TArget Clone™ Plus (TOYOBO), following the manufacturer's protocol. This allowed the separation of alleles according to parental origins. In addition, PCR samples of *DLA-88* were subjected to nested PCR with *DLA-88L* primers using the same thermal cycling conditions as previously described before subcloning. Randomly selected independent clones were then subjected to PCR with a mixture containing 0.4 units of KOD FX polymerase, 10 µl of 2×PCR buffer, 2 mM dNTPs, and 0.4 µM each primer [forward primer (T7-F): 5'-GTAATACGACTCACTATAGGGC-3', reverse primer: 5'-AATTAACCCTCACTAAAGGG-3'] for 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 90 s. The PCR products were purified using ExoSAP-IT and subjected to Sanger sequencing (Azenta) with the T7-F primer.

3. Results

3.1. DLA class I and class II alleles

In the DLA class I and class II genes, the genetic polymorphisms of *DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1* were identified as 11, 4, 1, 2, 1, 10, 6, and 7 alleles, respectively. The alleles of each gene in the DLA class I and II, along with their frequencies, are shown in Figure 2-1 and Figure 2-2, respectively. The alleles detected in each animal are listed in Table 2-3, Table 2-4, and Table 2-5. Notable findings are as follows: In the *DLA-88* gene, *DLA-88*nov-MS4* (accession number: LC797961) was discovered as a novel DLA class I allele in this study. For *DLA-DRB1* genes, all homozygous dogs detected with DRB1-F1 primer were re-analyzed with an alternative forward primer (DRB1-F2) reported by Wagner et al. [114], and consequently no allelic dropout was observed in the present study. *DLA-88*501:01* and *DLA-88*508:01*, which have been suggested to bind to the surface antigens of *L. donovani* [60], were found to be present at rates of 2.7% and 27.4%, respectively. No individuals were identified with *DLA-DRB1*015:02* which has been showento correlate with anti-*Leishmania* antibody levels in the epidemiological study of CVL with *L. infantum* [86].

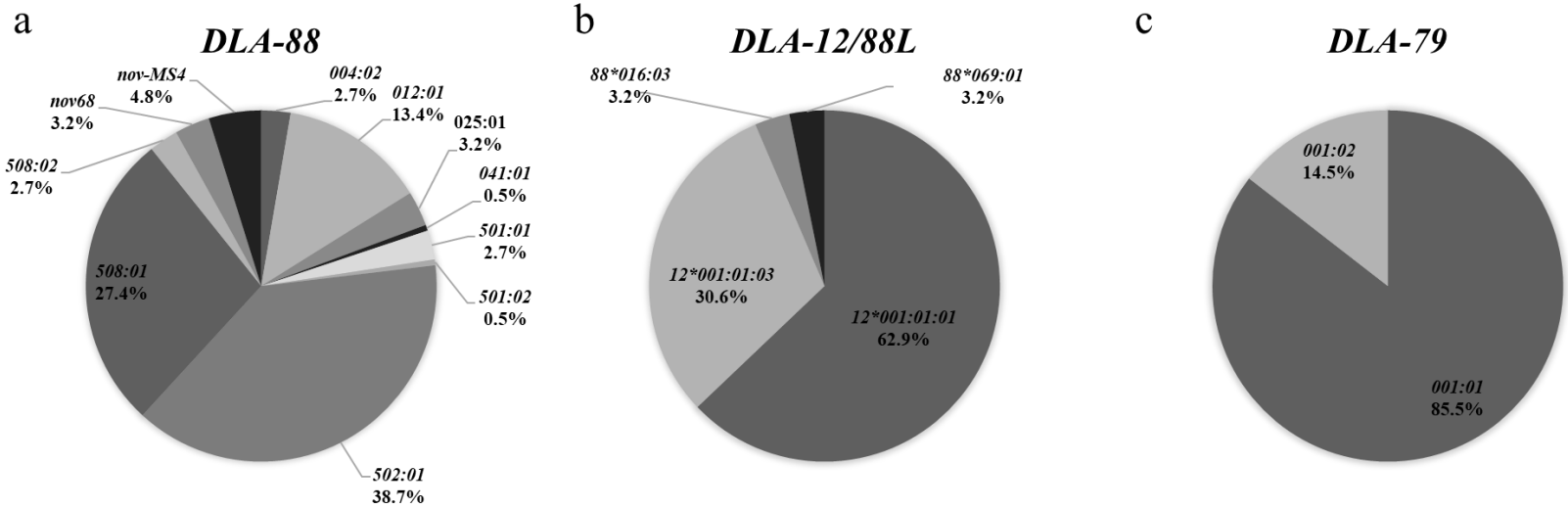

Figure 2-1. The distribution of DLA class I genes in laboratory beagle dogs.

Pie charts describe (a) *DLA-88*, (b) *DLA-12/88L*, and (c) *DLA-79* alleles detected and their allele frequencies in 93 beagle dogs.

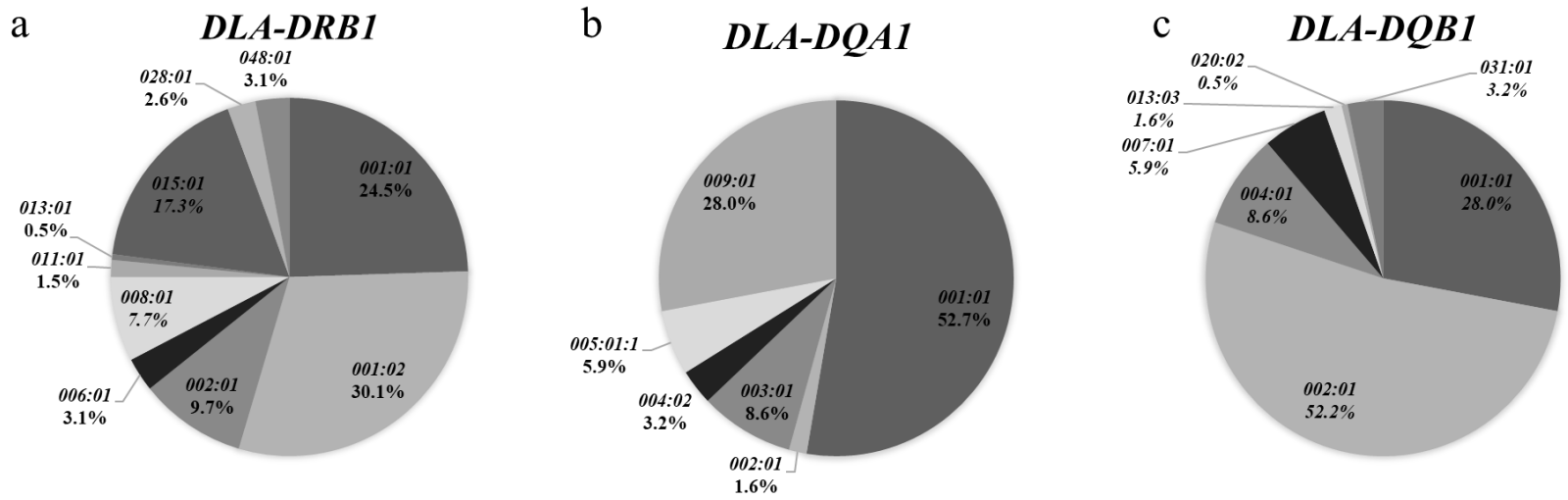

Figure 2-2. The distribution of DLA class II genes in laboratory beagle dogs.

Pie charts describe (a) *DLA-DRB1*, (b) *DLA-DQA1*, and (c) *DLA-DQB1* alleles detected and their allele frequencies in 93 beagle dogs.

Table 2-3. Alleles of DLA class I and class II genes in all tested animals

Animal ID	Strain	*DLA-88*	*DLA-12/88L*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*	Haplotype	*DLA-88*	*DLA-12/88L*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*	Haplotype
1	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	508:01	001:01:03	001:01	001:01	002:01	#2
2	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	002:01	009:01	001:01	#4
3	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	502:01	001:01:01	001:02	001:01	002:01	#1
4	TOYO	502:01	001:01:01	008:01	003:01	004:01	#5	502:01	001:01:01	008:01	003:01	004:01	#5
5	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	001:02	001:01	002:01	#1
6	TOYO	501:01	001:01:01	001:01	001:01	002:01	#9	502:01	001:01:01	001:01	001:01	002:01	#6
7	TOYO	nov-MS4	001:01:01	015:01	009:01	001:01	#7	502:01	001:01:01	008:01	003:01	004:01	#5
8	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	011:01	002:01	013:03	#13
9	TOYO	502:01	001:01:01	001:01	001:01	002:01	#6	502:01	001:01:01	001:01	001:01	002:01	#6
10	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	502:01	001:01:01	001:02	001:01	002:01	#1
11	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	001:02	001:01	002:01	#1
12	TOYO	502:01	001:01:01	008:01	003:01	004:01	#5	508:01	001:01:03	001:02	001:01	002:01	#14
13	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	502:01	001:01:01	001:02	001:01	002:01	#1
14	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	001:02	001:01	002:01	#1
15	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	508:01	001:01:03	001:01	001:01	002:01	#2
16	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	002:01	009:01	001:01	#4
17	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	002:01	009:01	001:01	#4
18	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	508:02	001:01:03	002:01	009:01	001:01	#10
19	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	502:01	001:01:01	001:02	001:01	002:01	#1
20	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	008:01	003:01	004:01	#5
21	TOYO	nov-MS4	001:01:01	015:01	009:01	001:01	#3	502:01	001:01:01	008:01	003:01	004:01	#5
22	TOYO	508:01	001:01:03	002:01	009:01	001:01	#4	508:02	001:01:03	002:01	009:01	001:01	#10
23	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	008:01	003:01	004:01	#5
24	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	001:02	001:01	002:01	#1
25	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	508:01	001:01:03	001:01	001:01	002:01	#2
26	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	nov-MS4	001:01:01	015:01	009:01	001:01	#7
27	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	001:01	001:01	002:01	#2
28	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	001:01	001:01	002:01	#2
29	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	012:01	001:01:01	015:01	009:01	001:01	#3
30	TOYO	502:01	001:01:01	008:01	003:01	004:01	#5	508:01	001:01:03	001:02	001:01	002:01	#14
31	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	002:01	009:01	001:01	#4
32	Marshall	508:01	001:01:03	002:01	009:01	001:01	#4	nov68	88*069:01	048:01	004:02	031:01	#8

33	Marshall	508:01	001:01:03	002:01	009:01	001:01	#4	025:01	88*016:03	028:01	005:01:1	007:01	#12
34	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	012:01	001:01:01	015:01	009:01	001:01	#3
35	TOYO	nov-MS4	001:01:01	015:01	009:01	001:01	#7	502:01	001:01:01	001:02	001:01	002:01	#1
36	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	008:01	003:01	004:01	#5
37	TOYO	041:01	001:01:01	013:01	003:01	004:01	other	502:01	001:01:01	008:01	003:01	004:01	#5
38	TOYO	nov-MS4	001:01:01	015:01	009:01	001:01	#7	502:01	001:01:01	001:02	001:01	002:01	#1
39	TOYO	025:01	88*016:03	008:01	003:01	004:01	other	502:01	001:01:01	008:01	003:01	004:01	#5
40	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	508:02	001:01:03	002:01	009:01	001:01	#10
41	TOYO	501:01	001:01:01	001:01	001:01	002:01	#9	502:01	001:01:01	001:02	001:01	002:01	#1
42	TOYO	508:01	001:01:03	002:01	009:01	001:01	#4	508:01	001:01:03	011:01	002:01	013:03	#13
43	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	001:02	001:01	002:01	#1
44	TOYO	502:01	001:01:01	001:01	001:01	002:01	#6	502:01	001:01:01	001:02	001:01	002:01	#1
45	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	501:01	001:01:01	001:01	001:01	002:01	#9
46	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	001:01	001:01	002:01	#2
47	TOYO	012:01	001:01:01	015:01	009:01	001:01	#3	025:01	88*016:03	028:01	005:01:1	007:01	#12
48	TOYO	501:01	001:01:01	001:01	001:01	002:01	#9	502:01	001:01:01	001:01	001:01	002:01	#6
49	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	508:02	001:01:03	002:01	009:01	001:01	#10
50	Marshall	502:01	001:01:01	001:01	001:01	002:01	#6	nov68	88*069:01	048:01	004:02	031:01	#8
51	Marshall	004:02	001:01:01	006:01	005:01:1	007:01	#11	nov68	88*069:01	048:01	004:02	031:01	#8
53	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	001:01	001:01	002:01	#2
54	TOYO	502:01	001:01:01	001:01	001:01	002:01	#6	508:02	001:01:03	002:01	009:01	001:01	#10
55	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	001:01	001:01	002:01	#2
56	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	001:01	001:01	002:01	#2
57	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	001:01	001:01	002:01	#2
58	TOYO	502:01	001:01:01	001:01	001:01	002:01	#6	502:01	001:01:01	001:02	001:01	002:01	#1
59	TOYO	502:01	001:01:01	001:01	001:01	002:01	#6	508:01	001:01:03	002:01	009:01	001:01	#4
60	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	001:01	001:01	002:01	#2
61	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	502:01	001:01:01	001:02	001:01	002:01	#1
62	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	001:01	001:01	002:01	#2
63	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	004:02	001:01:01	006:01	005:01:1	007:01	#11
64	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	nov68	88*069:01	048:01	004:02	031:01	#8
65	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	001:01	001:01	002:01	#2
66	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	004:02	001:01:01	006:01	005:01:1	007:01	#11

67	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	001:01	001:01	002:01	#2
68	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	001:01	001:01	002:01	#2
69	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	502:01	001:01:01	001:02	001:01	002:01	#1
70	Marshall	nov-MS4	001:01:01	015:01	009:01	001:01	#7	508:01	001:01:03	001:01	001:01	002:01	#2
71	Marshall	508:01	001:01:03	001:01	001:01	002:01	#2	508:01	001:01:03	001:01	001:01	002:01	#2
72	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	002:01	009:01	001:01	#4
74	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	004:02	001:01:01	006:01	005:01:1	007:01	#11
75	Marshall	nov-MS4	001:01:01	015:01	009:01	001:01	#7	nov68	88*069:01	048:01	004:02	031:01	#8
76	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	502:01	001:01:01	001:02	001:01	002:01	#1
77	Marshall	501:01	001:01:01	001:01	001:01	002:01	#9	508:01	001:01:03	015:01	001:01	020:02	other
78	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	508:01	001:01:03	001:01	001:01	002:01	#2
79	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	508:01	001:01:03	001:01	001:01	002:01	#2
80	Marshall	025:01	88*016:03	028:01	005:01:1	007:01	#12	508:01	001:01:03	002:01	009:01	001:01	#4
81	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	012:01	001:01:01	015:01	009:01	001:01	#3
82	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	025:01	88*016:03	028:01	005:01:1	007:01	#12
83	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	004:02	001:01:01	006:01	005:01:1	007:01	#11
84	Marshall	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	002:01	009:01	001:01	#4
85	Marshall	012:01	001:01:01	015:01	009:01	001:01	#3	508:01	001:01:03	002:01	009:01	001:01	#4
86	Marshall	508:01	001:01:03	002:01	009:01	001:01	#4	nov68	88*069:01	048:01	004:02	031:01	#8
87	Marshall	nov-MS4	001:01:03	015:01	009:01	001:01	other	502:01	001:01:01	001:02	001:01	002:01	#1
88	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	008:01	003:01	004:01	#5
89	TOYO	502:01	001:01:01	001:01	001:01	002:01	#6	502:01	001:01:01	001:02	001:01	002:01	#1
90	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	508:01	001:01:03	011:01	002:01	013:03	#13
91	TOYO	502:01	001:01:01	008:01	003:01	004:01	#5	025:01	88*016:03	028:01	005:01:1	007:01	#12
92	TOYO	502:01	001:01:01	001:01	001:01	002:01	#6	502:01	001:01:01	001:02	001:01	002:01	#1
93	TOYO	502:01	001:01:01	001:02	001:01	002:01	#1	502:01	001:01:01	008:01	003:01	004:01	#5

Animal ID	*DLA-79*	*DLA-79*	*DLA-64*	*DLA-64*	*DLA-DRA*	*DLA-DRA*	hom cl I&II	hom cl I	hom cl II
1	001:01	001:02	001:01	001:01	001:01	001:01			
2	001:02	001:02	001:01	001:01	001:01	001:01			
3	001:01	001:01	001:01	001:01	001:01	001:01			
4	001:01	001:02	001:01	001:01	001:01	001:01	y	y	y
5	001:01	001:02	001:01	001:01	001:01	001:01	y	y	y
6	001:01	001:02	001:01	001:01	001:01	001:01			y
7	001:01	001:01	001:01	001:01	001:01	001:01			
8	001:02	001:02	001:01	001:01	001:01	001:01			
9	001:01	001:02	001:01	001:01	001:01	001:01	y	y	y
10	001:01	001:02	001:01	001:01	001:01	001:01			
11	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
12	001:01	001:01	001:01	001:01	001:01	001:01			
13	001:01	001:01	001:01	001:01	001:01	001:01			
14	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
15	001:01	001:01	001:01	001:01	001:01	001:01			
16	001:01	001:01	001:01	001:01	001:01	001:01			
17	001:01	001:02	001:01	001:01	001:01	001:01			
18	001:01	001:01	001:01	001:01	001:01	001:01			
19	001:01	001:01	001:01	001:01	001:01	001:01			
20	001:01	001:01	001:01	001:01	001:01	001:01		y	
21	001:01	001:01	001:01	001:01	001:01	001:01			
22	001:01	001:02	001:01	001:01	001:01	001:01			y
23	001:01	001:02	001:01	001:01	001:01	001:01		y	
24	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
25	001:01	001:01	001:01	001:01	001:01	001:01			
26	001:01	001:01	001:01	001:01	001:01	001:01			y
27	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
28	001:01	001:01	001:01	001:01	001:01	001:01			
29	001:01	001:02	001:01	001:01	001:01	001:01	y	y	y
30	001:01	001:01	001:01	001:01	001:01	001:01			
31	001:01	001:01	001:01	001:01	001:01	001:01		y	
32	001:01	001:01	001:01	001:01	001:01	001:01			
33	001:01	001:01	001:01	001:01	001:01	001:01			
34	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
35	001:01	001:02	001:01	001:01	001:01	001:01			
36	001:01	001:01	001:01	001:01	001:01	001:01		y	
37	001:01	001:01	001:01	001:01	001:01	001:01			
38	001:01	001:01	001:01	001:01	001:01	001:01			

39	001:01	001:02	001:01	001:01	001:01	001:01			y
40	001:01	001:01	001:01	001:01	001:01	001:01			
41	001:01	001:02	001:01	001:01	001:01	001:01			
42	001:01	001:01	001:01	001:01	001:01	001:01		y	
43	001:01	001:02	001:01	001:01	001:01	001:01	y	y	y
44	001:01	001:01	001:01	001:01	001:01	001:01		y	
45	001:01	001:01	001:01	001:01	001:01	001:01			
46	001:02	001:02	001:01	001:01	001:01	001:01			
47	001:01	001:02	001:01	001:01	001:01	001:01			
48	001:01	001:01	001:01	001:01	001:01	001:01			y
49	001:01	001:01	001:01	001:01	001:01	001:01			
50	001:01	001:01	001:01	001:01	001:01	001:01			
51	001:01	001:01	001:01	001:01	001:01	001:01			
53	001:01	001:01	001:01	001:01	001:01	001:01			
54	001:01	001:02	001:01	001:01	001:01	001:01			
55	001:01	001:01	001:01	001:01	001:01	001:01			
56	001:01	001:01	001:01	001:01	001:01	001:01			
57	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
58	001:01	001:02	001:01	001:01	001:01	001:01		y	
59	001:01	001:02	001:01	001:01	001:01	001:01			
60	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
61	001:01	001:01	001:01	001:01	001:01	001:01			
62	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
63	001:01	001:01	001:01	001:01	001:01	001:01			
64	001:01	001:01	001:01	001:01	001:01	001:01			
65	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
66	001:01	001:01	001:01	001:01	001:01	001:01			
67	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
68	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
69	001:01	001:01	001:01	001:01	001:01	001:01			
70	001:01	001:01	001:01	001:01	001:01	001:01			
71	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
72	001:01	001:01	001:01	001:01	001:01	001:01			
74	001:01	001:01	001:01	001:01	001:01	001:01			
75	001:01	001:01	001:01	001:01	001:01	001:01			
76	001:01	001:01	001:01	001:01	001:01	001:01			
77	001:01	001:01	001:01	001:01	001:01	001:01			
78	001:01	001:01	001:01	001:01	001:01	001:01			
79	001:01	001:02	001:01	001:01	001:01	001:01			
80	001:01	001:01	001:01	001:01	001:01	001:01			
81	001:01	001:01	001:01	001:01	001:01	001:01	y	y	y
82	001:01	001:01	001:01	001:01	001:01	001:01			
83	001:01	001:01	001:01	001:01	001:01	001:01			

84	001:01	001:01	001:01	001:01	001:01	001:01	
85	001:01	001:01	001:01	001:01	001:01	001:01	
86	001:01	001:01	001:01	001:01	001:01	001:01	
87	001:01	001:01	001:01	001:01	001:01	001:01	
88	001:01	001:02	001:01	001:01	001:01	001:01	y
89	001:01	001:01	001:01	001:01	001:01	001:01	y
90	001:01	001:01	001:01	001:01	001:01	001:01	
91	001:01	001:01	001:01	001:01	001:01	001:01	
92	001:01	001:02	001:01	001:01	001:01	001:01	y
93	001:01	001:01	001:01	001:01	001:01	001:01	y

y: homozygous

Animal ID	Strain	*DLA-88*	*DLA-12/88L*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*	Haplotype	*DLA-79*	*DLA-64*	*DLA-DRA*
52[#]	Marshall	501:02	001:01:01	001:01	001:01	002:01	NE	001:01	001:01	001:01
		508:01	001:01:03	006:01	005:01:1	007:01	NE	001:01	001:01	001:01
	Ambiguity									
73[#]	Marshall	012:01	001:01:01	001:02	001:01	002:01	NE	001:01	001:01	001:01
		nov-MS4	001:01:01	015:01	009:01	001:01	#3 or #7	001:01	001:01	001:01
	Ambiguity			A-5	A-9	A-10				

[#]: Dogs whose haplotype could not be estimated

NE: Not estimated

Table 2-4. Allele combinations causing ambiguity of DLA class I and class II genes

Ambiguity type	Candidate alleles combination 1	Candidate alleles combination 2	Candidate alleles combination 3
A-1	*DLA-88*501:01* vs *DLA-88*nov27*	*DLA-88*502:01* vs *DLA-88*508:01*	
A-2	*DLA-88*004:02* vs *DLA-88*502:01*	*DLA-88*004:03* vs *DLA-88*501:01*	
A-3	*DLA-88*004:02* vs *DLA-88*012:01*	*DLA-88*012:02* vs *DLA-88*nov51*	
A-4	*DLA-DRB1*001:01* vs *DLA-DRB1*002:03*	*DLA-DRB1*001:02* vs *DLA-DRB1*002:01*	
A-5	*DLA-DRB1*001:01* vs *DLA-DRB1*015:04*	*DLA-DRB1*001:02* vs *DLA-DRB1*015:01*	
A-6	*DLA-DRB1*002:01* vs *DLA-DRB1*048:01*	*DLA-DRB1*002:02* vs *DLA-DRB1*048:03*	
A-7	*DLA-DRB1*002:01* vs *DLA-DRB1*015:01*	*DLA-DRB1*002:02* vs *DLA-DRB1*015:02*	
A-8	*DLA-DRB1*002:01* vs *DLA-DRB1*011:01*	*DLA-DRB1*002:03* vs *DLA-DRB1*011:03*	
A-9	*DLA-DQA1*001:01* vs *DLA-DQA1*009:01*	*DLA-DQA1*014:01:1* vs *DLA-DQA1*015:01*	
A-10	*DLA-DQB1*001:01* vs *DLA-DQB1*002:01*	*DLA-DQB1*008:02* vs *DLA-DQB1*060:01*	
A-11	*DLA-DQB1*002:01* vs *DLA-DQB1*013:03*	*DLA-DQB1*002:02* vs *DLA-DQB1*013:01*	*DLA-DQB1*013:04* vs *DLA-DQB1*nov5*
A-12	*DLA-DQB1*001:0*1 vs *DLA-DQB1*031:01*	*DLA-DQB1*023:01* vs *DLA-DQB1*030:01*	

Table 2-5. Allele ambiguity of DLA class I and class II genes in all tested animals

Animal ID	*DLA-88*	*DLA-12/88L*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*
1	-	-	-	A-9	A-10
2	A-1	-	A-4	A-9	A-10
3	-	-	A-5	A-9	A-10
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	A-1	-	-	-	A-11
9	-	-	-	-	-
10	-	-	A-5	A-9	A-10
11	-	-	-	-	-
12	A-1	-	-	-	-
13	-	-	A-5	-	A-10
14	-	-	-	-	-
15	-	-	A-5	A-9	A-10
16	A-1	-	A-4	A-9	A-10
17	A-1	-	A-4	A-9	A-10
18	-	-	A-4	A-9	A-10
19	-	-	A-5	A-9	A-10
20	-	-	-	-	-
21	-	-	-	-	-
22	-	-	-	-	-
23	-	-	-	-	-
24	-	-	-	-	-
25	-	-	A-5	A-9	A-10
26	-	-	-	-	-
27	-	-	-	-	-
28	A-1	-	-	-	-
29	-	-	-	-	-
30	A-1	-	-	-	-
31	-	-	-	A-9	A-10
32	-	-	A-6	-	A-12
33	-	-	-	-	-
34	-	-	-	-	-
35	-	-	A-5	A-9	A-10
36	-	-	-	-	-
37	-	-	-	-	-
38	-	-	A-5	A-9	A-10
39	-	-	-	-	-
40	-	-	A-7	-	-

41	-	-	-	-	-
42	-	-	A-8	-	-
43	-	-	-	-	-
44	-	-	-	-	-
45	-	-	-	A-9	A-10
46	A-1	-	-	-	-
47	-	-	-	-	-
48	-	-	-	-	-
49	-	-	A-4	A-9	A-10
50	-	-	-	-	-
51	-	-	-	-	-
53	A-1	-	-	-	-
54	-	-	-	-	A-10
55	A-1	-	-	-	-
56	A-1	-	-	-	-
57	-	-	-	-	-
58	-	-	-	-	-
59	A-1	-	-	A-9	A-10
60	-	-	-	-	-
61	-	-	A-5	A-9	A-10
62	-	-	-	-	-
63	A-2	-	-	-	-
64	-	-	-	-	-
65	-	-	-	-	-
66	-	-	-	-	-
67	-	-	-	-	-
68	-	-	-	-	-
69	-	-	A-5	A-9	A-10
70	-	-	-	A-9	A-10
71	-	-	-	-	-
72	A-1	-	-	A-9	A-10
74	A-3	-	-	-	-
75	-	-	-	-	A-12
76	-	-	A-5	A-9	A-10
77	-	-	-	-	-
78	-	-	-	A-9	A-10
79	-	-	-	A-9	A-10
80	-	-	-	-	-
81	-	-	-	-	-
82	-	-	-	-	-
83	A-2	-	-	-	-
84	A-1	-	A-4	A-9	A-10
85	-	-	A-7	-	-

86	-	-	A-6	-	A-12
87	-	-	A-5	A-9	A-10
88	-	-	-	-	-
89	-	-	-	-	-
90	A-1	-	-	-	A-11
91	-	-	-	-	-
92	-	-	-	-	-
93	-	-	-	-	-

3.2. DLA haplotype and homozygosity

The haplotypes in the DLA class I or class II, along with their frequencies, are shown in Figure 2-3. A total of 14 haplotypes in all the DLA class I and class II genes were estimated to be present in two or more different individuals (Table 2-6). Since haplotypes in all the DLA class I and class II genes could not be identified in two animals, they were identified in a total of 91 dogs. Of these, five different haplotypes were detected in homozygous dogs. The number of dogs with each haplotype homozygous was as follows: #1; 5 dogs, #2; 8 dogs, #3; 3 dogs, #5; 1 dog, and #6; 1 dog. The six major haplotypes that occurred at a frequency of over 5% in 88 of the 91 dogs consisted only of those with *DLA-12* genes, and none of them included those with *DLA-88L* genes. However, haplotype #8 and #12, including the *DLA-88L* gene, were identified at frequencies of 3.3% and 2.7% from six and five dogs each, respectively. This study found that the levels of homozygosity varied among the different genes (Table 2-7).

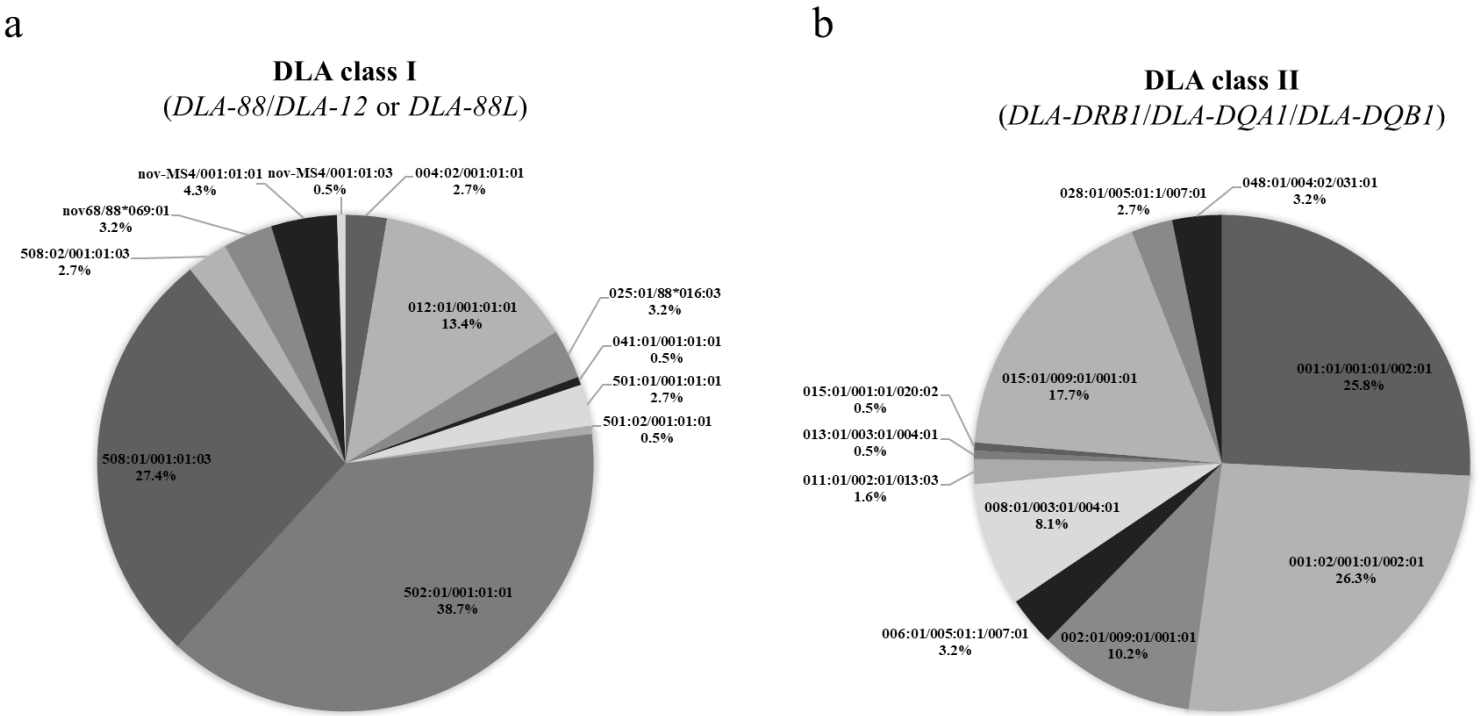

Figure 2-3. The haplotypes of DLA class I and class II genes in laboratory beagle dogs.

Pie charts describe (a) DLA class I (*DLA-88* and *DLA-12/88L*) and (b) DLA class II (*DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) haplotypes detected and their frequencies in 93 beagle dogs.

Table 2-6. Frequency of haplotypes of DLA class I and class II genes in 91 beagle dogs

Haplotype ID	*DLA-88*	*DLA-12/88L*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*	Haplotype frequency (%)*	Number of dogs who had each haplotype
#1	*502:01*	*001:01:01*	*001:02*	*001:01*	*002:01*	25.8%	42
#2	*508:01*	*001:01:03*	*001:01*	*001:01*	*002:01*	16.5%	22
#3	*012:01*	*001:01:01*	*015:01*	*009:01*	*001:01*	13.7%	22
#4	*508:01*	*001:01:03*	*002:01*	*009:01*	*001:01*	7.7%	14
#5	*502:01*	*001:01:01*	*008:01*	*003:01*	*004:01*	7.7%	13
#6	*502:01*	*001:01:01*	*001:01*	*001:01*	*002:01*	6.0%	10
#7	*nov-MS4*	*001:01:01*	*015:01*	*009:01*	*001:01*	3.3%	6
#8	*nov68*	*88*069:01*	*048:01*	*004:02*	*031:01*	3.3%	6
#9	*501:01*	*001:01:01*	*001:01*	*001:01*	*002:01*	2.7%	5
#10	*508:02*	*001:01:03*	*002:01*	*009:01*	*001:01*	2.7%	5
#11	*004:02*	*001:01:01*	*006:01*	*005:01:1*	*007:01*	2.7%	5
#12	*025:01*	*88:016:03*	*028:01*	*005:01:1*	*007:01*	2.7%	5
#13	*508:01*	*001:01:03*	*011:01*	*002:01*	*013:03*	1.6%	3
#14	*508:01*	*001:01:03*	*001:02*	*001:01*	*002:01*	1.1%	2
Other haplotypes						2.2%	4

*The total number of estimated haplotypes is 182.

Table 2-7. Frequency of homozygosity of DLA genes in beagle dogs

DLA-88	*DLA-12/88L*	*DLA-64*	*DLA-79*	*DLA-DRA*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*
31.2%	54.8%	100.0%	77.4%	100.0%	24.7%	39.8%	38.7%
Class I: 31.2%				Class II: 24.7%			
All genes: 19.4%							

3.2. DLA haplotype and homozygosity

The distribution of DLA alleles was compared between TOYO and Marshall Beagles. Alleles with statistically significant differences between strains are described in Figure 2-4. The result of the frequency of haplotypes between strains is shown in Table 2-8. Statistically significant differences between strains were observed in haplotype #1, #2, #5, #6, #8, and #11. The frequency of haplotype #1 was 34.0% in TOYO Beagles and 17.0% in Marshall Beagles. In contrast, the frequency of haplotype #2 was 3.2% in TOYO Beagles and 30.7% in Marshall Beagles. Haplotypes #5 and #11 were exclusively observed in TOYO Beagles and haplotype #8 was exclusively observed in Marshall Beagles. Moreover, haplotype #6 occurred at a frequency of 10.6% in TOYO Beagles compared with a frequency of only 1.1% in Marshall Beagles.

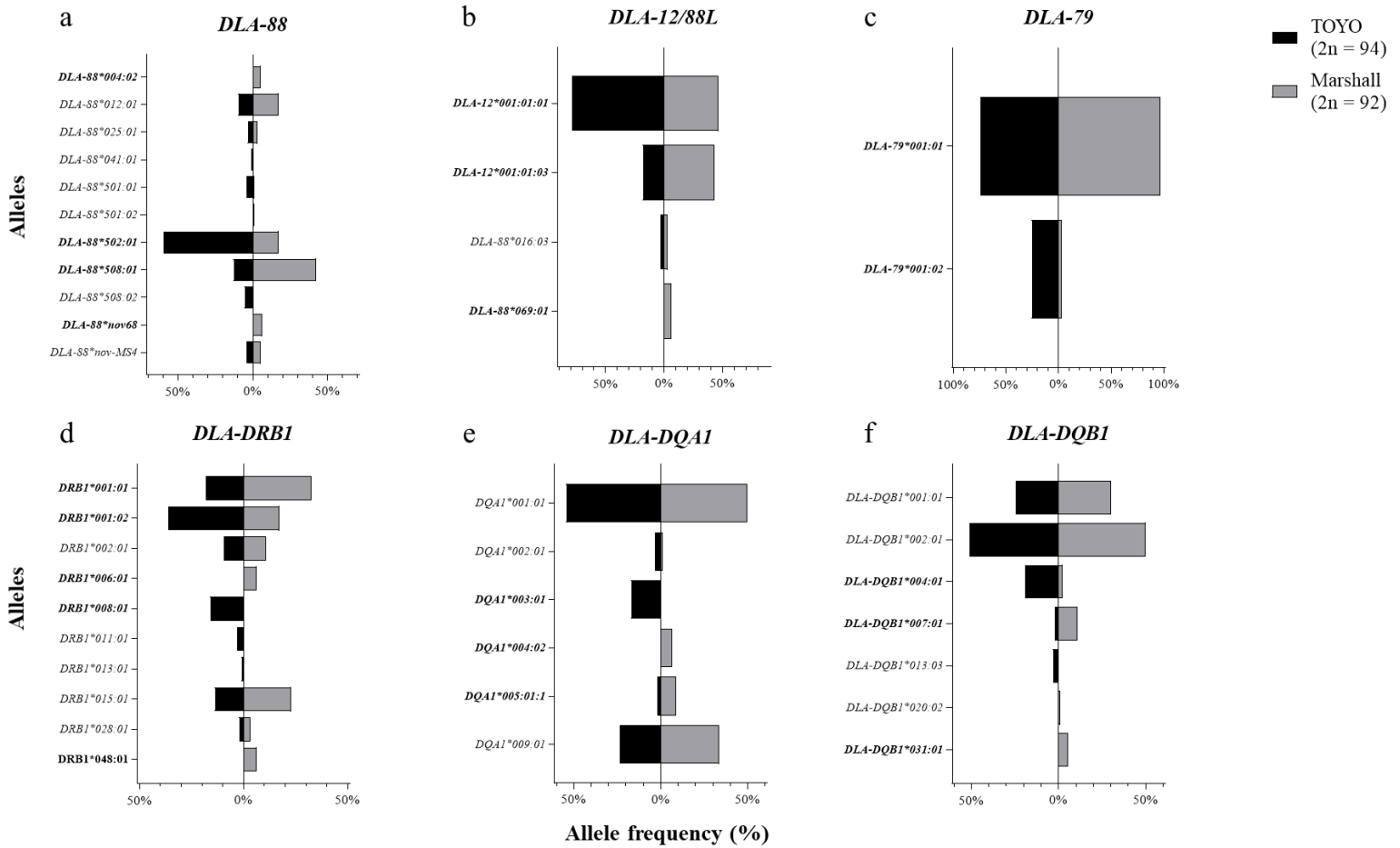

Figure 2-4. The differences of allele frequency in DLA class I and class II genes between two strains of laboratory beagle dogs. The allele frequencies in (a) *DLA-88*, (b) *DLA-12/88L*, (c) *DLA-79*, (d) *DLA-DRI* (e) *DLA-DQA1*, and (f) *DLA-DQB1* in TOYO (black) and Marshall (gray) Beagles are shown. Bold: Allele which show statistically difference of frequency between strains by Fisher's exact test ($p<0.05$).

Table 2-8. Frequency of haplotypes between strains in beagle dogs

	Haplotype (Number of homozygotes for each haplotype)															
Strain	**#1**	**#2**	#3	#4	**#5**	**#6**	#7	**#8**	#9	#10	**#11**	#12	#13	#14	Other	Total
TOYO	32	3	10	4	14	10	3	0	4	5	0	2	3	2	2	94
	(5)	(0)	(0)	(0)	(1)	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	
Marshall	15	27	15	10	0	1	3	6	1	0	5	3	0	0	2	88
	(0)	(8)	(3)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	
Total	47	30	25	14	14	11	6	6	5	5	5	5	3	2	4	182

Bold: Haplotype which show statistically difference of frequency between strains by Fisher's exact test ($p < 0.05$).

4. Discussion

In Chapter 2, genotyping of DLA genes across class I and II loci in 93 beagle dogs was conducted. The results revealed the presence and frequency of each allele in six genes, excluding *DLA-64* and *DLA-DRA*. This analysis provided insights into the genetic polymorphisms of a wide range of DLA genes in beagle dogs, which are commonly used in various biomedical research. The haplotypes of DLA genes located on chromosome 12 were also estimated. To our knowledge, no reports have been published regarding DLA haplotypes for almost all DLA class I and II genes, using a sample size of animals as large as in this study. Furthermore, since in the present study genotyping was performed using two different strains, TOYO and Marshall Beagles, it was also possible to compare these strains.

Regarding the distribution of DLA class I alleles (Figure 2-1), *DLA-88*012:01*, *DLA-88*502:01*, and *DLA-88*508:01* were observed at a high frequency (>10%) in the *DLA-88* gene. These alleles were also detected in a study involving a comprehensive examination of DLA genotyping in various dog breeds (ranked 3rd, 5th, and 6th by frequency, respectively) [74]. In detail, *DLA-88*012:01* was observed in Maltese terriers while *DLA-88*508:01* was observed in Chihuahuas and Labrador retrievers, whereas *DLA-88*502:01* was observed frequently only in beagle dogs. *DLA-12*001:01:01* and *DLA-12*001:01:03*, detected primarily in the *DLA-12/88L* gene in this study, were also observed in the previous study mentioned above (ranked 1st and 3rd, respectively). These results suggest that *DLA-88*502:01*, which was found at a high frequency in this study, maybe the predominant allele in beagle dogs. In the *DLA-79* gene, *DLA-79*001:01* was detected with the highest allele frequency, followed by *DLA-79*001:02*. This result is consistent with previous research conducted on various dog breeds, including beagle dogs [112].

Compared to DLA class I genes, the polymorphisms of DLA class II genes have received much attention in both the broader canine population and specifically in beagle dogs. In terms of the distribution of DLA class II alleles (Figure 2-2), the major alleles (>10%) in the *DLA-DRB1* gene for beagle dogs in this study were *DLA-DRB1*001:01*, *DLA-DRB1*001:02*, and *DLA-DRB1*015:01*. Meanwhile, in a study by Kennedy et al. [54] on pet beagle dogs, *DLA-DRB1*002:01* and *DLA-DRB1*008:01* were reported as the main alleles, in addition to the three alleles mentioned above. This is consistent with our findings

in beagles, in which these two alleles were the next most frequent after the three most frequent ones. Soutter et al. [104] also obtained similar results in laboratory beagle dogs. Considering the entire canine population, while *DLA-DRB1*008:01* is considered to be an allele with a relatively low frequency, it is likely to be one of the predominant alleles in beagle dogs [54,74]. In this study, the major alleles (>10%) of *DLA-DQA1* were *DLA-DQA1*001:01* and *DLA-DQA1*009:01*, followed by *DLA-DQA1*003:01* (9%). In the case of the *DLA-DQB1* gene, the major alleles (>10%) were *DLA-DQB1*001:01*, *DLA-DQB1*002:01*, and *DLA-DQB1*004:01*, with *DLA-DQB1*007:01* accounting for 6%. These findings are consistent with those reported by Kennedy et al. [54], except for the frequency of *DLA-DQB1*004:01*. In the previous report, this frequency was only 3.3%, while it was 10.8% in the present study. This difference may be attributable to strain-specific variations in the frequency of allele expression.

The haplotypes of *DLA-88*, *DLA-12/88L*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1* genes, all located on chromosome 12, were estimated (Table 2-3). The haplotype #1 was the most common haplotype (haplotype frequency, 25.8%). In a previous study, a haplotype consisting of *DLA-88*502:01–DLA-12*001:01:01–DLA-DRB1*001:02* was exclusively observed in beagle dogs with a high frequency (94.7%) among 59 dog breeds [74]. Additionally, the class II haplotype (*DLA-DRB1*001:02–DLA-DQA1*001:01–DLA-DQB1*002:01*) was also identified in beagle dogs [105]. Furthermore, other major haplotypes, such as *DLA-88–DLA-12/88L–DLA-DRB1* and *DLA-DRB1–DLA-DQA1–DLA-DQB1*, have been reported in canines and beagle dogs, respectively [74,104,105]. These findings suggest that the results of our analysis align with previous reports on individual DLA gene polymorphisms in beagle dogs, serving as a bridge between the studies. However, the class II haplotype *DLA-DRB1*008:01–DLA-DQA1*003:01–DLA-DQB1*004:01* was found in pet beagles but not in laboratory beagle dogs [104]. This clear discrepancy in haplotypes from a previous report may indicate the existence of differences between strains or populations of beagles.

Epidemiological studies have suggested that the presence of *DLA-DRB1*015:02* is associated with increased susceptibility to CVL infection [86]. This allele has been reported to have an expression frequency of approximately 8% in dogs [74], but, consistent with previous reports, no individuals with this allele were identified in beagle dogs in this study.

This suggests that the distribution of DLA alleles may account for the lack of significant changes in T-cell subsets during immunophenotyping in Chapter 1, potentially having influenced the immune response or the establishment of infection in this model. Additionally, the haplotypes including of *DLA-DRB1*001:001–DLA-DQA1*001:01–DLA-DQB1*002:01* were detected in approximately one-quarter of beagle dogs, of which approximately 96% were haplotype #1 (*DLA-88*502:01–DLA-12*001:01:01–DLA-DRB1*001:02–DLA-DQA1*001:01–DLA-DQB1*002:01*), suggesting that Haplotype #1, which includes *DLA-88* and *DLA-12*, may be a risk factor for CVL[105]. On the other hand, haplotype #1 showed different frequencies depending on the strain (34.0% in TOYO but 17.0% in Marshall), suggesting that strain differences may also influence the response to experimental CVL infection in dogs.

The differences in DLA diversity between TOYO and Marshall Beagles were analyzed in this study. The results showed that the type and frequency of DLA alleles differed between these two breeds. Given that the beagles studied here are laboratory animals, it is likely that their raising in closed colonies played a significant role in accentuating the differences between them. It is well known that even colonies of the same species and strain can exhibit variations in standing genetic variation [15]. It has also been reported that the types and allele frequencies of MHC genes differ among populations in humans [9] and among colonies in other animals [99]. A high frequency of MHC alleles that are associated with a particular immune-related disease results in an increased risk of developing the disease in the population. For example, *HLA-DRB1*04:05*, which is associated with the development of drug-induced interstitial lung disease, is one of the most common *HLA-DRB1* alleles in the Japanese population, increasing the risk of this disease in humans [47]. The association between various diseases and DLA has also been reported in dogs. For example, haplotype #2 and #6 components *DLA-DRB1*001:01–DLA-DQA1*001:01–DLA-DQB1*002:01* were shown to confer a risk of developing Addison's disease in West Highland terriers and Leonbergers [44], while *DLA-DQB1*001:01* and *DLA-DQA1*009:01* that were identified in this study were shown to be associated with sudden acquired retinal degeneration syndrome [107]. When conducting studies on immune-related diseases associated with DLA genes in beagle dogs, it would be desirable to use a strain with a high frequency of corresponding alleles.

In conclusion, DLA genotyping across all four DLA class I and four DLA class II genes in 93 beagle dogs housed in our laboratory was conducted. Polymorphisms were identified in *DLA-88*, *DLA-12/88L*, *DLA-79*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*, but no variation were found in *DLA-64* and *DLA-DRA*. Furthermore, our study provided information on the haplotypes, including all DLA class I and II genes located on chromosome 12. Differences between the TOYO and Marshall strains of laboratory beagle dogs were also analyzed, which revealed variations in the type and frequency of DLA alleles between them. Consolidation of DLA allele and haplotype information on laboratory beagle dogs is essential to advance research on the disease and infectious models, including CVL in dogs.

5. Summary

DLA polymorphisms are associated with inter-individual variations in the risk, susceptibility, and severity of immune-related phenomena. While DLA class II genes have been extensively studied, less research has been performed on the polymorphisms of DLA class I genes, especially in beagle dogs commonly used as laboratory animals for safety evaluations in drug development. Four DLA class I genes and four DLA class II genes were genotyped by locus-specific Sanger sequencing using 93 laboratory beagle dogs derived from two different strains: TOYO and Marshall. The results showed that, for DLA class I genes, 11, 4, 1, and 2 alleles, including a novel allele, were detected in *DLA-88*, *DLA-12/88L*, *DLA-64*, and *DLA-79*, while, for DLA class II genes, 1, 10, 6, and 7 alleles were detected in *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*, respectively. It was estimated that there were 14 DLA haplotypes, six of which had a frequency of ≥5%. Furthermore, when comparing the DLA diversity between TOYO and Marshall strains, the most common alleles and haplotypes differed between them. This is the first study to genotype all DLA loci and determine DLA haplotypes, including all DLA class I and class II genes in dogs. Integrating information on the DLA diversity of laboratory beagle dogs should reinforce their benefit as an animal model for understanding various diseases associated with a specific DLA allele.

Chapter 3.

PacBio long-read amplicon sequencing of full length of dog leukocyte antigen genes

1. Introduction

MHC genotyping was performed using methods like serological reagents and PCR-based techniques, which often provided partial sequence data, particularly around PBS on MHC molecules. Nevertheless, the new alleles continue to be discovered via advances in genotyping technologies. The development of NGS methods, followed by the emergence of long-read sequencing technologies, such as PacBio SMRT and Oxford Nanopore Technologies sequencing, has transformed MHC genotyping by enabling the acquisition of more comprehensive, full-length sequence information [96,110]. PacBio sequencers, utilizing long-read sequencing technology, are particularly advantageous over conventional short-read NGS, as they can generate highly accurate long reads [68]. This allows for unambiguous determination of full-length allele sequences and accurate haplotype phasing, which is often challenging with short-read NGS due to the high polymorphisms and repetitive nature of the MHC region [111]. In dogs, recent research has also intensified investigations into DLA mutations by using NGS [37,85]. However, to the best of our knowledge, no reports on DLA genotyping utilizing long-read sequencing technology have been published.

To address these limitations, the development of a novel DLA genotyping assay utilizing long-range PCR amplification with specific primers for eight DLA genes (*DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) and PacBio SMRT sequencing was attempted in order to obtain comprehensive sequence information covering the entire lengths of the DLA class I and class II genes. The long-read sequencing results were validated by comparing with the allele assignments with established Sanger sequencing-based typing reported in Chapter 2 [59]. This full-length analysis approach enabled the investigation of polymorphisms outside the PBS region, allowing us to attempt further subdivision of alleles and haplotypes that could not be distinguished using only the PBS region. Polymorphisms in regulatory regions, such as the 5' upstream and cytosine-phosphate-guanine (CpG) island regions, that could potentially influence gene expression were also investigated. Using this new method, we analyzed the genotypes of 83 beagle dogs from two distinct strains (TOYO and Marshall) housed in our laboratory, and the resulting full-length sequences were used to elucidate differences in the genetic backgrounds between these strains, providing crucial data for the standardization and interpretation of the beagle dogs as a biomedical research model

2. Materials and methods

2.1 Animals and genomic DNA samples

Eighty-three beagle dogs were used in this study, including a subset of individuals previously analyzed in Chapter 2. A total of 42 TOYO Beagles were obtained from Oriental Yeast Co., Ltd., while 41 Marshall Beagles were obtained from Marshall Bioresources Japan. Blood collection and DNA extraction were performed using the same procedures as described in Chapter 2. Briefly, blood samples were collected from the jugular vein of 83 unrelated beagle dogs and stored in EDTA-2K tubes. DNA was extracted using the QIAamp Blood Mini Kit according to the manufacturer's instructions. Consistent with the ethical standards detailed in Chapter 2, all animal procedures were conducted in accordance with the relevant Japanese laws and the guidelines of the Animal Care and Use Committee of Daiichi Sankyo Co., Ltd., under the approval of the institutional Ethics Review Committee.

2.2 Long-range PCR amplification, barcoding, and pooling

The specific primer sets were used for amplifying *DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1* genes (product sizes: 4.0, 5.6, 4.1, 3.9, 5.8, 13.3, 7.0, and 6.0 kb, respectively) (Table 3-1 and Figure 3-1). While the primers for *DLA-88* and *DLA-12/88L* were obtained from previously reported sequences, those for the remaining six genes were newly designed in this study. These novel primers were positioned in the conserved regions, based on the canine reference genomes, to ensure complete coverage of all exons and introns for each target locus. Figure 3-2 provides a comprehensive overview of the method flow from sample preparation to data analysis.

Table 3-1. Locus specific primers used for long-range PCR

DLA locus	Primer name	Primer sequence (5' to 3')	Strand	Position	Product size	Reference
DLA-88	88-seg-F	AGGGGACAATGGGACAGGAACTTGGA	-	chr12:1,031,323-1,035,288	4.0 kb	Miyamae et al (2018).
	88-seg-R2	AGGACTCAGGGAGACAGTGCGACAAGA				Miyamae et al (2023).
DLA-12/88L	88L/12-seg-F	CATTTCTGTTGGGATATGTGGTAA	+	chr12:1,071,361-1,077,013	5.6 kb	Miyamae et al (2018).
	88L/12-seg-R	CATCAAGGGATAAGGTGAAAGA				Miyamae et al (2018).
DLA-64	64-seg-F2	GTAAGTAAGCAAGTCAGTCGGTGGAGG	+	chr12:1,122,425-1,126,542	4.1 kb	This study*
	64-seg-R2	CTGGAAGGTTCTCAGGGCTTCCATTT				This study*
DLA-79	79-seg-F	GGAGAAGAGGGGTCAAGGACAAAG	+	chr18:41,564,034-41,567,965	3.9 kb	This study*
	79-seg-R	CAGCAATCTGGCAGATTCTGGAAGG				This study*
DLA-DRA	DRA-seg-F2	CTCTGTCACCCACTACTGCCAAGAATC	+	chr12:2,287,298-2,293,136	5.8 kb	This study*
	DRA-seg-R2	CCACTCTCATCTGCTCCCCATCATC				This study*
DLA-DRB1	DRB1-seg-F1-4	GTGATGGTGCAGCAAGGTCCAGAGC	-	chr12:2,305,906-2,319,484	13.3 kb	This study*
	DRB1-seg-R2-4	AAACTCAAGAAGGGGGAATTGTGAGC				This study*
DLA-DQA1	DQA1-seg-F	GGAGTTAAGGATTGCCAAGGAGGGG	+	chr12:2,375,211-2,382,308	7.0 kb	This study*
	DQA1-seg-R2	CACAGGGATACACAGGGATTCAGTGAG				This study*
DLA-DQB1	DQB1-seg-F	GTTTCTTAAACCCCTAAGGCATTCAATC	-	chr12:2,399,306-2,405,299	6.0 kb	This study*
	DQB1-seg-R	GGTTTCCTGCCTCCACCCAGTTAAG				This study*

*: newly designed for this study

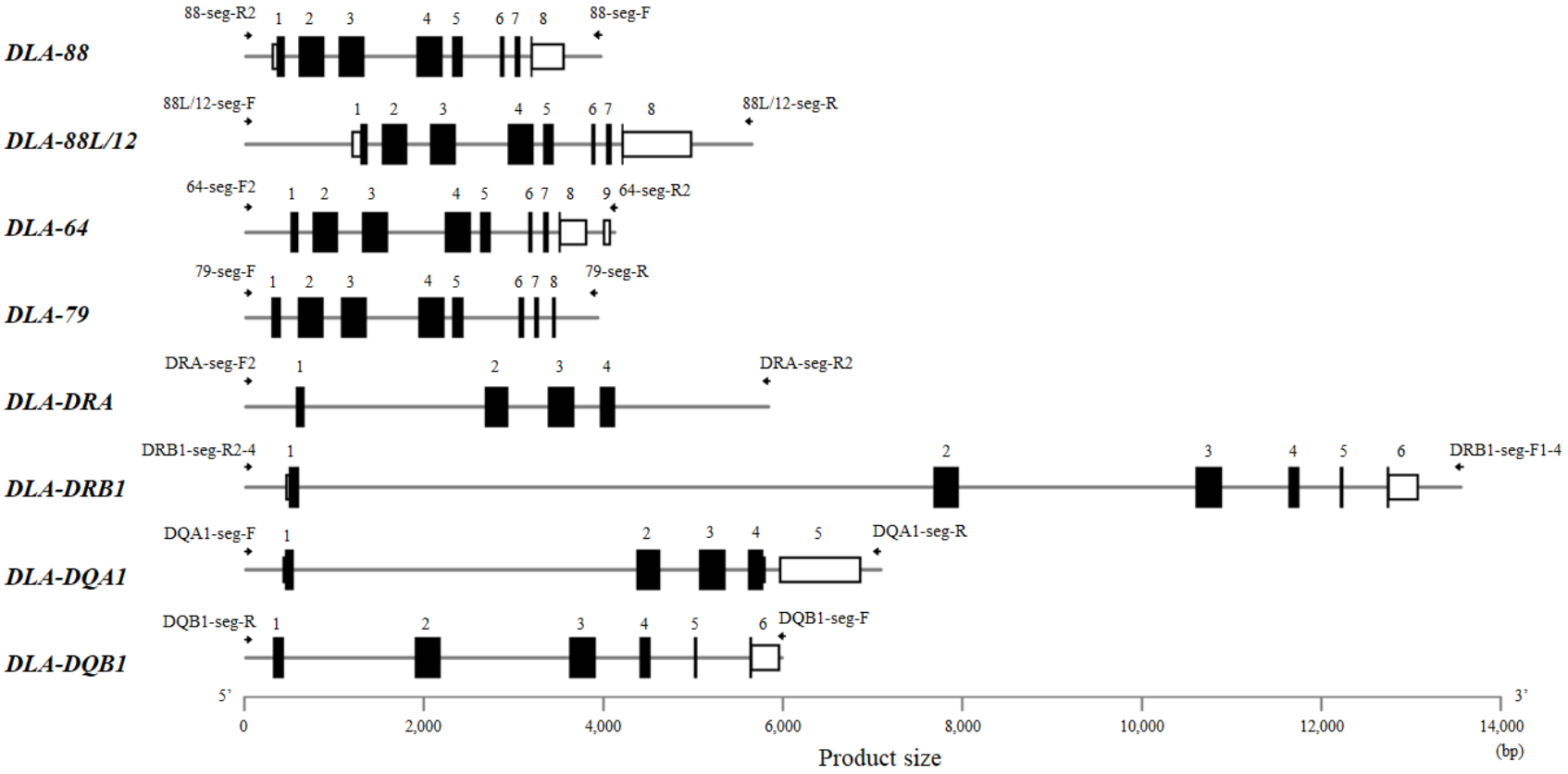

Figure 3-1. DLA genes and primers in the region of interest for long-range PCR.

Black box: exon, white box: utr, arrow: primer

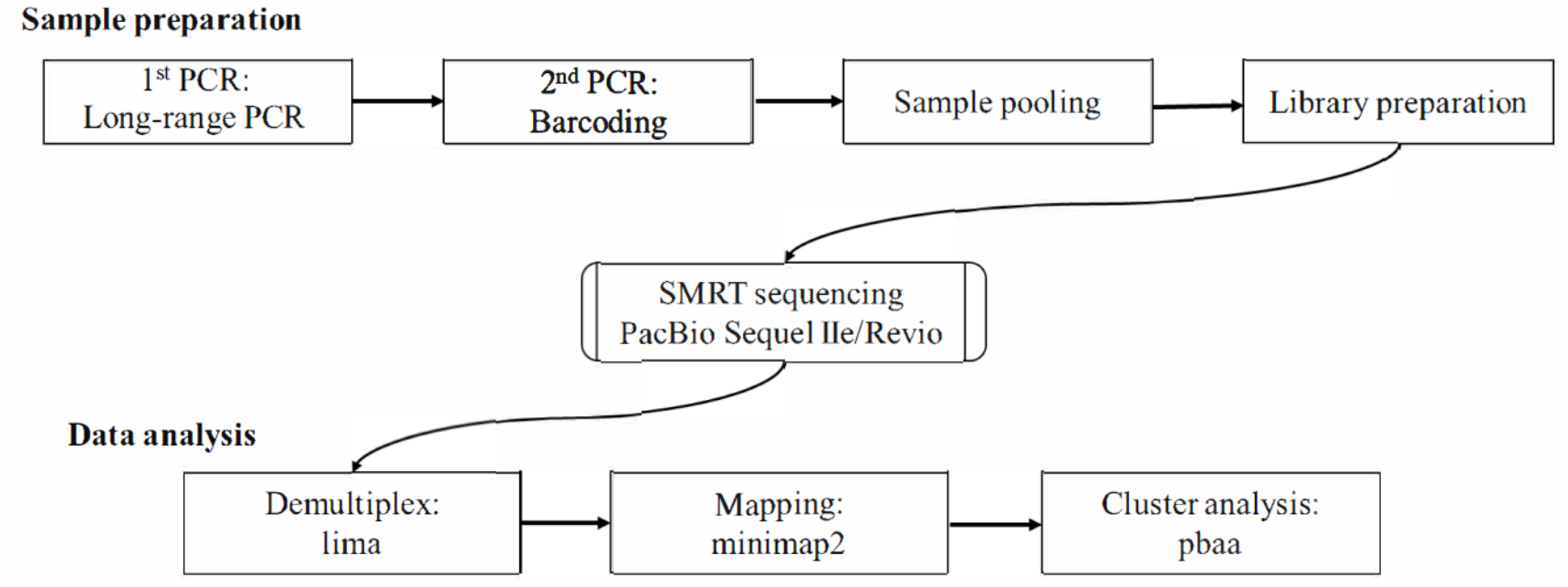

Figure 3-2. The flowchart of the current experiment from sample preparation to sequencing and data analysis

The primers for all eight loci were modified to identify individuals by adding a 5 block and a tail representing the PacBio universal sequences (forward: 5'-GCAGTCGAACATGTAGCTGACTCAGGTCAC-3', reverse: 5'-TGGATCACTTGTGCAAGCATCACATCGTAG-3') for the Barcoded Universal F/R Primers Plate 96 v2 (Pacific Biosciences, Menlo Park, CA, USA). Since insufficient data were obtained at four loci (*DLA-88*, *DLA-12/88L*, *DLA-64*, and *DLA-DRB1*), additional data acquisition was performed for these genes. The primers, apart from those for *DLA-DRB1*, were modified by adding a 5' block and a tail representing the PacBio universal sequences for the Barcoded M13 Primer Plate (Pacific Biosciences). The primers for *DLA-DRB1*, which have the longest sequence (approximately 13.3 kb) among the eight loci, were modified by directly adding a 5' block and a tail representing the PacBio barcode sequences of the Barcoded M13 Primer Plate to avoid the need for a second round of PCR amplification.

To amplify the target region by long-range PCR in both experiments, in the first round of PCR amplification, the PCR mixture for seven genes except for *DLA-DRB1*, with a total volume of 50 μl, contained 20 ng of DNA, 1 unit of KOD FX polymerase, 25 μl of 2×PCR buffer, 10 μl of 2 mM dNTP, and 15 μM of each primer. The PCR mixture for *DLA-DRB1*, with a total volume of 50 μl, contained 200 ng of DNA, 1.25 units of PrimeSTAR GXL DNA polymerase (Takara Bio, Otsu, Japan), 10 μl of 5×PCR buffer, 4 μl of 2.5 mM dNTP, and 15 μM of each primer. The thermal cycling conditions for these genes were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of 98 °C for 10 s, 63 °C for 30 s, and 68 °C for 1 min/kb for *DLA-88* and *DLA-79*; 98 °C for 10 s, 58 °C for 30 s, and 68 °C for 1 min/kb for *DLA-12/88L*; 98 °C for 10 s, 65 °C for 30 s, and 68 °C for 1 min/kb for *DLA-64*; 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 1 min/kb for *DLA-DQA1*; and 98 °C for 10 s and 68 °C for 1 min/kb for *DLA-DRA*, *DLA-DRB1*, and *DLA-DQB1*.

The second round of amplification was then performed using the Barcoded Universal F/R Primers Plate-96 or the Barcoded M13 Primer Plate. For barcode assignment, the same PCR mixture as for the first round of PCR was used together with 1 μl of the first PCR sample (25 μl in total). The Barcoded Universal F/R Primers Plate-96 or Barcoded M13 Primer Plate used 2 or 3 μM of the barcode-indexed primer, respectively. The PCR program was set for 20 cycles, instead of 30 cycles as in the first round of PCR. All PCR

products were purified using AMPure XP beads and were verified by visible bands on the electrophoresis gel. DNA was then quantified using a Qubit HS dsDNA kit (Thermo Fisher Scientific). Samples using the Barcoded Universal F/R Primers Plate-96 or the Barcoded M13 Primer Plate were then pooled equimolarly to give a final DNA amount of approximately 4 µg (40 ng/µl) or 1.4 µg (117 ng/µl), respectively.

2.3. Library preparation and sequencing

We used two different PacBio sequencers: the Sequel IIe and the Revio. The initial sequencing run involved all eight DLA loci (*DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) on the Sequel IIe. However, the Sequel IIe sequencing did not yield a sufficient number of HiFi reads required for high-confidence allele calling for all target loci across all samples. Therefore, the amplicons of four key, highly polymorphic loci that suffered from insufficient read depth (*DLA-88*, *DLA-12/88L*, *DLA-64*, and *DLA-DRB1*) were re-sequenced using the Revio system to ensure robust genotype determination by achieving the necessary sequencing depth.

Library preparation for Sequel IIe was performed with the SMRTbell Express Template Prep Kit 2.0 and AMPure PB (Pacific Biosciences), in accordance with the PacBio protocol (PN 101-791-700, version 06). Briefly, the library was prepared from pooled amplicon DNA by the following reactions: repair of DNA damage such as nicks in the dsDNA, end-repair and A-tailing, ligation with unbarcoded SMRTbell adapter, and purification of SMRTbell ligated products. Preparation for sequencing followed the sample set-up instructions in SMRT Link v11.0 with selection of the application of amplicons longer than 3 kb using a Sequel II Binding Kit 2.0 and Sequencing Primer v4 (Pacific Biosciences). The library was sequenced on Sequel IIe (ICS v11.0 and SMRT Link v11.0) with the Sequel II SMRT Cell 8M Tray and Sequel II Sequencing Kit 2.0. Cells were loaded by diffusion loading with a loading concentration of 180 pM with pre-extension time of 1.3 h and movie time of 30 h. Circular consensus sequencing (CCS) analysis was performed on-instrument to create HiFi data.

Library preparation for Revio was performed with SMRTbell prep Kit 3.0 (Pacific Biosciences), in accordance with the PacBio protocol (PN 102-359-000 REV03 DEC2023). Briefly, the library was prepared from pooled amplicon DNA by the following reactions:

repair of DNA damage, end-repair and A-tailing, ligation with unbarcoded SMRTbell adapter, nuclease treatment to degrade unligated products, and purification of SMRTbell ligated products. A library of reads longer than 1 kb was selected using BluePippin (Sage Science, Beverly, MA, USA) and then purified with AMPure PB (Pacific Biosciences). Preparation for sequencing followed the sample set-up instructions in SMRT Link v13.0 with selection of the application of amplicons longer than 3 kb using Revio polymerase kit (Pacific Biosciences). The library was sequenced on Revio (ICS v13.0 and SMRT Link v13.0) with a Revio SMRT cell tray and Revio sequencing plate. Cells were loaded by adaptive loading with a loading concentration of 200 pM and movie time of 30 h. CCS analysis with DeepConsensus was performed on-instrument to create HiFi data.

2.4. Bioinformatics processing and allele construction

Demultiplexing of reads and removal of the PCR primer sequence were performed using lima (SMRT Tools v12.0 or v13.0) with the CCS preset and the following options: --min-sc–e 80 --min-end-sc–e 50 --min-ref-span 0.75. A Fastq file was generated from the demultiplexed bam file using bam2fastq (SMRT Tools v12.0 or v13.0). Positions of the target loci were determined using the dog reference genome (canFam4) and PCR primer positions. Reads from Sequel IIe and Revio were assigned to the loci according to the top-scoring hits [alignment tool, Minimap2 (v2.26-r1175)]. Differences between Sequel IIe and Revio did not affect the quality of the analysis, since both HiFi read sequencers use the same chemistry. Reads with mean quality values (QV) of less than 20 were excluded for most loci. However, given the extremely long amplicon length of *DLA-DRB1*, its QV distribution showed a concentration of reads above QV>15. Therefore, the exclusion threshold for *DLA-DRB1* was adjusted to QV<15. In addition, each read was aligned to the corresponding locus using Minimap2, and alignment coverage (total number of matches and mismatches / locus length) and sequence identity were calculated. Note that the loci were defined as the region from the 5' UTR end to the 3' UTR end. Reads with alignment coverage <91% or sequence identity <80% were excluded. For each locus and individual, reads were clustered, and consensus sequences were constructed using pbAA (https://github.com/PacificBiosciences/pbAA) (v1.0.3; “cluster” subcommand) with the default parameters except for *DLA-DRB1*, for which the minimum quality value and the

number of reads for clustering were set to 15 and 3, respectively (--min-read–v 15 --min-cluster-read-count 3). In this setting, pbAA initially sampled 500 reads, consensus sequences with a number of reads ≥5 (>3 for *DLA-DRB1*) were retained, and the sequences with the top two or one highest read count were extracted as full-length allele sequences of each sample. These sequences were then aligned to the reference loci using BLASTN (v2.14.0; options: -task blastn -dust no -qcov_hsp_perc 50 -perc_identity 80 max_hsps 1 -outfmt "6 std qlen slen qseq sseq"). The alignment results were parsed by the in-house program to call single-nucleotide variants (SNVs) and insertions-deletions (indels).

2.5. Allele identification

To identify the DLA allele, representative clustered sequences were compared with DLA allele sequences publicly available in the IPD-MHC database and NCBI databases using GENETYX-SV/RC Ver. 13.1.1 (Genetyx Corporation). Only full-length sequences that showed 100% identity across multiple individuals were used as extended alleles by CD-HIT-EST v4.6 (options: -c 1.0 -aL 1) [39]. The accuracy of gene typing using the consensus sequence was evaluated by comparing it with the PBS sequences obtained using Sanger sequencing (focusing on highly variable exons 2 and 3 in DLA class I genes and exon 2 in DLA class II genes), following a previously reported method [59]. To ascertain the haplotypes of seven DLA genes (*DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) located on chromosome 12, the haplotypes of homozygous dogs were initially identified, after which the haplotypes of heterozygous dogs were estimated manually based on the data from the homozygous dogs. Alleles and haplotypes were compared between TOYO and Marshall strains.

2.6. Haplotype estimation and allele comparison between strains

For haplotyping and strain comparisons, the presence of loci where extended alleles could not be identified due to large variations in the number of repeat sequences, particularly on *DLA-DRB1* locus, made accurate analysis difficult. Therefore, we additionally defined repeat-masked alleles as full-length allele sequences without repeats on introns. The repetitive elements (RepeatMasker annotations on canFam4 downloaded from the University of California, Santa Cruz genome browser) focusing on intron regions using

Liftoff (v1.6.3; option: -cds). The repeat-masked sequences were aligned for all individuals, and the regions of the allele sequences that were not masked due to mutation were identified using trimAl (v1.5; option: -nogaps) [18] and masked. To ascertain the haplotypes of seven DLA genes (*DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) located on chromosome 12, the haplotypes of homozygous dogs were initially identified, after which the haplotypes of heterozygous dogs were estimated manually based on the data from the homozygous dogs. Alleles and haplotypes were compared between TOYO and Marshall strains. To statistically compare the frequencies of haplotypes between strains, Fisher's exact test was performed using EXSUS software version 10.1.8 (EPS Corporation). Bonferroni adjustments for multiple comparisons were not performed, as recommended in similar studies [84,107]. To compare the level of genetic differentiation between the TOYO and Marshall strains, Analysis of Molecular Variance (AMOVA) was computed based on the diversity of alleles that were assigned by PBS (PBS alleles) or repeat-masked alleles using the software GenAlEx 6.5 [83]. The proportion of total genetic variation attributable to differences between the strains was measured using the pairwise differentiation index (PhiPT) by locus.

2.7. Analysis of potential regulatory elements

We examined the 5' UTR sequences including the promoter region of the extended alleles to identify mutations within the known core promoter elements (S, X1, X2, Y, and TATA) [113,114,115]. The sequences of all extended alleles were analyzed using the web server GC-Profile 2.0 to detect the CpG island (CGI) according to the established criteria [43,62]. Specifically, a region was defined as a CGI if it was at least 200 base pairs (bp) in length, possessed a GC content of 50% or greater, and exhibited an Observed/Expected CpG Ratio of 0.6 or higher.

2.8. Data availability

Reads generated in this study have been deposited in the DDBJ/ENA/GenBank Sequence Read Archive under BioProject ID PRJDB20367.

3. Results

3.1. Data available to identify alleles

The long-read sequencing strategy successfully generated high-quality data, and extended allele assignment was achieved for all 83 samples in *DLA-79*, *DLA-DRA*, *DLA-DQA1*, and *DLA-DQB1* using Sequel IIe data, and for *DLA-12/88L* and *DLA-64* using the Revio data. For the most complex loci, *DLA-88* and *DLA-DRB1*, successful allele calls were obtained for 67 and 69 of the 83 total samples, respectively. The obtained read counts for the *DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1* genes were listed in Table 3-2. The read lengths constituting each cluster in each gene, calculated by multiplying the read count by the full length, are shown in Figure 3-3. The accuracy of allele identification was partially validated by comparing the PBS sequences obtained from PacBio consensus sequences with Sanger sequencing data. The PBS sequences in all samples of *DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, and *DLA-DQA1* perfectly matched the Sanger sequencing results. The full-length region analysis revealed a substantial increase in the number of DLA alleles compared to conventional PBS-based typing, as summarized in Table 3-3, demonstrating that various nucleotide substitutions exist in exons outside of PBS, as well as within the intron sequences. There were a few cases in which the corresponding allele cluster could not be identified due to insufficient read counts (*DLA-DRB1*011:01* [1 sample]), and allele drops (*DLA-DQB1*004:01* [6 samples], and *DLA-DQB1*007:01* [4 samples]).

Table 3-2. Summary of read counts obtained for DLA genes

Locus	Median read count (minimum count–maximum count)	
	Homozygous	Heterozygous
DLA-88	500 (12–500)	146 (5–259)
DLA-12	500 (496–500)	164 (86–332)
DLA-64	500 (499–500)	145 (8–296)
DLA-79	498 (477–500)	210 (133–288)
DLA-DRA	474 (424–500)	186 (122–239)
DLA-DRB1	155 (75–289)	56 (13–239)
DLA-DQA1	497 (242–500)	215 (57–334)
DLA-DQB1	492 (82–500)	209 (56–376)

Table 3-3. The number of alleles classified based on designated nucleotide sequence region

	Number of alleles		
DLA class I locus	Exons 2 and 3 sequence	Coding sequence	Full-length sequence
DLA-88	9	9	9
DLA-12/88L	4	6	7
DLA-64	2	4	6
DLA-79	2	2	8
DLA class II locus	Exon 2 sequence	Coding sequence	Full-length sequence
DLA-DRA	1	2	11
DLA-DRB1	6	6	18
DLA-DQA1	6	6	12
DLA-DQB1	5	5	8

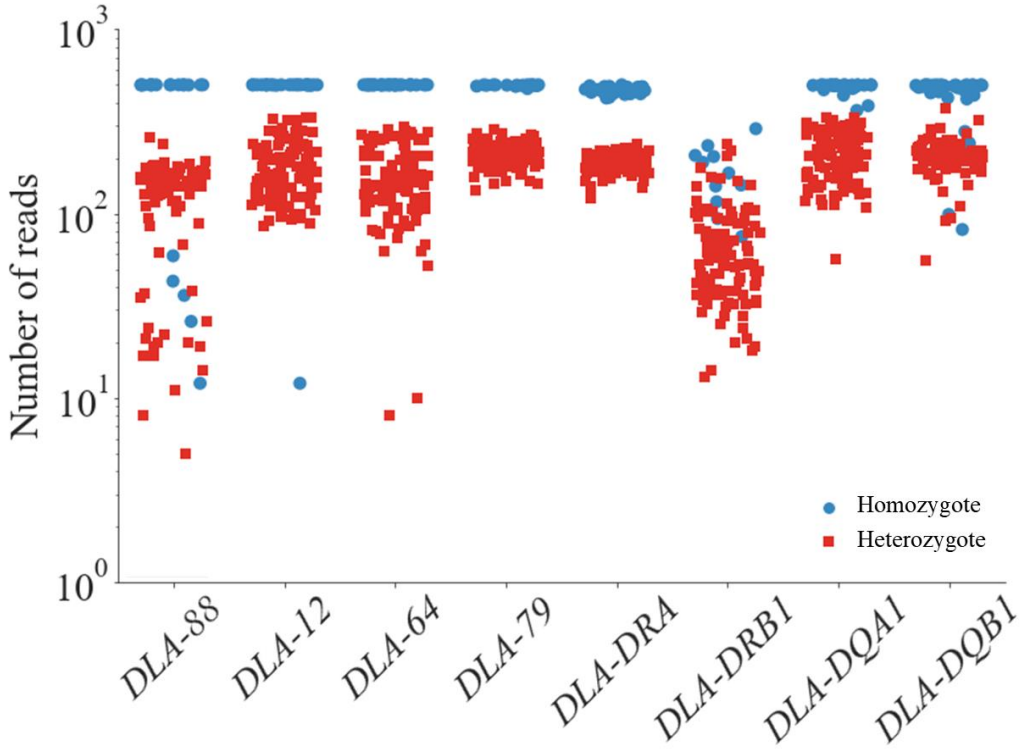

Figure 3-3. The read numbers that consist of each cluster in DLA genes.

circle: homozygous allele, square: heterozygous allele

3.2. Discovery of novel extended alleles by full-length region analysis

In the present study, 36 extended allele candidates for DLA class I genes (*DLA-88*: 10, *DLA-12*: 9, *DLA-88L*: 2, *DLA-64*: 7, and *DLA-79*: 8) and 117 extended allele candidates for DLA class II genes (*DLA-DRA*: 17, *DLA-DRB1*: 66, *DLA-DQA1*: 18, and *DLA-DQB1*: 16) were identified at full-length level. Of these sequences, 30 alleles for DLA class I genes (*DLA-88*: 9, *DLA-12*: 5, *DLA-88L*: 2, *DLA-64*: 6, and *DLA-79*: 8) and 49 alleles for DLA class II genes (*DLA-DRA*: 11, *DLA-DRB1*: 18, *DLA-DQA1*: 12, and *DLA-DQB1*: 8) were identified in two or more individuals. These alleles were subsequently designated as "extended alleles" at the full-length level in this analysis. The individual genotyping results and accession numbers were provided in Table 3-4 and Table 3-5, respectively. The classification and nomenclature for extended alleles, repeat-masked alleles, and PBS alleles, and the sequences of these extended alleles are listed in Table 3-6. Although no polymorphisms had previously been reported in *DLA-DRA* at PBS level, the current study revealed novel *DLA-DRA* extended alleles, which comprised one non-synonymous SNV in exon 1 and 14 SNVs and 14 indels within the introns. Conversely, the number of alleles for *DLA-88* did not increase from PBS alleles to extended alleles, suggesting *DLA-88* sequences have high intra-allelic linkage disequilibrium across the full-length level, but high inter-allelic polymorphisms, as 113 SNVs were found comparing nine *DLA-88* extended alleles.

Table 3-4. Summary of extended allele in beagle dogs

Animal ID	Strain	Sex	*DLA-88*		*DLA-12*	
1	TOYO	Male	012:01_ext1	501:01_ext1	001:01:01_ext4	001:01:01_ext1
2			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext1
3			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext1
4			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext1
5			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext2
6			012:01_ext1	508:01_ext1	001:01:01_ext4	001:01:03_ext1
7			502:01_ext1	041:01	001:01:01_ext1	002:03
8			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext1
9			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext1
10			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext1
11			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext2
12			502:01_ext1	508:01_ext1	001:01:01_ext1	001:01:03_ext1
13			502:01_ext1	508:01_ext1	001:01:01_ext2	001:01:03_ext1
14			502:01_ext1	508:01_ext1	001:01:01_ext2	001:01:03_ext1
15			502:01_ext1	508:01_ext1	001:01:01_ext2	001:01:03_ext1
16			N/A (502:01)	N/A (502:01)	001:01:01_ext1	001:01:01_ext1
17			N/A (502:01)	N/A (502:01)	001:01:01_ext1	001:01:01_ext1
18			N/A (502:01)	N/A (502:01)	001:01:01_ext1	001:01:01_ext1
19			N/A (502:01)	N/A (508:01)	001:01:01_ext1	001:01:03_ext1
20			N/A (nov-MS4)	N/A (502:01)	001:01:01_ext1	001:01:01_ext4
21		Female	012:01_ext1	025:01_ext1	001:01:01_ext4	88*016:03_ext1
22			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext1
23			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext1
24			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext2
25			012:01_ext1	508:01_ext1	001:01:01_ext4	001:01:03_ext1
26			012:01_ext1	508:02_ext1	001:01:01_ext4	001:01:03_ext1
27			025:01_ext1	502:01_ext1	88*016:03_ext1	001:01:01_ext1
28			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext1
29			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext2
30			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext2
31			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext2
32			502:01_ext1	502:01_ext1	001:01:01_ext1	001:01:01_ext2
33			502:01_ext1	508:02_ext1	001:01:01_ext1	001:01:03_ext1
34			502:01_ext1	508:02_ext1	001:01:01_ext1	001:01:03_ext1
35			502:01_ext1	508:02_ext1	001:01:01_ext1	001:01:03_ext1
36			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
37			508:01_ext1	508:02_ext1	001:01:03_ext1	001:01:03_ext1
38			N/A (012:01)	N/A (012:01)	001:01:01_ext4	001:01:01_ext4
39			N/A (025:01)	N/A (502:01)	88*016:03_ext1	001:01:01_ext1
40			N/A (501:01)	N/A (502:01)	001:01:01_ext1	001:01:01_ext2
41			N/A (502:01)	N/A (502:01)	001:01:01_ext1	001:01:01_ext1
42			N/A (502:01)	N/A (502:01)	001:01:01_ext1	001:01:01_ext2

43	Marshall	Male	012:01_ext1	012:01_ext1	001:01:01_ext4	001:01:01_ext4
44			012:01_ext1	502:01_ext1	001:01:01	001:01:01
45			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext2
46			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext2
47			012:01_ext1	508:01_ext1	001:01:01_ext4	001:01:03_ext1
48			012:01_ext1	508:01_ext1	001:01:01_ext4	001:01:03_ext1
49			012:01_ext1	508:01_ext1	001:01:01_ext4	001:01:03_ext1
50			502:01_ext1	025:01_ext1	001:01:01_ext1	88*016:03_ext1
51			502:01_ext1	508:01_ext1	001:01:01_ext2	001:01:03_ext1
52			502:01_ext1	508:01_ext1	001:01:01_ext2	001:01:03_ext1
53			502:01_ext1	508:01_ext1	001:01:01_ext2	001:01:03_ext1
54			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
55			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
56			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
57			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
58			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
59			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
60			N/A (502:01)	N/A (nov68)	001:01:01_ext1	88*069:01_ext1
61			nov-68_ext1	004:02_ext1	88*069:01_ext1	001:01:01_ext3
62			nov-68_ext1	508:01_ext1	001:01:03_ext1	88*069:01_ext1
63			508:01_ext1	nov-68_ext1	001:01:03_ext1	88*069:01_ext1
64			nov-MS4_ext1	508:01_ext1	001:01:03_ext1	004:01
65		Female	012:01_ext1	004:02_ext1	001:01:01_ext4	001:01:01_ext3
66			502:01_ext1	004:02_ext1	001:01:01_ext2	001:01:01_ext3
67			012:01_ext1	012:01_ext1	001:01:01_ext4	001:01:01_ext4
68			012:01_ext1	502:01_ext1	001:01:01_ext4	001:01:01_ext1
69			012:01_ext1	nov-MS4_ext1	001:01:01_ext4	001:01:01_ext4
70			025:01_ext1	508:01_ext1	88*016:03_ext1	001:01:03_ext1
71			025:01_ext1	508:01_ext1	88*016:03_ext1	001:01:03_ext1
72			501:01_ext1	508:01_ext1	001:01:01	001:01:03_ext1
73			N/A (502:01)	N/A (508:01)	001:01:01_ext1	001:01:03_ext1
74			502:01_ext1	508:01_ext1	001:01:01_ext1	001:01:03_ext1
75			502:01_ext1	508:01_ext1	001:01:01_ext1	001:01:03_ext1
76			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
77			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
78			508:01_ext1	508:01_ext1	001:01:03_ext1	001:01:03_ext1
79			012:01_ext1	nov-68_ext1	001:01:01_ext4	88*069:01_ext1
80			N/A (012:01)	N/A (508:01)	001:01:01_ext4	001:01:03_ext1
81			N/A (508:01)	N/A (501:02)	001:01:03_ext1	001:01:01_ext3
82			N/A (nov68)	N/A (508:01)	001:01:03_ext1	88*069:01_ext1
83			N/A (502:01)	N/A (nov-MS4)	001:01:01_ext1	001:01:01_ext4

Samples for which a full-length sequence was obtained but rejected due to its uniqueness was indicated with the PBS allele designation.

N/A: No applicable, ext: extended allele

Table 3-4. Summary of extended allele in beagle dogs (continued)

Animal ID	Strain	Sex	*DLA-64*		*DLA-79*	
1	TOYO	Male	001:01:01_ext1	001:01_ext1	001:01_ext1	001:01_ext2
2			001:01:01_ext1	001:01_ext1	001:01_ext1	001:01_ext4
3			001:01:01_ext1	001:01_ext1	001:01_ext4	001:01_ext4
4			001:01:01_ext1	001:01_ext1	001:01_ext4	001:01_ext5
5			001:01:01_ext1	001:01_ext1	001:01_ext3	001:01_ext5
6			001:01:01_ext1	001:01_ext2	001:01_ext6	001:02_ext1
7			001:01_ext1	001:01:01	001:01_ext1	001:01_ext6
8			001:01_ext1	001:01_ext1	001:01_ext1	001:01_ext4
9			001:01_ext1	001:01_ext1	001:01_ext1	001:02_ext2
10			001:01_ext1	001:01_ext1	001:01_ext3	001:01_ext5
11			001:01_ext1	001:01_ext1	001:01_ext3	001:02_ext2
12			001:01_ext1	001:01_ext2	001:02_ext2	001:02_ext2
13			001:01_ext1	001:01_ext2	001:01_ext2	001:02_ext2
14			001:01_ext1	001:01_ext2	001:02_ext1	001:02_ext2
15			001:01_ext1	001:01_ext2	001:02_ext2	001:02_ext2
16			001:01_ext1	001:01_ext1	001:01_ext1	001:01_ext1
17			001:01_ext1	001:01_ext1	001:01_ext1	001:01_ext5
18			001:01_ext1	001:01_ext1	001:01_ext4	001:02_ext2
19			001:01_ext1	001:01_ext2	001:01_ext4	001:01_ext6
20			001:01_ext1	001:01:01_ext1	001:01_ext6	001:02_ext1
21		Female	001:01:01_ext1	001:01:01_ext3	001:01_ext4	001:02_ext2
22			001:01:01_ext1	001:01_ext1	001:01_ext1	001:02_ext2
23			001:01:01_ext1	001:01_ext1	001:01_ext4	001:01_ext6
24			001:01:01_ext1	001:01_ext1	001:01_ext1	001:01_ext1
25			001:01:01_ext1	001:01_ext2	001:01_ext4	001:01_ext4
26			001:01:01_ext1	001:01_ext2	001:01_ext1	001:01_ext3
27			001:01:01_ext3	001:01_ext1	001:01_ext5	001:02_ext1
28			001:01_ext1	001:01_ext1	001:01_ext1	001:01_ext3
29			001:01_ext1	001:01_ext1	001:01_ext1	001:01_ext1
30			001:01_ext1	001:01_ext1	001:01_ext2	001:02_ext2
31			001:01_ext1	001:01_ext1	001:01_ext4	001:01_ext6
32			001:01_ext1	001:01_ext1	001:01_ext6	001:02_ext1
33			001:01_ext1	001:01_ext2	001:01_ext1	001:01_ext4
34			001:01_ext1	001:01_ext2	001:01_ext2	001:01_ext4
35			001:01_ext1	001:01_ext2	001:01_ext4	001:02_ext2
36			001:01_ext2	001:01_ext2	001:01_ext6	001:01_ext6
37			001:01_ext2	001:01:01_ext1	001:01_ext5	001:02_ext2
38			001:01:01_ext1	001:01:01_ext1	001:01_ext5	001:02_ext2
39			001:01:01_ext3	001:01_ext1	001:01_ext1	001:01_ext4
40			001:01_ext1	001:01_ext1	001:01_ext1	001:02_ext2
41			001:01_ext1	001:01_ext1	001:01_ext3	001:02_ext2
42			001:01_ext1	001:01_ext1	001:01_ext1	001:01_ext2

43	Marshall	Male	001:01:01_ext1	001:01:01_ext1	001:01_ext1	001:01_ext5
44			001:01:01_ext1	001:01_ext1	001:01_ext3	001:01_ext6
45			001:01:01_ext1	001:01_ext1	001:01_ext1	001:01_ext1
46			001:01:01_ext1	001:01_ext1	001:01_ext1	001:01_ext3
47			001:01:01_ext1	001:01_ext2	001:01_ext1	001:02_ext2
48			001:01:01_ext1	001:01_ext2	001:01_ext3	001:01_ext5
49			001:01:01_ext1	001:01_ext2	001:01_ext1	001:01_ext3
50			001:01_ext1	001:01:01_ext3	001:01_ext3	001:01_ext5
51			001:01_ext1	001:01_ext2	001:01_ext1	001:01_ext3
52			001:01_ext1	001:01_ext2	001:01_ext3	001:01_ext3
53			001:01_ext1	001:01_ext2	001:01_ext3	001:01_ext5
54			001:01_ext2	001:01_ext2	001:01_ext1	001:01_ext1
55			001:01_ext2	001:01_ext2	001:01_ext1	001:01_ext3
56			001:01_ext2	001:01_ext2	001:01_ext1	001:01_ext5
57			001:01_ext2	001:01_ext2	001:01_ext1	001:01_ext5
58			001:01_ext2	001:01_ext2	001:01_ext3	001:01_ext6
59			001:01_ext2	001:01_ext2	001:01_ext3	001:01_ext6
60			001:01_ext1	001:01_ext3	001:01_ext1	001:01_ext1
61			001:01_ext3	001:01:01_ext2	001:01_ext1	001:01_ext5
62			001:01_ext2	001:01_ext3	001:01_ext1	001:01_ext5
63			001:01_ext2	001:01_ext3	001:01_ext1	001:01_ext6
64			001:01_ext2	001:01:01_ext1	001:01_ext1	001:01_ext6
65		Female	001:01:01_ext1	001:01:01_ext2	001:01_ext1	001:01_ext1
66			001:01_ext1	001:01:01_ext2	001:01_ext1	001:01_ext5
67			001:01:01_ext1	001:01_ext2	001:01_ext1	001:01_ext1
68			001:01:01_ext1	001:01_ext1	001:01_ext1	001:01_ext6
69			001:01:01_ext1	001:01:01_ext1	001:01_ext1	001:01_ext3
70			001:01:01_ext3	001:01_ext2	001:01_ext1	001:01_ext3
71			001:01:01_ext3	001:01_ext2	001:01_ext3	001:01_ext3
72			001:01_ext1	001:01_ext2	001:01_ext1	001:01_ext1
73			001:01_ext1	001:01_ext2	001:01_ext1	001:01_ext1
74			001:01_ext1	001:01_ext2	001:01_ext1	001:01_ext5
75			001:01_ext1	001:01_ext2	001:01_ext3	001:01_ext6
76			001:01_ext2	001:01_ext2	001:01_ext3	001:01_ext5
77			001:01_ext2	001:01_ext2	001:01_ext3	001:01_ext5
78			001:01_ext2	001:01_ext2	001:01_ext3	001:01_ext5
79			001:01:01_ext1	001:01_ext3	001:01_ext1	001:01_ext3
80			001:01:01_ext1	001:01_ext2	001:01_ext1	001:01_ext1
81			001:01_ext2	001:01:01_ext2	001:01_ext3	001:01_ext5
82			001:01_ext2	001:01_ext3	001:01_ext1	001:01_ext3
83			001:01_ext1	001:01:01_ext1	001:01_ext3	001:01_ext6

Samples for which a full-length sequence was obtained but rejected due to its uniqueness was indicated with the PBS allele designation.

N/A: No applicable, ext: extended allele

Table 3-4. Summary of extended allele in beagle dogs (continued)

Animal ID	Strain	Sex	*DLA-DRA*		*DLA-DRB1*	
1	TOYO	Male	001:01_ext1	001:01_ext12	015:01_ext1	001:01_ext1
2			001:01	001:01_ext5	015:01_ext2	008:01_ext2
3			001:01_ext1	001:01_ext14	015:01_ext1	001:02_ext2
4			001:01_ext1	001:01_ext5	015:01_ext1	008:01_ext2
5			001:01_ext1	001:01_ext12	015:01	001:02_ext1
6			001:01_ext1	001:01_ext13	015:01_ext1	001:01_ext3
7			001:01	001:01_ext5	008:01_ext5	013:01
8			001:01_ext5	001:01_ext12	001:02	008:01_ext3
9			001:01_ext12	001:01_ext12	001:01	001:01
10			001:01_ext12	001:01_ext12	001:01	001:02_ext1
11			001:01_ext12	001:01_ext12	001:02_ext1	001:02_ext1
12			001:01_ext2	001:01_ext14	001:02_ext1	002:01
13			001:01_ext2	001:01_ext12	001:02	002:01_ext2
14			001:01_ext12	001:01_ext12	001:02	001:01_ext7
15			001:01_ext7	001:01_ext12	001:02_ext1	N/A# (011:01)
16			001:01_ext5	001:01_ext13	001:02_ext1	008:01
17			001:01_ext12	001:01_ext12	001:01	001:02_ext1
18			001:01_ext5	001:01_ext12	001:02_ext1	008:01_ext3
19			001:01_ext7	001:01_ext12	001:02	011:01
20			001:01_ext1	001:01_ext13	001:02_ext2	015:01_ext1
21		Female	001:01_ext1	001:01_ext10	015:01_ext1	028:01
22			001:01_ext1	001:01_ext14	015:01_ext1	001:02_ext3
23			001:01_ext1	001:01_ext12	015:01	001:02
24			001:01_ext1	001:01_ext12	015:01_ext1	001:02
25			001:01_ext1	001:01_ext13	N/A (015:01)	N/A (001:01)
26			001:01_ext1	001:01_ext2	015:01_ext2	002:01
27			001:01	001:01_ext10	028:01	008:01_ext1
28			001:01_ext12	001:01_ext12	001:02_ext1	001:02_ext5
29			001:01_ext13	001:01_ext13	001:02_ext4	001:02_ext4
30			001:01_ext12	001:01_ext12	001:01_ext7	001:01_ext7
31			001:01_ext13	001:01_ext13	001:02	001:02_ext5
32			001:01_ext13	001:01_ext13	001:02	001:02_ext1
33			001:01_ext2	001:01_ext13	001:02_ext1	002:01
34			001:01_ext2	001:01_ext12	001:02_ext1	002:01
35			001:01_ext2	001:01_ext12	001:01	002:01_ext1
36			001:01_ext2	001:01_ext7	002:01	011:01
37			001:01	001:01	002:01_ext1	002:01_ext1
38			001:01_ext1	001:01_ext1	015:01_ext1	015:01_ext1
39			001:01_ext5	001:01_ext10	028:01	008:01_ext5
40			001:01_ext12	001:01_ext12	001:01_ext1	001:02_ext1
41			001:01_ext12	001:01_ext12	001:01_ext7	001:02
42			001:01_ext5	001:01_ext12	001:02_ext2	008:01_ext1

43	Marshall	Male	001:01_ext1	001:01_ext1	N/A (015:01)	N/A (015:01)
44			001:01_ext1	001:01_ext13	015:01_ext2	001:02
45			001:01_ext1	001:01_ext12	015:01_ext1	001:02_ext1
46			001:01_ext1	001:01_ext13	015:01_ext1	001:01
47			001:01_ext1	001:01_ext12	N/A (015:01)	N/A (001:01)
48			001:01_ext1	001:01_ext13	N/A (015:01)	N/A (001:01)
49			001:01_ext1	001:01_ext12	015:01_ext2	001:01_ext1
50			001:01_ext10	001:01_ext12	N/A (001:02)	N/A (028:01)
51			001:01	001:01	001:02_ext1	001:01
52			001:01_ext12	001:01_ext12	001:02_ext1	001:01_ext2
53			001:01_ext12	001:01_ext12	001:02	001:01_ext1
54			001:01_ext2	001:01_ext13	001:01	002:01
55			001:01_ext12	001:01_ext12	001:01_ext1	001:01_ext1
56			001:01_ext12	001:01_ext12	001:01	001:01_ext1
57			001:01_ext13	001:01_ext13	001:01_ext1	001:01_ext2
58			001:01_ext12	001:01_ext12	001:01	001:01
59			001:01_ext12	001:01_ext12	001:01_ext1	001:01_ext1
60			001:01_ext8	001:01_ext13	001:01_ext3	048:01_ext1
61			001:01_ext3	001:01_ext9	048:01	006:01
62			001:01_ext2	001:01_ext9	002:01	048:01
63			001:01_ext8	001:01_ext14	001:01_ext1	048:01
64			001:01_ext1	001:01_ext13	001:01_ext1	015:01_ext1
65		Female	001:01_ext3	001:01_ext1	N/A (015:01)	N/A (006:01)
66			001:01_ext3	001:01_ext12	N/A (001:02)	N/A (006:01)
67			001:01_ext1	001:01_ext1	015:01_ext2	015:01_ext2
68			001:01_ext1	001:01_ext13	N/A (015:01)	N/A (001:02)
69			001:01_ext1	001:01_ext1	015:01_ext1	015:01_ext1
70			001:01_ext2	001:01_ext10	028:01	002:01
71			001:01	001:01_ext2	N/A (028:01)	N/A (002:01)
72			001:01	001:01_ext12	N/A (001:01)	N/A (015:01)
73			001:01_ext2	001:01_ext12	N/A (001:02)	N/A (002:01)
74			001:01_ext2	001:01_ext12	001:02	002:01_ext2
75			001:01_ext12	001:01_ext12	001:02_ext3	001:01_ext1
76			001:01_ext12	001:01_ext12	001:01	001:01
77			001:01_ext12	001:01_ext12	001:01_ext2	001:01_ext1
78			001:01_ext13	001:01_ext13	001:01_ext1	001:01_ext1
79			001:01_ext1	001:01_ext8	N/A (015:01)	N/A (048:01)
80			001:01_ext1	001:01_ext2	N/A (015:01)	N/A (002:01)
81			001:01_ext3	001:01_ext12	N/A (001:01)	N/A (006:01)
82			001:01_ext2	001:01_ext8	002:01	048:01_ext1
83			001:01_ext1	001:01_ext13	001:02	015:01_ext1

Samples for which a full-length sequence was obtained but rejected due to its uniqueness was indicated with the PBS allele designation.

N/A#: No consistency with the allele based on short read sequencing, N/A: No applicable, ext: extended allele

Table 3-4. Summary of extended allele in beagle dogs (continued)

Animal ID	Strain	Sex	*DLA-DQA1*		*DLA-DQB1*	
1	TOYO	Male	009:01_ext3	001:01	001:01_ext1	002:01_ext5
2			009:01_ext1	003:01_ext2	001:01_ext1	N/A# (004:01)
3			009:01_ext1	001:01_ext1	001:01_ext1	002:01_ext1
4			009:01_ext3	003:01_ext2	001:01_ext1	N/A# (004:01)
5			009:01_ext1	001:01_ext3	001:01_ext1	002:01_ext1
6			009:01_ext1	001:01_ext3	001:01	002:01_ext1
7			003:01_ext1	003:01	004:01	004:01
8			001:01_ext1	003:01_ext2	002:01_ext1	N/A# (004:01)
9			001:01_ext2	001:01_ext2	002:01_ext5	002:01_ext5
10			001:01_ext2	001:01_ext1	002:01_ext1	002:01_ext5
11			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
12			001:01_ext1	009:01_ext2	002:01_ext1	001:01_ext1
13			001:01_ext1	009:01_ext2	002:01_ext1	001:01_ext1
14			001:01_ext3	001:01_ext3	002:01_ext1	002:01_ext1
15			001:01_ext1	002:01_ext1	002:01_ext1	013:03_ext1
16			001:01_ext3	003:01_ext1	002:01_ext1	N/A# (004:01)
17			001:01_ext3	001:01_ext3	002:01_ext1	002:01_ext1
18			003:01_ext1	001:01_ext1	002:01_ext1	N/A# (004:01)
19			001:01_ext3	002:01_ext1	002:01_ext3	013:03_ext1
20			001:01_ext3	009:01_ext1	002:01_ext1	001:01_ext1
21		Female	009:01	005:01_ext1	001:01_ext1	007:01_ext1
22			009:01_ext1	001:01_ext3	001:01_ext1	002:01_ext1
23			009:01_ext1	001:01_ext1	001:01_ext1	002:01_ext1
24			009:01_ext1	001:01_ext3	001:01_ext1	002:01_ext2
25			009:01_ext1	001:01_ext1	001:01_ext1	002:01_ext1
26			009:01_ext3	009:01_ext3	001:01_ext1	001:01_ext1
27			005:01_ext1	003:01_ext1	007:01_ext1	004:01
28			001:01_ext3	001:01_ext3	002:01_ext1	002:01_ext1
29			001:01_ext3	001:01_ext3	002:01_ext1	002:01_ext1
30			001:01_ext3	001:01_ext4	002:01_ext1	002:01_ext5
31			001:01_ext3	001:01_ext3	002:01_ext2	002:01_ext2
32			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
33			001:01_ext1	009:01_ext3	002:01_ext1	001:01_ext1
34			001:01_ext1	009:01_ext2	002:01_ext1	001:01_ext1
35			001:01_ext3	009:01_ext3	002:01_ext1	001:01_ext1
36			009:01_ext2	002:01	001:01_ext1	013:03_ext1
37			009:01_ext2	009:01_ext2	001:01_ext1	001:01_ext1
38			009:01_ext1	009:01_ext1	001:01_ext1	001:01_ext1
39			005:01_ext1	003:01_ext1	007:01	004:01
40			001:01	001:01_ext3	002:01_ext1	002:01_ext5
41			001:01_ext3	001:01_ext4	002:01_ext1	002:01_ext5
42			001:01_ext1	003:01_ext1	002:01_ext1	N/A# (004:01)

43	Marshall	Male	009:01_ext1	009:01_ext1	001:01_ext1	001:01_ext1
44			009:01_ext1	001:01_ext3	001:01_ext1	002:01_ext1
45			009:01_ext1	001:01_ext1	001:01_ext1	002:01_ext1
46			009:01_ext1	001:01_ext3	001:01_ext1	002:01_ext1
47			009:01_ext3	001:01_ext3	001:01_ext1	002:01_ext1
48			009:01_ext1	001:01_ext1	001:01_ext1	002:01_ext1
49			009:01_ext1	001:01_ext3	001:01_ext1	002:01_ext1
50			001:01_ext3	005:01_ext1	002:01_ext1	007:01_ext1
51			001:01_ext3	001:01_ext3	002:01_ext1	002:01_ext1
52			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
53			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
54			001:01_ext1	009:01_ext2	002:01_ext1	001:01_ext1
55			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
56			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
57			001:01_ext1	001:01_ext1	002:01_ext3	002:01_ext3
58			001:01_ext3	001:01_ext3	002:01_ext1	002:01_ext1
59			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
60			001:01_ext1	004:02_ext1	002:01_ext1	031:01_ext1
61			004:02_ext1	005:01_ext1	031:01_ext1	N/A# (007:01)
62			009:01_ext2	004:02_ext1	001:01_ext1	031:01_ext1
63			001:01_ext3	004:02_ext1	002:01_ext1	031:01_ext1
64			001:01_ext1	009:01	002:01_ext1	001:01_ext1
65		Female	009:01_ext1	005:01_ext1	001:01_ext1	N/A# (007:01)
66			001:01_ext3	005:01_ext1	002:01_ext1	N/A# (007:01)
67			009:01_ext1	009:01_ext1	001:01_ext1	001:01_ext1
68			009:01_ext1	001:01_ext1	001:01_ext1	002:01_ext1
69			009:01_ext1	009:01_ext1	001:01_ext1	001:01_ext1
70			005:01_ext1	009:01_ext2	007:01	001:01_ext1
71			005:01_ext1	009:01_ext2	007:01_ext1	001:01_ext1
72			001:01_ext1	001:01_ext1	002:01_ext1	020:02
73			001:01_ext3	009:01_ext2	002:01_ext1	001:01_ext1
74			001:01_ext3	009:01_ext2	002:01_ext1	001:01_ext1
75			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
76			001:01_ext3	001:01_ext3	002:01	002:01
77			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
78			001:01_ext1	001:01_ext1	002:01_ext1	002:01_ext1
79			009:01_ext1	004:02_ext1	001:01_ext1	031:01_ext1
80			009:01_ext3	009:01_ext3	001:01_ext1	001:01_ext1
81			001:01_ext3	005:01_ext1	002:01_ext1	N/A# (007:01)
82			009:01_ext2	004:02_ext1	001:01_ext1	031:01_ext1
83			001:01_ext1	009:01_ext1	002:01_ext1	001:01_ext1

Samples for which a full-length sequence was obtained but rejected due to its uniqueness was indicated with the PBS allele designation.

N/A#: No consistency with the allele based on short read sequencing, N/A: No applicable, ext: extended allele

Table 3-5. List of accession number for each sample and each DLA genes

Animal ID	*DLA-88*	*DLA-12/88L*	*DLA-64*	*DLA-79*	*DLA-DRA*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*
1	DRR797068	DRR797064	DRR797066	DRR797067	DRR797071	DRR797072	DRR797069	DRR797070
2	DRR796850	DRR796846	DRR796848	DRR796849	DRR796853	DRR796854	DRR796851	DRR796852
3	DRR796831	DRR796827	DRR796829	DRR796830	DRR796834	DRR796835	DRR796832	DRR796833
4	DRR796734	DRR796731	DRR796732	DRR796733	DRR796737	DRR796739	DRR796735	DRR796736
5	DRR796999	DRR796995	DRR796997	DRR796998	DRR797002	DRR797003	DRR797000	DRR797001
6	DRR796696	DRR796692	DRR796694	DRR796695	DRR796699	DRR796700	DRR796697	DRR796698
7	DRR796989	DRR796985	DRR796987	DRR796988	DRR796992	DRR796993	DRR796990	DRR796991
8	DRR796840	DRR796837	DRR796838	DRR796839	DRR796843	DRR796844	DRR796841	DRR796842
9	DRR796752	DRR796749	DRR796750	DRR796751	DRR796755	DRR796756	DRR796753	DRR796754
10	DRR797058	DRR797054	DRR797056	DRR797057	DRR797061	DRR797062	DRR797059	DRR797060
11	DRR796725	DRR796722	DRR796723	DRR796724	DRR796728	DRR796730	DRR796726	DRR796727
12	DRR796706	DRR796702	DRR796704	DRR796705	DRR796709	DRR796710	DRR796707	DRR796708
13	DRR797194	DRR797190	DRR797192	DRR797193	DRR797197	DRR797198	DRR797195	DRR797196
14	DRR797078	DRR797074	DRR797076	DRR797077	DRR797081	DRR797082	DRR797079	DRR797080
15	DRR796744	DRR796740	DRR796742	DRR796743	DRR796747	DRR796748	DRR796745	DRR796746
16	NA	DRR796975	DRR796977	DRR796978	DRR796982	DRR796983	DRR796980	DRR796981
17	NA	DRR797437	DRR797439	DRR797440	DRR797443	DRR797444	DRR797441	DRR797442
18	NA	DRR797429	DRR797431	DRR797432	DRR797435	DRR797436	DRR797433	DRR797434
19	NA	DRR797446	DRR797448	DRR797449	DRR797452	DRR797453	DRR797450	DRR797451
20	NA	DRR796965	DRR796967	DRR796968	DRR796972	DRR796973	DRR796970	DRR796971
21	DRR797088	DRR797084	DRR797086	DRR797087	DRR797091	DRR797092	DRR797089	DRR797090
22	DRR796762	DRR796758	DRR796760	DRR796761	DRR796765	DRR796766	DRR796763	DRR796764
23	DRR796716	DRR796712	DRR796714	DRR796715	DRR796719	DRR796720	DRR796717	DRR796718
24	DRR796782	DRR796778	DRR796780	DRR796781	DRR796785	DRR796786	DRR796783	DRR796784
25	DRR796880	DRR796876	DRR796878	DRR796879	DRR796883	NA	DRR796881	DRR796882
26	DRR797019	DRR797015	DRR797017	DRR797018	DRR797022	DRR797023	DRR797020	DRR797021
27	DRR797009	DRR797005	DRR797007	DRR797008	DRR797012	DRR797013	DRR797010	DRR797011
28	DRR796792	DRR796788	DRR796790	DRR796791	DRR796795	DRR796796	DRR796793	DRR796794
29	DRR796772	DRR796768	DRR796770	DRR796771	DRR796775	DRR796776	DRR796773	DRR796774
30	DRR797184	DRR797180	DRR797182	DRR797183	DRR797187	DRR797188	DRR797185	DRR797186
31	DRR796870	DRR796866	DRR796868	DRR796869	DRR796873	DRR796874	DRR796871	DRR796872
32	DRR797048	DRR797044	DRR797046	DRR797047	DRR797051	DRR797052	DRR797049	DRR797050
33	DRR797098	DRR797094	DRR797096	DRR797097	DRR797101	DRR797102	DRR797099	DRR797100
34	DRR796821	DRR796817	DRR796819	DRR796820	DRR796824	DRR796825	DRR796822	DRR796823
35	DRR797144	DRR797140	DRR797142	DRR797143	DRR797147	DRR797149	DRR797145	DRR797146
36	DRR797038	DRR797034	DRR797036	DRR797037	DRR797041	DRR797042	DRR797039	DRR797040
37	DRR796860	DRR796856	DRR796858	DRR796859	DRR796863	DRR796864	DRR796861	DRR796862
38	NA	DRR796916	DRR796918	DRR796919	DRR796922	DRR796923	DRR796920	DRR796921
39	NA	DRR797454	DRR797455	DRR797456	DRR797459	DRR797460	DRR797457	DRR797458
40	NA	DRR797025	DRR797026	DRR797027	DRR797031	DRR797032	DRR797029	DRR797030
41	NA	DRR797461	DRR797462	DRR797463	DRR797466	DRR797467	DRR797464	DRR797465
42	NA	DRR797469	DRR797470	DRR797471	DRR797474	DRR797475	DRR797472	DRR797473

43	DRR797374	DRR797370	DRR797372	DRR797373	DRR797377	NA	DRR797375	DRR797376
44	DRR797214	DRR797210	DRR797212	DRR797213	DRR797217	DRR797218	DRR797215	DRR797216
45	DRR797274	DRR797270	DRR797272	DRR797273	DRR797277	DRR797278	DRR797275	DRR797276
46	DRR797304	DRR797300	DRR797302	DRR797303	DRR797307	DRR797308	DRR797305	DRR797306
47	DRR797358	DRR797355	DRR797356	DRR797357	DRR797361	NA	DRR797359	DRR797360
48	DRR797350	DRR797346	DRR797348	DRR797349	DRR797353	NA	DRR797351	DRR797352
49	DRR796802	DRR796798	DRR796800	DRR796801	DRR796805	DRR796806	DRR796803	DRR796804
50	DRR797383	DRR797379	DRR797381	DRR797382	DRR797386	NA	DRR797384	DRR797385
51	DRR797155	DRR797151	DRR797153	DRR797154	DRR797158	DRR797159	DRR797156	DRR797157
52	DRR797134	DRR797130	DRR797132	DRR797133	DRR797137	DRR797138	DRR797135	DRR797136
53	DRR796910	DRR796906	DRR796908	DRR796909	DRR796913	DRR796914	DRR796911	DRR796912
54	DRR796929	DRR796925	DRR796927	DRR796928	DRR796932	DRR796933	DRR796930	DRR796931
55	DRR797224	DRR797220	DRR797222	DRR797223	DRR797227	DRR797228	DRR797225	DRR797226
56	DRR797294	DRR797290	DRR797292	DRR797293	DRR797297	DRR797298	DRR797295	DRR797296
57	DRR797264	DRR797260	DRR797262	DRR797263	DRR797267	DRR797268	DRR797265	DRR797266
58	DRR797204	DRR797200	DRR797202	DRR797203	DRR797207	DRR797208	DRR797205	DRR797206
59	DRR796900	DRR796896	DRR796898	DRR796899	DRR796903	DRR796904	DRR796901	DRR796902
60	NA	DRR797103	DRR797105	DRR797106	DRR797110	DRR797111	DRR797108	DRR797109
61	DRR797117	DRR797113	DRR797115	DRR797116	DRR797120	DRR797121	DRR797118	DRR797119
62	DRR796939	DRR796935	DRR796937	DRR796938	DRR796942	DRR796943	DRR796940	DRR796941
63	DRR797234	DRR797230	DRR797232	DRR797233	DRR797237	DRR797238	DRR797235	DRR797236
64	DRR797284	DRR797280	DRR797282	DRR797283	DRR797287	DRR797288	DRR797285	DRR797286
65	DRR797314	DRR797310	DRR797312	DRR797313	DRR797317	NA	DRR797315	DRR797316
66	DRR797392	DRR797388	DRR797390	DRR797391	DRR797395	NA	DRR797393	DRR797394
67	DRR796959	DRR796955	DRR796957	DRR796958	DRR796962	DRR796963	DRR796960	DRR796961
68	DRR797332	DRR797328	DRR797330	DRR797331	DRR797335	NA	DRR797333	DRR797334
69	DRR796890	DRR796886	DRR796888	DRR796889	DRR796893	DRR796894	DRR796891	DRR796892
70	DRR796949	DRR796945	DRR796947	DRR796948	DRR796952	DRR796953	DRR796950	DRR796951
71	DRR797365	DRR797362	DRR797363	DRR797364	DRR797368	NA	DRR797366	DRR797367
72	DRR797341	DRR797337	DRR797339	DRR797340	DRR797344	NA	DRR797342	DRR797343
73	NA	DRR797397	DRR797399	DRR797400	DRR797404	NA	DRR797402	DRR797403
74	DRR796812	DRR796808	DRR796810	DRR796811	DRR796815	DRR796816	DRR796813	DRR796814
75	DRR797164	DRR797161	DRR797162	DRR797163	DRR797167	DRR797168	DRR797165	DRR797166
76	DRR797244	DRR797240	DRR797242	DRR797243	DRR797247	DRR797248	DRR797245	DRR797246
77	DRR797254	DRR797250	DRR797252	DRR797253	DRR797257	DRR797258	DRR797255	DRR797256
78	DRR797174	DRR797170	DRR797172	DRR797173	DRR797177	DRR797178	DRR797175	DRR797176
79	DRR797323	DRR797319	DRR797321	DRR797322	DRR797326	NA	DRR797324	DRR797325
80	NA	DRR797406	DRR797408	DRR797409	DRR797412	NA	DRR797410	DRR797411
81	NA	DRR797122	DRR797123	DRR797124	DRR797127	NA	DRR797125	DRR797126
82	NA	DRR797413	DRR797415	DRR797416	DRR797419	DRR797420	DRR797417	DRR797418
83	NA	DRR797421	DRR797423	DRR797424	DRR797427	DRR797428	DRR797425	DRR797426

NA: not applicable

Table 3-6. The nomenclature for DLA alleles assigned based on the designated nucleotide sequence

DLA class I gene	Allele assigned by PBS	Repeat-masked allele	Extended allele	Frequency	Number of animals
DLA-88	**004:02**	**004:02_rm1**	**004:02_ext1**	**3**	**3**
	012:01	**012:01_rm1**	**012:01_ext1**	**26**	**23**
	025:01	**025:01_rm1**	**025:01_ext1**	**5**	**4**
	041:01	041:01_rm1	041:01_ext1	1	1
	501:01	**501:01_rm1**	**501:01_ext1**	**2**	**2**
	502:01	**502:01_rm1**	**502:01_ext1**	**46**	**37**
	508:01	**508:01_rm1**	**508:01_ext1**	**42**	**32**
	508:02	**508:02_rm1**	**508:02_ext1**	**5**	**5**
	nov-68	**nov-68_rm1**	**nov-68_ext1**	**4**	**4**
	nov-MS4	**nov-MS4_rm1**	**nov-MS4_ext1**	**2**	**2**
DLA-12	**001:01:01**	**001:01:01_rm1**	**001:01:01_ext1**	**45**	**37**
		001:01:01_rm2	**001:01:01_ext2**	**18**	**18**
		001:01:01_rm3	**001:01:01_ext3**	**4**	**4**
		001:01:01_rm4	**001:01:01_ext4**	**31**	**27**
		001:01:01_rm5	001:01:01_ext5	2	1
		001:01:01_rm6	001:01:01_ext6	1	1
	001:01:03	**001:01:03_rm1**	**001:01:03_ext1**	**51**	**40**
	002:03	002:03_rm1	002:03_ext1	1	1
	004:01	004:01_rm1	004:01_ext1	1	1
DLA-88L	**016:03**	**016:03_rm1**	**016:03_ext1**	**6**	**6**
	069:01	**069:01_rm1**	**069:01_ext1**	**6**	**6**
DLA-64	**001:01**	**001:01_rm1**	**001:01_ext1**	**65**	**50**
		001:01_rm2	**001:01_ext2**	**51**	**41**
		001:01_rm3	**001:01_ext3**	**6**	**6**
	001:01:01	**001:01:01_rm1**	**001:01:01_ext1**	**33**	**30**
		001:01:01_rm2	**001:01:01_ext2**	**4**	**4**
		001:01:01_rm3	**001:01:01_ext3**	**6**	**6**
		001:01:01_rm4	001:01:01_ext4	1	1

DLA-79	**001:01**	**001:01_rm1**	**001:01_ext1**	**54**	**43**
		001:01_rm2	**001:01_ext2**	**5**	**5**
		001:01_rm3	**001:01_ext3**	**30**	**28**
		001:01_rm4	**001:01_ext4**	**16**	**14**
		001:01_rm5	**001:01_ext5**	**21**	**21**
		001:01_rm6	**001:01_ext6**	**17**	**16**
	001:02	**001:02_rm1**	**001:02_ext1**	**5**	**5**
		001:02_rm2	**001:02_ext2**	**18**	**16**

Bold: Alleles identified as extended alleles because they showed 100% identity among multiple individuals based on the full-length sequence

rm: repeat-masked allele, ext: extended allele

Table 3-6. The nomenclature for DLA alleles assigned based on the designated nucleotide sequence (continued)

DLA class II gene	Allele assigned by PBS	Repeat-masked allele	Extended allele	Frequency	Number of animals
DLA-DRA	**001:01**	**001:01_rm1**	**001:01_ext1**	**32**	**28**
			001:01_ext2	**15**	**15**
			001:01_ext3	**4**	**4**
			001:01_ext4	1	1
		001:01_rm2	**001:01_ext5**	**8**	**8**
			001:01_ext6	2	1
		001:01_rm3	**001:01_ext7**	**3**	**3**
		001:01_rm4	**001:01_ext8**	**4**	**4**
			001:01_ext9	**2**	**2**
		001:01_rm5	**001:01_ext10**	**5**	**4**
			001:01_ext11	1	1
		001:01_rm6	**001:01_ext12**	**57**	**40**
			001:01_ext13	**23**	**18**
			001:01_ext14	**4**	**4**
			001:01_ext15	2	1
		001:01_rm7	001:01_ext16	1	1
		001:01_rm8	001:01_ext17	1	1
DLA-DRB1	**001:01**	**001:01_rm1**	**001:01_ext1**	**16**	**13**
			001:01_ext2	**3**	**3**
			001:01_ext3	**2**	**2**
			001:01_ext4	1	1
			001:01_ext5	1	1
			001:01_ext6	1	1
		001:01_rm2	**001:01_ext7**	**4**	**3**
			001:01_ext8	1	1
			001:01_ext9	1	1
			001:01_ext10	1	1
			001:01_ext11	1	1
		001:01_rm3	001:01_ext12	1	1

		001:01_ext13	1	1
	001:01_rm4	001:01_ext14	2	1
	001:01_rm5	001:01_ext15	2	1
	001:01_rm6	001:01_ext16	1	1
001:02	**001:02_rm1**	**001:02_ext1**	**17**	**16**
		001:02_ext2	**3**	**3**
		001:02_ext3	**2**	**2**
		001:02_ext4	**2**	**2**
		001:02_ext5	**2**	**2**
		001:02_ext6	1	1
		001:02_ext7	1	1
		001:02_ext8	1	1
		001:02_ext9	1	1
		001:02_ext10	1	1
		001:02_ext11	1	1
	001:02_rm2	001:02_ext12	1	1
		001:02_ext13	1	1
		001:02_ext14	1	1
		001:02_ext15	1	1
	001:02_rm3	001:02_ext16	1	1
		001:02_ext17	1	1
	001:02_rm4	001:02_ext18	1	1
002:01	**002:01_rm1**	**002:01_ext1**	**3**	**2**
		002:01_ext2	**2**	**2**
		002:01_ext3	1	1
	002:01_rm2	002:01_ext4	1	1
		002:01_ext5	1	1
	002:01_rm3	002:01_ext6	1	1
		002:01_ext7	1	1
	002:01_rm3	002:01_exy8	1	1
	002:01_rm4	002:01_ext9	1	1
	002:01_rm5	002:01_ext10	1	1

		002:01_rm6	002:01_ext11	1	1
	006:01	006:01_rm1	006:01_ext1	1	1
	008:01	**008:01_rm1**	**008:01_ext1**	**2**	**2**
			008:01_ext2	**2**	**2**
			008:01_ext3	**2**	**2**
			008:01_ext4	1	1
		008:01_rm2	**008:01_ext5**	**2**	**2**
	011:01	011:01_rm1	011:01_ext1	1	1
		011:01_rm2	011:01_ext2	1	1
	013:01	013:01_rm1	013:01_ext1	1	1
	015:01	**015:01_rm1**	**015:01_ext1**	**16**	**14**
		015:01_rm2	**015:01_ext2**	**6**	**5**
		015:01_rm3	015:01_ext3	1	1
		015:01_rm4	015:01_ext4	1	1
	028:01	**028:01_rm1**	028:01_ext1	1	1
			028:01_ext2	1	1
		028:01_rm2	028:01_ext3	1	1
	048:01	**048:01_rm1**	**048:01_ext1**	**2**	**2**
		048:01_rm2	048:01_ext2	1	1
			048:01_ext3	1	1
		048:01_rm3	048:01_ext4	1	1
DLA-DQA1	**001:01**	**001:01_rm1**	**001:01_ext1**	**43**	**31**
		001:01_rm2	**001:01_ext2**	**3**	**2**
		001:01_rm3	**001:01_ext3**	**37**	**29**
			001:01_ext4	**2**	**2**
		001:01_rm4	001:01_ext5	1	1
		001:01_rm5	001:01_ex6	1	1
	002:01	**002:01_rm1**	**002:01_ext1**	**2**	**2**
		002:01_rm2	002:01_ext2	1	1
	003:01	**003:01_rm1**	**003:01_ext1**	**6**	**6**
		003:01_rm2	**003:01_ext2**	**3**	**3**

		003:01_rm3	003:01_ext3	1	1
	004:02	**004:02_rm1**	**004:02_ext1**	**6**	**6**
	005:01	**005:01_rm1**	**005:01_ext1**	**10**	**9**
	009:01	**009:01_rm1**	**009:01_ext1**	**26**	**22**
		009:01_rm2	**009:01_ext2**	**13**	**12**
		009:01_rm3	**009:01_ext3**	**9**	**7**
		009:01_rm4	009:01_ext4	1	1
		009:01_rm5	009:01_ext5	1	1
DLA-DQB1	**001:01**	**001:01_rm1**	**001:01_ext1**	**49**	**42**
			001:01_ext2	1	1
	002:01	**002:01_rm1**	**002:01_ext1**	**71**	**55**
			002:01_ext2	**3**	**2**
			002:01_ext3	**3**	**2**
			002:01_ext4	2	1
		002:01_rm2	**002:01_ext5**	**7**	**6**
	004:01	**004:01_rm1**	004:01_ext1	2	1
			004:01_ext2	1	1
			004:01_ext3	1	1
	007:01	**007:01_rm1**	**007:01_ext1**	**4**	**4**
			007:01_ext2	1	1
			007:01_ext3	1	1
	013:03	**013:03_rm1**	**013:03_ext1**	**3**	**3**
	020:02	020:02_rm1	020:02_ext1	1	1
	031:01	**031:01_rm1**	**031:01_ext1**	**6**	**6**

Bold: Alleles identified as extended alleles because they showed 100% identity among multiple individuals based on the full-length sequence

rm: repeat-masked allele, ext: extended allele

3.3. Haplotype estimation and population differentiation

Haplotypes of the seven DLA genes located on chromosome 12 (*DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) were estimated based on repeat-masked alleles. *DLA-79* was excluded from haplotype estimation as it was located on chromosome 18. The incorporation of repeat-masked alleles resulted in a substantial increase in the number of estimated haplotypes, subdividing the 10 haplotypes based on PBS alleles into a total of 25 haplotypes based on repeat-masked alleles, as detailed in Table 3-7. The diversity of haplotypes between the TOYO and Marshall Beagles was significantly different in Hp.4-2 and Hp.6-1 (Table 3-8).

Table 3-7. The estimated haplotypes based on repeat-masked alleles

Haplotype ID	*DLA-88*	*DLA-12/88L*	*DLA-64*	*DLA-DRA*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*
Hp.1	012:01	001:01:01	001:01:01	001:01	015:01	009:01	001:01
Hp.1-1	012:01_rm1	001:01:01_rm4	001:01:01_rm1	001:01_rm1	015:01_rm1	009:01_rm1	001:01_rm1
Hp.1-2	-	-	-	-	015:01_rm2	-	-
Hp.1-3	-	-	-	-	-	009:01_rm3	-
Hp.2	025:01	88*016:03	001:01:01	001:01	028:01	005:01	007:01
Hp.2-1	025:01_rm1	88*016:03_rm1	001:01:01_rm3	001:01_rm5	028:01_rm1	005:01_rm1	007:01_rm1
Hp.3	502:01:00	001:01:01	001:01	001:01	001:01	001:01	002:01
Hp.3-1	502:01_rm1	001:01:01_rm1	001:01_rm1	001:01_rm6	001:01_rm2	001:01_rm3	002:01_rm1
Hp.3-2	-	001:01:01_rm2	-	-	-	-	002:01_rm2
Hp.4	502:01	001:01:01	001:01	001:01	001:02	001:01	002:01
Hp.4-1	502:01_rm1	001:01:01_rm1	001:01_rm1	001:01_rm5	001:02_rm1	001:01_rm1	002:01_rm1
Hp.4-2	-	-	-	-	-	001:01_rm3	-
Hp.4-3	-	-	-	-	001:02_rm2	-	-
Hp.4-4	-	-	-	-	001:02_rm3	-	-
Hp.4-5	-	001:01:01_rm2	-	-	-	-	-
Hp.4-6	-	001:01:01_rm2	-	-	-	001:01_rm3	-
Hp.5	502:01	001:01:01	001:01	001:01	008:01	003:01	004:01
Hp.5-1	502:01_rm1	001:01:01_rm1	001:01_rm1	001:01_rm2	008:01_rm1	003:01_rm1	004:01_rm1
Hp.5-2	-	-	-	-	008:01_rm2	-	-
Hp.6	508:01:00	001:01:03	001:01	001:01	001:01	001:01	002:01
Hp.6-1	508:01_rm1	001:01:03_rm1	001:01_rm2	001:01_rm6	001:01_rm1	001:01_rm1	002:01_rm1
Hp.6-2	-	-	-	-	-	001:01_rm3	
Hp.6-3	-	-	-	-	001:01_rm2	-	-
Hp.6-4	-	-	-	-	001:01_rm2	001:01_rm3	-
Hp.7	508:01	001:01:03	001:01	001:01	002:01	009:01	001:01
Hp.7-1	508:01_rm1	001:01:03_rm1	001:01_rm2	001:01_rm1	002:01_rm1	009:01_rm2	001:01_rm1
Hp.7-2	-	-	-	-	002:01_rm3	-	-
Hp.8	508:02	001:01:03	001:01	001:01	002:01	009:01	001:01
Hp.8-1	508:02_rm1	001:01:03_rm1	001:01_rm2	001:01_rm1	002:01_rm1	009:01_rm3	001:01_rm1
Hp.8-2	-	-	-	-	002:01_rm2	-	-
Hp.9	nov-68	88*069:01	001:01	001:01	048:01	004:02	031:01
Hp.9-1	nov-68_rm1	88*069:01_rm1	001:01_rm3	001:01_rm1	048:01_rm2	004:02_rm1	031:01_rm1
Hp.9-2	-	-	-	001:01_rm4	-	-	-
Hp.10	nov-MS4	001:01:01	001:01:01	001:01	015:01	009:01	001:01
Hp.10-1	nov-MS4_rm1	001:01:01_rm4	001:01_rm1	001:01_rm1	015:01_rm1	009:01_rm1	001:01_rm1

Within each haplotype group, only the differing repeat-masked alleles from the topmost haplotype were listed, and identical repeat-masked alleles were indicated with a hyphen. rm: repeat-masked allele:

Table 3-8. Frequency of haplotypes in two different strains in laboratory beagle dogs

Strain	Haplotype (Number of animals for each haplotype)																								
	Hp.1			Hp.2	Hp.3		Hp.4						Hp.5		Hp.6				Hp.7		Hp.8		Hp.9		Hp.10
	Hp. 1-1	Hp. 1-2	Hp. 1-3	Hp. 2-1	Hp. 3-1	Hp. 3-2	Hp. 4-1	**Hp. 4-2**	Hp. 4-3	Hp. 4-4	Hp. 4-5	Hp. 4-6	Hp. 5-1	Hp. 5-2	**Hp. 6-1**	Hp. 6-2	Hp. 6-3	Hp. 6-4	Hp. 7-1	Hp. 7-2	Hp. 8-1	Hp. 8-2	Hp. 9-1	Hp. 9-2	Hp. 10-1
TOYO	4 (4)	3 (3)	2 (2)	1 (1)	2 (2)	1 (1)	8 (6)	**9 (6)**	2 (2)	1 (1)	0 (0)	0 (0)	1 (1)	1 (1)	**0 (0)**	1 (1)	0 (0)	1 (1)	1 (1)	0 (0)	2 (2)	1 (1)	0 (0)	0 (0)	0 (0)
Marshall	3 (3)	4 (3)	0 (0)	1 (1)	0 (0)	0 (0)	2 (2)	**2 (2)**	0 (0)	0 (0)	2 (2)	1 (1)	0 (0)	0 (0)	**16 (10)**	5 (4)	1 (1)	0 (0)	2 (2)	1 (1)	0 (0)	0 (0)	1 (1)	1 (1)	1 (1)

Bold: haplotype which show statistically difference of frequency between strains by Fisher's exact test ($p < 0.05$).

Haplotype analysis based on PBS alleles identified 13 haplotypes reported previously [20], including alleles identified only by Sanger sequencing. The lower number of haplotypes based on PBS alleles listed in Table 3-7 was primarily due to samples for which data could not be successfully obtained in *DLA-88* and *DLA-DRB1*. Furthermore, AMOVA analysis using both PBS and repeat-masked alleles to compare genetic differentiation between the strains showed that statistically significant moderate genetic differentiation was observed in *DLA-88* and *DLA-DRB1* ($p < 0.05$). Although the PhiPT value of repeat-masked alleles in *DLA-12* was low and did not suggest genetic differentiation, it was statistically significant ($p = 0.048$) (Table 3-9).

Table 3-9. Genetic differences between two strains based on the PBS alleles or repeat-masked alleles at each locus

Allele type	Index	*DLA-88*	*DLA-12*	*DLA-64*	*DLA-79*	*DLA-DRA*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*	Total
PBS allele	PhiPT value	0.060	0.069	-0.016	0.094	-0.010	0.091	0.038	0.008	0.045
	P value	0.007	0.034	0.572	0.043	0.637	0.003	0.042	0.254	0.014
Repeat-masked allele	PhiPT value	0.060	0.035	0.028	0.020	-0.010	0.060	0.003	0.012	0.028
	P value	0.008	0.048	0.092	0.101	0.595	0.008	0.283	0.183	0.012

PBS: peptide-binding site, PhiPT: the pairwise population differentiation

3.4. Characterization of polymorphisms in the upstream and CpG islands regions

The nucleotide sequences of the upstream region, including multiple transcription factor binding sites (S, X1, X2, Y, and TATA) within the core promoter region were analyzed for the extended alleles of each DLA class I and DLA class II gene. As a result, polymorphisms were observed only between extended alleles in *DLA-DQB1*. Compared to the reference sequence, chr12:2405143 A>G mutation was observed in the X1 box in *DLA-DQB1*001:01_ext1* and *DLA-DQB1*031:01_ext1*, and chr12:2405159 T>C mutation was observed in the X2 box in all sequences except *DLA-DQB1*013:03_ext1* (Figure 3-4).

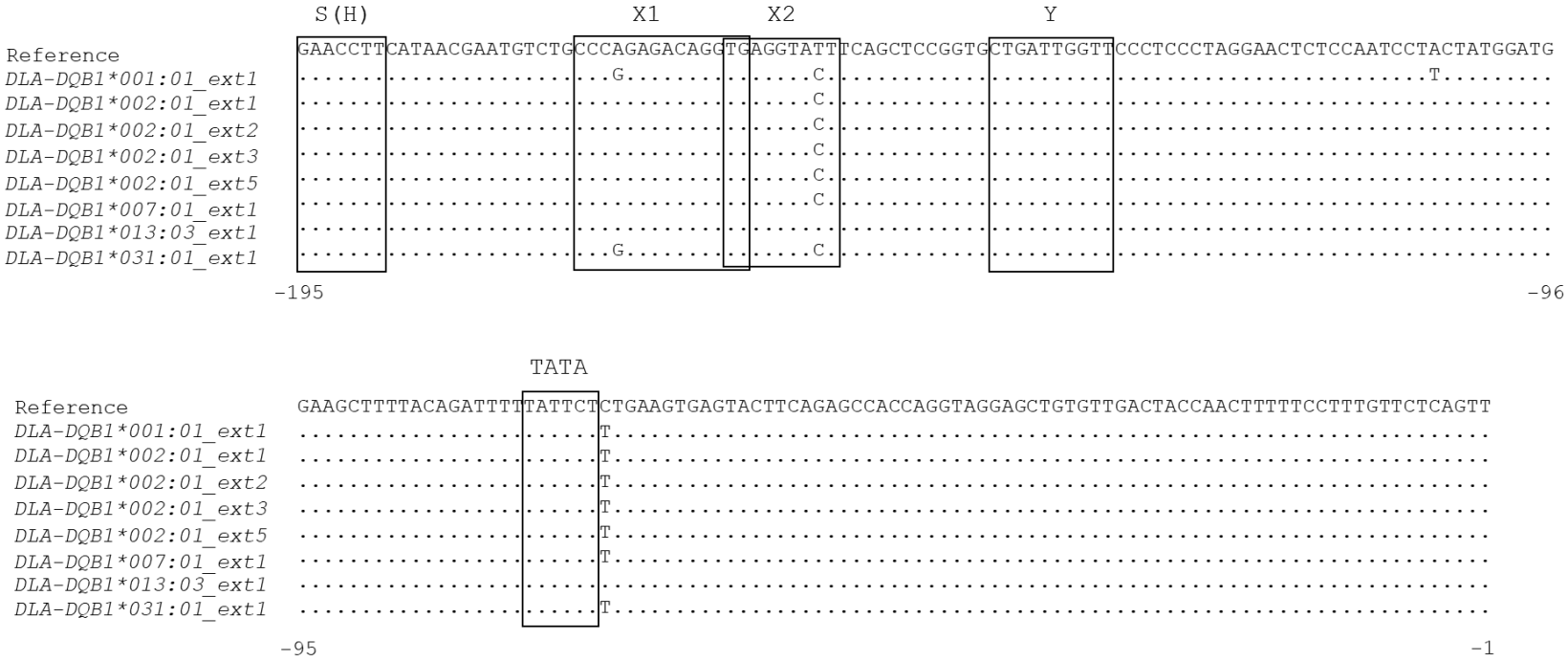

Figure 3-4. Sequences of the promoter region of extended alleles in *DLA-DQB1*

CGI analysis was performed on the full-length sequences, leading to the positional and length estimation of CGIs in all eight loci. The results indicated that all loci contained CGIs primarily composed of exon 2 and surrounding intron regions, suggesting a non-canonical regulatory organization. The differences in CGI length were less than 10% across most alleles within the same locus with the exception of *DLA-DQB1*. The difference in CGI length between the two major *DLA-DQB1* extended alleles (*DLA-DQB1*001:01_ext1* and *DLA-DQB1*002:01_ext1*) was 21.8% (217 bp) with lengths of 1,213 bp and 996 bp, respectively (Figure 3-5). There was a substantial difference between these extended alleles, suggesting considerable inter-allelic diversity in this potential regulatory element.

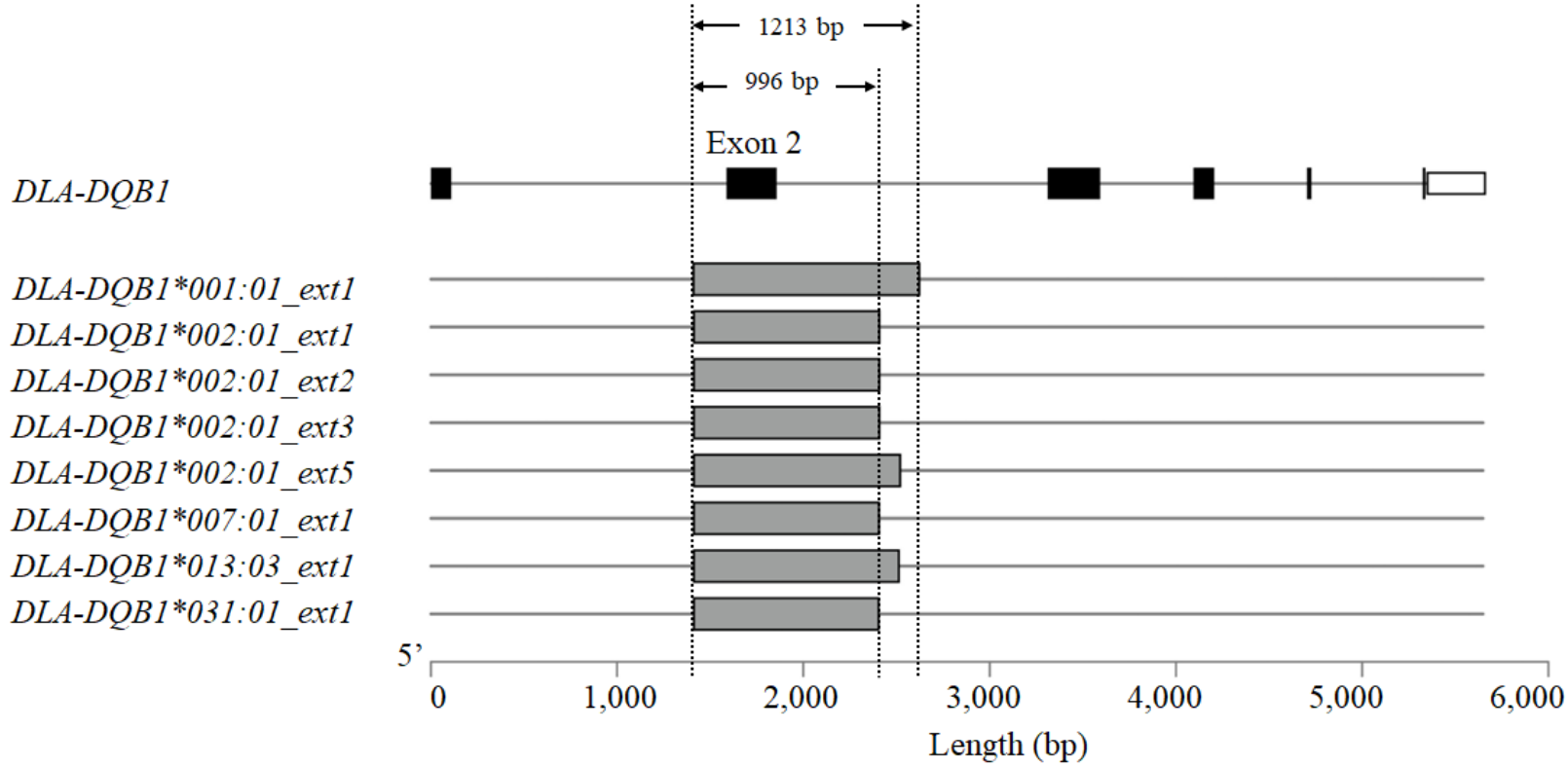

Figure 3-5. CpG islands predicted for each extended allele on the *DLA-DQB1*

Black box: exon, white box utr, gray box: predicted CpG island

4. Discussion

In Chapter 3, the polymorphisms of eight loci in the DLA class I and II genes (*DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) were identified and analyzed across their full gene length by amplifying the target regions using long-range PCR and then performing PacBio SMRT sequencing. Using this advanced method, the genotypes of 83 beagle dogs housed in our laboratory were analyzed. Full-length sequence information was successfully obtained for all loci, including outside antigen-binding regions that have not previously been a focus of analysis. This advance has enabled the precise classification of genetic differences that were overlooked by conventional methods.

The standard method of DLA genotyping is based on Sanger sequencing, but this has a limitation in the size of regions that can be analyzed simultaneously, conferring lower throughput for the analysis of long sequences. Although protocols for dogs have been developed, such as target amplification sequencing [56,89], NGS [85], and *de novo* assembly methods for the specific gene [37], to the best of our knowledge, no comprehensive full-length analysis across DLA class I and class II has been reported. On the other hand, HLA typing using long-read sequencing technology has already been successfully performed, enabling high-resolution HLA genotyping [78]. Long-read technology is particularly applicable for DLA genotyping given its various advantages, including faster library preparation, real-time base calling, shorter sequencing times, and potentially lower costs for massive samples. Furthermore, the design of the primers utilized in this study referenced the reference genomes from other dog breeds. Therefore, the possibility of these primers being strictly specific only to beagle dogs is extremely low, suggesting the method's broad applicability. The major advantage of performing a full-length analysis of DLA genes lies in their potential to clarify the pathological and functional effects of mutations located outside the PBS. This study revealed SNVs located outside the PBS across the eight loci, including *DLA-88*, *DLA-12*, *DLA-88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*. For instance, the SNV rs22195405 (chr12:1073877 A>G), located in intron 3 of *DLA-12* and previously reported as a potential risk variant for early-onset acquired myasthenia gravis, was identified within the extended alleles of *DLA-12*001:03* and *DLA-88*016:03* (classified as a *DLA-88L* allele) [76,116]. Similarly, rs8511444693

(chr18:41564611 T>G), an SNV in intron 1 of *DLA-79* known to be a risk allele for immune-mediated diseases such as canine immune-mediated polyarthritis and atopic dermatitis, as it is linked to *DLA-79* 001:02*. This study was shown to further subdivide *DLA-79*001:02* into two distinct extended alleles based on other SNVs [38]. This ability to classify genetic differences in more detail than previous risk analyses is expected to contribute significantly to the precise identification of true DLA risk alleles.

In the *DLA-DRB1* genotyping, 47 candidate extended alleles were not identified as extended alleles, primarily due to the complexity of the repeat sequences in this study. This was attributed to the observation of numerous mutations or indels within the multiple Short interspersed nuclear element and simple repeat sequences predominantly located within intron 1 of *DLA-DRB1*. In humans, the intron repeat sequences of *HLA-DRB1* are highly variable, and it has been reported that the degree of microsatellite diversity varies even within specific alleles [13,35], raising expectations for tracing allele origins and evolutionary processes. To clarify these points in *DLA-DRB1*, analysis of more individuals using this method or additional analysis incorporating verification of individual repeat sequences into this method is necessary.

The polymorphism analysis of the upstream region confirmed the same mutations as previously reported on the promoter in *DLA-DQB1* [12,115] and revealed a relationship with a new extended allele (Table 3-6). The analysis of CGIs, made possible by the identification of the full-length sequences, revealed a significant difference in length between *DLA-DQB1* alleles compared to other gene loci. Haplotypes containing *DLA-DQB1*001:01*, which have a mutation on X1 box and the longest CGI in the gene, have been suggested to offer protective effects against canine chronic enteropathy [80], Doberman hepatitis [32], and infectious diseases such as leishmaniasis [105], relative to haplotypes with other alleles. The mRNA expression levels differed due to methylation of the promoter region in *HLA-A* [87], and the polymorphism of promoter and CGIs affected mRNA expression through DNA methylation in mammals [8,100,103]. Consequently, these findings suggest that the protective effect of *DLA-DQB1*001:01* may be brought about not only by antigen specificity in PBS but also by regulatory differences determined by polymorphisms in the upstream region and CGI length. Verification of this hypothesis requires functional studies, such as *in vitro*

reporter gene assays or analysis of DNA methylation status in the *DLA-DQB1* promoter region.

Comparison of the genetic diversity between TOYO and Marshall Beagles revealed significant frequency differences in two haplotypes (Table 3-8). While the class I gene clusters constituting these haplotypes were identified as *DLA-88*502:01/DLA-12*001:01:01* and *DLA-88*508:01/DLA-12*001:01:03* in Chapter 2, it was newly revealed that the different *DLA-64* alleles, *DLA-64-rm1* and *DLA-64-rm2*, linked to the Hp.4-2 and Hp.6-1, respectively. In addition, significant genetic differentiation was also suggested in *DLA-88*, *DLA-12*, and *DLA-DRB1* from full-length sequence analysis (Table 3-9), which is generally considered less likely to identify differences due to noise from mutations in intron regions [108]. Collectively, these findings suggest that the two strains are genetically differentiated across a wide range of DLA genes located on chromosome 12. This differentiation is likely to be attributable to the closed colony breeding environment of the laboratory beagle dogs, which was likely a major factor contributing to the observed differences between the two strains. This fact reiterates the need for researchers to adequately consider DLA genetic diversity, especially when using beagle dogs in immunology-related and translational research.

While the SMRT sequencing method offers a highly accurate alternative to standard Sanger sequencing, several technical challenges remain for its establishment as a gold standard for DLA typing. Firstly, data loss can occur due to degradation of sample quality during the library preparation process, particularly during the PCR steps required for barcoding. Secondly, achieving balanced coverage remains difficult; the significantly longer *DLA-DRB1* locus consistently yielded clusters with low read counts even after attempts at equimolar library pooling. Thirdly, we observed allele dropouts in the *DLA-DQB1* locus of some samples. This may be due to variants at the primer site, as only one of the two alleles was confirmed with standard Sanger sequencing. This concern also suggests the potential for allelic dropout based on mutations outside the PBS region. Therefore, novel sequencing methods, such as those using Cas9-targeted sequence technology, have been developed to circumvent allele dropout and artifact generation by PCR amplification [45]. However, they currently have limitations in terms of throughput and require further technical refinement. Beyond these technical constraints, the necessity of full-length gene

analysis requires further verification. Reports correlating mutations outside the PBS region on DLA genes with individual differences in immune responses in dogs are extremely limited. Given that the PacBio sequencer has cost disadvantages for small-scale studies, the value of full-length DLA gene analysis must be strongly verified through large-scale studies. If such studies strongly indicate the need for full-length analysis, their application to veterinary clinical practice and other fields is expected to be transformative, potentially mirroring the 1-week turnaround time for HLA typing using PacBio SMRT sequencing.

The full-length DLA genotyping method developed in this study, which utilizes long-range PCR and PacBio SMRT sequencing, is anticipated to have wide-ranging applications in advancing our comprehension of canine immune response susceptibility. Firstly, in the field of veterinary medicine, this high-resolution DLA typing promises significant progress. For example, this methodology provides a robust foundation for accurately evaluating DLA compatibility between donors and recipients, which is indispensable in transplant medicine. High-resolution matching of DLA is expected to be vital for reducing the risk of graft versus host disease and transplant rejection in procedures such as hematopoietic stem cell transplantation and organ transplantation, as well as cases where using HLA compatibility through full-length sequencing has significantly improved transplant outcomes, which is deeply involved in rejection reactions [72]. Secondly, significant applications are also expected in biomedical research. Given that dogs are extensively used as models for human diseases and for safety assessment during drug development, analyzing the association between DLA alleles and drug-induced adverse effects using full-length sequences can provide valuable comparative data for clinical studies, thus contributing to improving safety prediction accuracy of non-clinical studies.

In conclusion, a new method was developed for identifying the full-length sequences of eight loci in canine DLA gene class I and II (*DLA-88*, *DLA-12/88L*, *DLA-64*, *DLA-79*, *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*), which involves amplifying the target regions using long-range PCR and then performing PacBio SMRT sequencing. This advanced method enabled the resolution of the full spectrum of genetic variation, which was unattainable by conventional PBS-based typing, revealing a substantial increase in allelic and haplotype complexity. Crucially, the full-length analysis facilitated the characterization of promoter and CGI length polymorphism in *DLA-DQB1*, suggesting a

potential regulatory mechanism that contributes to canine immune responses and disease susceptibility including CVL. Furthermore, the genetic differentiation observed between the TOYO and Marshall beagle strains underscores the necessity of considering DLA diversity in biomedical research. Our findings provide a powerful, high-resolution framework for future functional studies seeking to precisely link DLA polymorphisms outside the PBS to immunological phenotypes in dogs, thus opening new avenues for both veterinary and human translational medicine.

While the primary focus of Chapter 3 was the methodological development of a high-resolution DLA genotyping assay, this advancement is essential for a deeper understanding of the host-pathogen interactions observed in Chapter 1. The full-length sequence information, particularly concerning regulatory elements such as the *DLA-DQB1* promoter and CGI polymorphisms, may provide a missing link in explaining the variability of immune responses to *L. donovani* infection that cannot be achieved by conventional PBS-based typing employed in Chapter 2. In the context of the experimental CVL model, where clinical signs remained subclinical over eight months, the ability to subdivide haplotypes and identify SNVs in non-coding regions allows for more precise investigations into whether specific genetic signatures contribute to parasite persistence or delayed disease progression. Ultimately, the integration of the infection model from Chapter 1 with the advanced genomic framework established in Chapter 3 creates a powerful platform for future studies to pinpoint the risk alleles governing canine leishmaniasis susceptibility and to develop more effective immunological interventions.

5. Summary

DLA polymorphism is key to understanding immune mechanisms, but technical challenges have impeded its comprehensive genetic analysis. This study addressed these issues by using a novel DLA genotyping method combining long-range PCR and PacBio SMRT sequencing to analyze the full-length DLA class I and II gene sequences in 83 beagle dogs from two different strains (TOYO and Marshall), which are commonly used as laboratory animals. As a result of genotyping using the full-length sequences, 9, 5, 2, 6, and 8 extended alleles were newly discovered for the DLA class I genes in *DLA-88*, *DLA-12*, *DLA-88L*, *DLA-64*, and *DLA-79*, respectively. For the DLA class II genes, 11, 18, 12, and 8 extended alleles were newly discovered in *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*, respectively. There were 25 haplotypes consisting of extended alleles, in contrast to only 10 haplotypes classified using only PBS sequences. Furthermore, comparisons between the strains revealed differences in haplotype frequencies and genetic differentiation. The full-length analysis also provided preliminary insights into regulatory elements, such as promoter and CpG island polymorphisms in *DLA-DQB1*. The results of this research have important implications for the understanding of the relationship between DLA polymorphism at full length and individual immune responses in dogs.

Conclusion

Visceral leishmaniasis (VL), a zoonotic disease, is caused by the primary pathogens *Leishmania infantum* and *Leishmania donovani*. However, reports of canine VL (CVL) in dogs caused by *L. donovani* are limited, and the overall transmission route remains unclear. Similar to other infections, the risk of CVL infection is thought to be influenced not only by geographical factors but also by genetic factors on the host side. Therefore, understanding genetic factors that influence a host's immune response is essential for understanding CVL. However, unlike humans, major histocompatibility complex (MHC) gene polymorphisms, which are important risk factors for susceptibility to VL infection, have been limited to only a few genes and regions related to antigen presentation in dogs. Therefore, it is urgent to obtain accurate and comprehensive information on MHC gene polymorphisms in dogs.

In Chapter 1, an experimental CVL model with *L. donovani* infection was constructed using beagle dogs. During an 8-month observation period after parasite inoculation, no significant changes were observed in clinical signs, clinical pathology, or lymphocyte subset analysis in the three infected dogs. A similar pattern of the amount of mitochondrial kinetoplast DNA was observed in peripheral blood and liver by real-time polymerase chain reaction (PCR) analysis. In addition, parasite antigens were detected from the liver biopsy sections by immunohistochemical analysis, further supporting the existence of parasites in the liver. These results experimentally demonstrate that *L. donovani* can establish persistent infections in dogs, suggesting that dogs may serve as potential reservoirs for this pathogen.

In Chapter 2, the MHC polymorphisms, which are known to be associated with inter-individual variations in VL susceptibility and severity in both humans and dogs, were investigated. To address this, four dog leukocyte antigens (DLA) class I and four DLA class II genes were analyzed in 93 laboratory beagle dogs from two distinct strains (TOYO and Marshall) using locus-specific PCR and Sanger sequencing. The results showed that, for DLA class I genes, 11, 4, 1, and 2 alleles, including a novel allele, were detected in *DLA-88*, *DLA-12/88L*, *DLA-64*, and *DLA-79*, while for DLA class II genes, 1, 10, 6, and 7 alleles were detected in *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*, respectively. It was estimated that there were 14 DLA haplotypes. Haplotype analysis identified haplotypes across a broad range of DLA genes with CVL risk based on previously reported alleles.

Furthermore, when comparing the DLA diversity between TOYO and Marshall strains, the most common alleles and haplotypes differed between them. This is the first study to genotype all DLA loci and determine DLA haplotypes, including all DLA class I and class II genes in dogs. This comprehensive baseline of DLA diversity provides a fundamental genetic framework for investigating the associations between host genotypes and various immune-related conditions, including CVL.

In Chapter 3, these issues were addressed by using a novel DLA genotyping method combining long-range PCR and PacBio single-molecule real-time sequencing to analyze the full-length DLA class I and II gene sequences in 83 beagle dogs from two different strains (TOYO and Marshall). As a result of genotyping using the full-length sequences, 9, 5, 2, 6, and 8 extended alleles were newly discovered for the DLA class I genes in *DLA-88*, *DLA-12*, *DLA-88L*, *DLA-64*, and *DLA-79*, respectively. For the DLA class II genes, 11, 18, 12, and 8 extended alleles were newly discovered in *DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*, respectively. There were 25 haplotypes consisting of extended alleles, in contrast to only 10 haplotypes classified using only peptide binding site sequences. Furthermore, comparisons between the strains revealed differences in haplotype frequencies and genetic differentiation. The full-length analysis also provided preliminary insights into regulatory elements, such as promoter and cytosine-phosphate-guanine island polymorphisms in *DLA-DQB1*. The results of this research have important implications for the understanding of the relationship between DLA polymorphism at full-length and individual immune responses in dogs.

In conclusion, the results of this study demonstrate that beagle dogs, widely used as experimental animals, can be experimentally infected with *L. donovani*, indicating their potential to serve as a reservoir of the parasites. To investigate host genetic factors associated with CVL infection risk in dogs, comprehensive polymorphism analysis of DLA class I and class II gene clusters was performed, identifying allele and haplotype expression frequencies. To elucidate individual differences in immune responses mediated by MHC molecules, full-length nucleotide sequence information, not just antigen-binding sites, plays a crucial role. Therefore, the novel full-length analysis method established in this study supersedes conventional techniques by enabling the identification of extended alleles and haplotypes with unprecedented resolution. These findings provide a high-resolution genetic

foundation that is expected to facilitate the identification of CVL-susceptible individuals and advance the understanding of the pathogenesis of various canine immune-related diseases.

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Summary in Japanese

人獣共通感染症として知られる内臓リーシュマニア症（visceral leishmaniasis, VL）は、主要な病原体である *Leishmania infantum* と *L. donovani* によって引き起こされる。特に *L. donovani* によるイヌ内臓リーシュマニア症（canine VL, CVL）の感染報告は限られており、その感染経路の全体像は未解明である。他の感染症と同様に、CVL の感染リスクには地理的要因だけでなく、宿主側の遺伝的要因も関与している。そのため、宿主の免疫応答に影響を与える遺伝的素因を知ることは、CVL の感染感受性や病態進行のメカニズムを理解する上で不可欠である。しかし、VL 感染の感受性差に影響を及ぼす重要なリスクファクターである抗原提示に関わる主要組織適合性複合体（major histocompatibility complex, MHC）分子の遺伝子多型に関する研究は、イヌではこれまで一部の遺伝子や抗原提示に関わる領域のみに限られていた。そのため、イヌの MHC 多型情報を正確かつ幅広く得ることが急務である。

第一章では、ビーグル犬を用いて *L. donovani* による実験的な CVL モデルを構築した。*L. donovani* プロマスティゴートを静脈内接種することで感染後 8 か月の観察期間中、一般状態、血液学的検査、血液化学的検査、及びリンパ球サブセット解析では、顕著な変化は認められなかった。しかし、リアルタイム polymerase chain reaction（PCR）解析を実施したところ、末梢血および肝臓からキネトプラスト DNA が持続的に検出された。さらに、肝生検組織の免疫組織化学的解析により寄生虫抗原が検出され、肝臓内の寄生虫の存在を支持する結果であった。本実験モデルにより、*L. donovani* はイヌへ感染することが実験的に示され、イヌが CVL の潜在的な保虫宿主となり得る可能性が示された。

第二章では、ヒト及びイヌで VL のリスク、感受性、および重症度の個体差への関連性が示唆されている MHC 多型について、これまでイヌで広く研究されてきたイヌ MHC（dog leukocyte antigen, DLA）クラス II 遺伝子に加え、DLA クラス I 遺伝子の多型に関する研究を実施した。実験には、2 つの異なる系統（TOYO および Marshall）から由来する 93 頭の遺伝的均一性が高いビーグル犬を用い、遺伝座特異的な PCR およびサンガーシーケンスによって DLA クラス I 遺伝子 4 つと DLA クラス II 遺伝子 4 つの遺伝子型を決定した。結果として、DLA クラス I 遺伝子では、新規アレルを含む合計 11、4、1、2 のアレルがそれぞれ *DLA-88*、*DLA-12/88L*、*DLA-64*、*DLA-79* で確認された。また、DLA クラス II 遺

伝子では、*DLA-DRA*、*DLA-DRB1*、*DLA-DQA1*、および *DLA-DQB1* において、それぞれ 1、10、6、7 のアレルが検出され、全体で 14 種類の DLA ハプロタイプが存在することが判明した。ハプロタイプ解析では、DLA 遺伝子群の広範な領域におけるアレルおよびハプロタイプが推測された。また、系統間の比較により、そのアレルおよびハプロタイプの発現頻度が異なることも明らかにした。DLA 遺伝子多型に関する新たな情報が得られたことで、CVL を含む様々な疾患の感受性に関わる遺伝子素因が発見される可能性が示唆された。

第三章では、83 頭のビーグル犬を対象に、長鎖 PCR と PacBio Single-Molecule Real-Time シーケンスを組み合わせた新しい DLA 遺伝子型解析法を用いて、DLA クラス I および II 遺伝子の全長配列を詳細に解析した。結果として、*DLA-88*、*DLA-12*、*DLA-88L*、*DLA-64*、*DLA-79* においてそれぞれ 10、9、2、7、8 の全長配列に基づく新規アレルが発見された。DLA クラス II 遺伝子では、*DLA-DRA*、*DLA-DRB1*、*DLA-DQA1*、*DLA-DQB1* においてそれぞれ 11、18、12、8 の新規アレルが発見された。さらに、従来のペプチド結合部位配列に基づくアレル分類では 10 種類であった DLA ハプロタイプが、イントロン内の反復配列を除外した全長配列に基づくアレル分類では合計で 25 種類に細分化されることが推定された。全長解析は DLA 遺伝子多型のより詳細な分類を可能にするだけでなく、転写調節に関するプロモーターや CpG アイランド領域の多型の同定にも寄与した。

以上、本研究は *L. donovani* がイヌに感染し、保虫宿主として機能する可能性を実験的に証明した。さらに、新規開発した全長解析手法により、ビーグル犬における DLA 遺伝子群の極めて高度な多様性を明らかにした。本手法および多型情報は、CVL の感受性個体の特定のみならず、イヌの様々な免疫関連疾患の病態解明に向けた強力な基盤となることが期待される。