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ORIGINAL ARTICLE

Identification of novel polymorphisms and two distinct haplotype structures in dog leukocyte antigen class I genes: *DLA-88*, *DLA-12* and *DLA-64*

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Abstract The current information on the polymorphism variation and haplotype structure of the domestic dog *leukocyte antigen* (*DLA*) genes is limited in comparison to other experimental animals. In this paper, to better elucidate the degree and types of polymorphisms and genetic differences for *DLA-88*, *DLA-12* and *DLA-64*, we genotyped four families of 38 beagles and another 404 unrelated dogs representing 49 breeds by RT-PCR based Sanger sequencing. We also sequenced and analyzed the genomic organization of the *DLA-88* and *DLA-12* gene segments to better define these two-gene *DLA* haplotypes more precisely. We identified 45 alleles for *DLA-88*, 15 for *DLA-12* and six for *DLA-64*, of which 20, 14 and six, respectively, were newly described alleles. Therefore, this study shows that the *DLA-12* and *DLA-64* loci are far more polymorphic than previously reported. Phylogenetic analysis strongly supported that the *DLA-88*, *DLA-12* and *DLA-64* alleles were independently generated after the original divergence of the *DLA-79* alleles. Two distinct *DLA-88* and *DLA-12* haplotype structures, tentatively named *DLA-88–DLA-12* and *DLA-88–DLA-88L*, were identified, and the novel haplotype *DLA-88–DLA-88L* contributed to 32.7% of the unrelated dogs. Quantitative real-time PCR analysis showed that the gene expression levels of *DLA-88L* and *DLA-88* were similar, and that the gene expression level of *DLA-12* was significantly lower. In addition, haplotype frequency estimations using frequently occurring alleles revealed 45 different *DLA-class I* haplotypes (*88-88L/12-64*) overall, and 22 different *DLA-class I* haplotypes in homozygous dogs for 18 breeds and mongrels.

Keywords Dog · Major histocompatibility complex; MHC · Dog leukocyte antigen; *DLA* · Polymorphism · Haplotype structure · Gene expression

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Introduction

Experimental animal models have been developed to advance biomedical research on various aspects of the Major Histocompatibility Complex (MHC) such as gene expression and the mechanisms of peptide presentation in the mouse and rat, and diversity in the dog, pig and primate species (Shiba et al. 2016). As one of the experimental animal models, domestic dogs (*Canis lupus familiaris*) are often used for biomedical research for various diseases such as systemic lupus erythematosus-related disease complex (Wilbe et al. 2009), immune-mediate hematopoietic anemia (Friedenberg et al. 2016; Kennedy et al. 2006), diabetes (Kennedy et al. 2007),

chronic hepatitis (Bexfield et al. 2012), hypoadrenocorticism (Massey et al. 2013a), polymyositis (Massey et al. 2013b), exocrine pancreatic insufficiency (Tsai et al. 2013) and for transplantation studies such as using peripheral stem cells (Sandmaier et al. 1996), bone marrow cells (Shi et al. 1998; Storb et al. 1997) and mesenchymal stem cells (Arinzeh et al. 2003; Jung et al. 2009; Kim et al. 2013) in transplantation. Most of these diseases and treatment outcomes are influenced by polymorphisms within the MHC genomic region that encodes the MHC transplantation and immune regulatory molecules (Shiina et al. 2009; Shiina et al. 2004). Matching the MHC polymorphisms between donor and recipient is believed to be an essential factor to avoid acute graft rejection. However, transplantation studies so far have not been performed on canine donors and recipients that were matched for dog MHC (known as *Dog Leukocyte Antigen*: *DLA*) polymorphisms, although in some cases microsatellite markers located in the *DLA* genomic region were used to select donors and recipients for transplantation (Wagner et al. 1996). Hence, detailed information on the polymorphisms and haplotypes of the *DLA* genes are necessary to better understand major genetic factors of MHC-related diseases and to advance MHC matched canines in transplantation studies.

The human MHC (known as the Human Leucocyte Antigen; *HLA*) genomic region is located on the short arm of chromosome 6 with the class I region located at the telomeric end and the class II region located at the centrometric end. The *HLA* genomic regions encode the highly polymorphic gene complex of the *HLA-class I* and *HLA-class II* genes. The class I region is additionally divided into three genomic blocks, alpha, beta and kappa, that include duplicated *HLA* genes (Kulski et al. 2002) and two framework gene blocks that include well-conserved non-MHC genes in mammalian species (Kumanovics et al. 2003). *HLA-A, HLA-G* and *HLA-F* are in the alpha block, *HLA-B* and *HLA-C* are in the beta block, and *HLA-E* is in the kappa block (Fig. 1). The classical *HLA-class I* genes, *HLA-A, HLA-B* and *HLA-C*, and the classical *HLA-class II* genes, *HLA-DR*, *HLA-DQ* and *HLA-DP*, are distinguished by their extraordinary polymorphisms, whereas the non-classical *HLA-class I* genes, *HLA-E, HLA-F* and *HLA-G*, and the non-classical *HLA-class II* genes, *HLA-DM* and *HLA-DO*, are distinguished by their tissue-specific expression, their specific function and/or limited polymorphism (Shiina et al. 2009; Shiina et al. 2004).

In comparison to the *HLA*, the *DLA* genomic region is located on chromosome 12, except for one divergent *DLA-class I* gene *DLA-79* that is on chromosome 18. The major *DLA* genomic region on chromosome 12 contains three transcribed *DLA-class I* genes (*DLA-88, DLA-12* and *DLA-64*) (Burnett et al. 1997; Burnett and Geraghty 1995; Sarmiento and Storb 1990a; Wagner et al. 2005) and four *DLA-class II* genes (*DLA-DR*, *DLA-DQ, DLA-DM* and *DLA-DO*) (Debenham et al. 2005; Sarmiento et al. 1992; Sarmiento et al. 1993; Sarmiento and Storb 1990b). A small fragment of the *DLA* genomic region is also located on chromosome 35. All of the *DLA-class I* genes are located in the beta block that is orthologous to the *HLA-B* and *HLA-C* segment, whereas the *HLA-DP* orthologous gene is absent from the whole genome sequence of a female boxer breed (Lindblad-Toh et al. 2005) (Fig. 1). Therefore, the basic organizational structure of the MHC sub-region is largely conserved between the dog and the human with the exception that the dog class I region is distributed on three different chromosomes (Fig. 1) and that the class I and class II genes probably were remodeled between the species by "birth and death" evolution in response to environmental pathogens (Nei et al. 1997).

From the previous *DLA* polymorphism research, 73 *DLA-88*, one *DLA-12*, one *DLA-64* and six *DLA-79* were released in the IPD and NCBI databases. Of the class I genes, *DLA-88* is considered to have classical functions, and *DLA-12* and *DLA-64* are defined as non-classical genes in the *DLA* nomenclature (IPD-MHC Canines). However, comparative polymorphism and gene expression analyses of *DLA-88*, *DLA-12* and *DLA-64* have not been performed in much detail so far, although *DLA-79*, located on chromosome 18, is highly expressed in muscle and in various other tissues (Burnett and Geraghty 1995; Graumann et al. 1998; Ross et al. 2012; Venkataraman et al. 2013; Wagner et al. 2000). Furthermore, Ross et al. (2012) reported the possibility of gene duplication of two *DLA-88* genes, *DLA-88*028:03* and *DLA-88*029:01*, but they did not provide any detailed genomic and genetic analyses. A total of 16 *DLA* haplotypes that are composed of *DLA-class I* and *-class II* genes (*DLA-88–DLA-DRB1* or *DLA-88–DLA-DRB1–DLA-DQA1–DLA-DQB1*) were estimated from only two breeds, the Beagle and the German Shepherd (Hardt et al. 2006; Tsai et al. 2013). In addition, most of the *DLA-class I* polymorphisms and haplotypes have been limited to large-sized breeds and/or European breeds such as the Boxer, Doberman and Labrador Retriever, although genotyping of *DLA-88* using 428 dogs from 92 different breeds was reported (Kennedy et al. 2012). Hence, detailed comparison of polymorphisms and relative gene expression levels among the *DLA-class I* genes and estimation of the *DLA-class I* haplotypes (*DLA-88–DLA-12–DLA-64*) in small-sized breeds and/or Asian breeds such as Chihuahua, Dachshund and Pomeranian are still necessary for various MHC-controlled biomedical research in domestic dogs.

In this study, to better elucidate the degree and types of allele and haplotype diversity of the *DLA-class I* genes, we identified 45 *DLA-88*, 15 *DLA-12* and six *DLA-64* alleles of which 20 *DLA-88*, 14 *DLA-12* and six *DLA-64* alleles were novel alleles in 38 related Beagles (four families) and 404 unrelated animals by reverse transcriptase-polymerase chain reaction (RT-PCR) based Sanger sequencing. During the course of this study, we identified a newly duplicated *DLA-88* gene either as a gene replacement or gene conversion

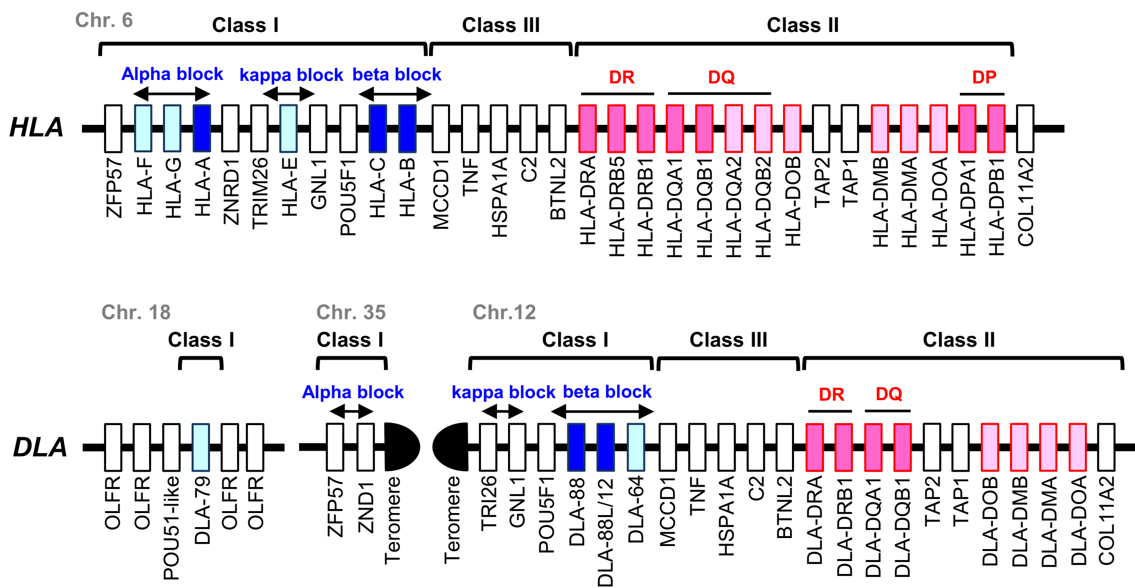

Fig. 1 Comparative map of the *HLA* and *DLA* genomic regions. The genetic maps are based on the genomic information of the NCBI map viewer. The regions are divided into three sub-regions, class I, class III, and class II. The class I region is separated into three blocks, the alpha, beta, and kappa blocks (Kulski et al. 2002; Kumanovics et al. 2003; Shiina et al. 2017), as indicated by blue letters and horizontal arrows. Dark and light blue boxes indicate classical and non-classical MHC class I genes, respectively, dark and light pink boxes indicate classical and non-classical MHC class II genes, respectively, and white boxes indicate the non-MHC genes that are the landmarks for defining the comparative MHC sub-regions and blocks between the *HLA* and *DLA* regions

within the *DLA-12* gene locus and named it unofficially as "*DLA-88* like" (*DLA-88L*) to distinguish it from *DLA-88* and *DLA-12*. To elucidate the genomic organization of the *DLA-88, DLA-88L* and *DLA-12* genes, we sequenced a complete genomic region of 95-kb including the *DLA-88, DLA-88L* and *DLA-12* genes, and identified two distinct *DLA-88* and *DLA-12* haplotype structures, tentatively named as *DLA-88–DLA-12* and *DLA-88–DLA-88L.* The former haplotype is composed of the originally known *DLA-88* and *DLA-12* genes and the latter novel haplotype is composed of duplicated *DLA-88* genes as described in this paper. We analyzed the diversity and variability of the allele and haplotype *DLA-class I* sequences by performing gene expression, amino acid variability, phylogenetic and population genetic analyses.

Material and methods

Terminology and gene nomenclature

We have used the terms 'major' and 'minor' alleles and haplotypes in this paper as synonyms with 'common' or 'frequent' and 'rare' alleles, respectively. In all cases, 'major' refers to the number of alleles and haplotypes that are present in more than three dogs and 'minor' refers to the number of alleles and haplotypes that are present in three dogs or less. The description 'official' allele refers to the *DLA* allele that has been released by IPD-MHC and/or by the GenBank databases.

The use of the gene names *DLA-88* and *DLA-12* refers to any gene sequence or gene allele previously designated as such. We have found in this study that some of the gene alleles that were previously designated as *DLA-88* are more likely to belong to the newly identified *DLA*-locus, *DLA-88L* ("*DLA-88* like"). We have used the official allele designations for all previously discovered alleles (e.g., *DLA-88*016:03*, *DLA-12*1*) and our own allele designations for the new alleles that we have identified and reported in this paper (e.g., *DLA-88*nov12*, *DLA-12*nov1-2*). In this paper, we refer to the previously reported *DLA-88L* allele in GenBank (HQ340122) as *DLA-88*L* in order to specify that it is part of the *DLA-88L* gene. The *DLA-12* and *DLA-88L* genes are hypothesized to be located at the same genomic position (*locus*), but they are different genes because of their differences in gene expression levels, evolutionary history and phylogenetic lineages that are described in the Results and Discussion sections.

Animals

Peripheral white blood cells (PWBCs) were obtained from 38 related dogs from four Beagle families (Oriental Yeast Co. Ltd. Tokyo, Japan) and 404 unrelated dogs from 49 breeds and mongrels (Animal Medical Center (ANMEC) at Nihon University and the Marble Veterinary Medical Center) (Table 1). The blood collection and dog studies were conducted in accordance with the guidelines for animal experiments specific to each location.

Table 1 Sample information of unrelated dogs used for this study

Breed	Animal num.	Breed	Animal num.
Akita	1		
Basenji	1	Miniature Pinscher	4
Beagle	6	Miniature Schnauzer	6
Bernese Mountain Dog	1	Papillon	9
Bichon Frize	2	Pomeranian	7
Brussel Griffon	1	Poodle: Standard	1
Bulldog	1	Poodle: Toy	42
Bull Terrier: Miniature	1	Pug	5
Bull Terrier: Staffordshire	1	Rottweiler	1
Chihuahua	36	Saluki	1
Chihuahua: Longcoat	3	Shetland Sheepdog	18
Chin	2	Shiba	37
Chinese Crested Dog	1	Shih Tzu	12
Collie	4	Shikoku	1
Dachshund: Kaninchen	3	Spaniel: American Cocker	3
Dachshund: Miniature	48	Spaniel: Cavalier King Charles	3
Dalmatian	2	Terrier: Boston	3
French Bulldog	12	Terrier: Jack Russell	6
Golden Retriever	3	Terrier: Lakeland	1
German Shepherd	1	Terrier: Norfolk	2
Husky	4	Terrier: Toy Manchester	1
Irish Setter	1	Terrier: Yorkshire	41
Japanese Spitz	3	Welsh Corgi	8
Kooikerhondje	3	Whippet	1
Labrador Retriever	12	Mongrel	29
Maltese	9	49 breeds and mongrel	404

Total RNA and genomic DNA isolation

Total RNA and genomic DNA samples were isolated from the PWBCs of each of the 442 dogs in this study by using the TRIzol LS Reagent (Invitrogen/Life Technologies/Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturer's instructions.

Primers for RT-PCR and DLA allele sequence determination

Three pairs of primers were used to amplify the *DLA-88, DLA-12* and *DLA-64* genes as previously reported (Ross et al. 2012) and new primers were designed incorporating sequences from exons 1 and 4 of *DLA-88* (PCR product size: 654–657 bp)*, DLA-12* (660 bp) and *DLA-64* (653 bp). The primer names, the 5′ to 3′ nucleotide sequence, and gene locations, and the PCR annealing temperatures that were used for RT-PCR amplification are presented in Supplementary table 1A. The exact primer locations and comparison of the primer sequences among the *DLA-class I* loci are shown in Supplementary figure 1. Of these three primer pairs, 88/88L/12-F and 88/88L/12-R were previously reported (Ross et al. 2012), and 12-F and 64-R were newly designed for this study. The three *DLA* genes, *DLA-88, DLA-12* and *DLA-64*, share high similarity, therefore it was not possible to simply design the specific forward and reverse primers individually for each gene. To amplify the *DLA-88* gene, we designed the forward primer (88/88L/64-F) to anneal with the *DLA-88* and *DLA-64* loci and the reverse primer (88/88L/12-R) to anneal with the *DLA-88* and *DLA-12* loci (Ross et al. 2012). We also designed a *DLA-12* gene specific forward primer (12-F) and a *DLA-64* gene specific reverse primer (64-R). The two primer pairs, 12-F and 88/88L/12-R, and 88/88L/64-F and 64-R, were used separately for the amplification of *DLA-12* and *DLA-64*, respectively. *DLA-88L* was amplified by the same primer pair that we used for *DLA-88* (88/88L/64-F and 88/88L/12-R), but its gene location was the same as the *DLA-12* locus. The *DLA-12* locus (RefSeq NM_001014378.1) was genotyped with the 12-F and 88/88L/12-R primer pair. All the alleles were identified and confirmed by Sanger sequencing the PCR products as described in the following sections. All the primer sequences have significant nucleotide differences with the *DLA-79* RefSeq (NM_001020810.1).

Reverse-transcriptase (RT) reaction and RT-PCR amplification

cDNA was synthesized with the oligo-dT primer using the RevaTra Ace reverse transcriptase reaction (TOYOBO, Osaka, Japan) after treatment of the isolated RNA with DNase I (Invitrogen/Life Technologies/Thermo Fisher Scientific, Carlsbad, CA). In brief, the 20 μl amplification reaction contained 10 ng of cDNA, 0.4 units of KOD FX polymerase (TOYOBO, Osaka, Japan), 1 × PCR buffer, 2 mM of each dNTP and 0.4 μM of each primer. The cycling parameters were as follows: an initial denaturation of 94 °C/2 min followed by 33 cycles of 98 °C/10 s, 63 °C/30 s and 68 °C/45 s for *DLA-88, DLA-12* and *DLA-64*. PCR reactions were performed with the thermal cycler GeneAmp PCR system 9700 (Applied Biosystems/Life Technologies/Thermo Fisher Scientific, Foster City, CA). The specificity of the primers was confirmed by Sanger sequencing of the PCR products.

Sub-cloning and Sanger sequencing

RT-PCR products were cloned into the pTA2 cloning vector with the TA cloning kit according to the protocol provided by the manufacturer (TOYOBO, Osaka, Japan) and sequenced by using the ABI3130 genetic analyzer (Applied Biosystems/Life Technologies/Thermo Fisher Scientific, Foster City, CA) in accordance with the protocol of the Big Dye terminator method. To avoid PCR and sequencing artifacts generated by polymerase errors, eight to 32 clones per dog were sequenced. The nucleotide sequences of all the dogs' cDNA also were sequenced by direct-sequencing of the RT-PCR products using the primers that we used for PCR amplification.

Determination of the DLA-class I allele sequences

Allele sequences were determined using Sequencher Ver. 5.0.1 DNA sequence assembly software (Gene Code Co., Ann Arbor, MI) and by comparing them with known *DLA-class I* allele sequences released in the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the IPD-MHC Canines databases. Allele sequences were also assigned using the MHC allele assignment software, Assign ATF ver. 1.0.2.45 (Conexio, Australia) from direct sequencing data. Published and newly determined *DLA-class I* sequences were used as references. When allele sequences were found to be unique by both methods, we confirmed the sequences of the new alleles by sub-cloning and Sanger sequencing them again.

Genomic sequencing of the DLA-88–DLA-88–DLA-64 haplotype (LC271133)

The 95 kb genomic segment including the *DLA-88* duplicated haplotype, *DLA-88–DLA-88–DLA-64*, was independently amplified by long-range PCR method using ten kinds of long-range primer pairs (Fig. 2a and Supplementary table 1)B1 and one *DLA-class I* homozygous dog that has the *DLA-88*028:03–DLA-88*029:01–DLA-64*nov2* haplotype (LC271133). For PCR amplification, the 20 μL PCR amplification-reaction-volume contained 25 ng of genomic DNA, 1 unit of PrimeSTAR GXL DNA polymerase (TaKaRa Bio, Shiga, Japan), 4.0 μL of 5 × PrimeSTAR GXL Buffer (5 mM Mg^{2+}), 1.6 μL of 2 mM of each dNTP and 0.4 μM of each primer. The cycling parameters were as follows: primary denaturation 94 °C/2 min, followed by 30 cycles for 98 °C/10 s and 68 °C/10 min using the GeneAmp PCR system 9700 (Applied Biosystems/Life Technologies/Thermo Fisher Scientific). The PCR products were purified by the Agencourt AMPure XP (Beckman Coulter, Fullerton, CA) and quantified by the PicoGreen assay (Invitrogen/Life Technologies/Thermo Fisher Scientific) with a Fluoroskan Ascent micro-plate fluorometer (Thermo Fisher Scientific, Waltham, MA). One hundred nanograms of the pooled PCR products was used for the preparation of the DNA library that was prepared with an Ion Xpress Plus Fragment Library Kit according to the manufacturer's protocol for 400 base-read sequencing (Life Technologies/Thermo Fisher Scientific). Emulsion PCR (emPCR) was performed using the library with the Ion 520 & 530 Kit - OT2 on an Ion OneTouch 2 automated system (Life Technologies/Thermo Fisher Scientific). After the emulsion was automatically broken with the OneTouch 2 instrument, the beads carrying the single-stranded DNA templates were enriched according to the manufacturer's recommendation. Sequencing was performed using the Ion S5 and an Ion 530 Chip (Life Technologies/Thermo Fisher Scientific). The raw data processing and base-calling, trimming and output of quality-filter sequence reads were all performed with the Torrent Suite 4.2.1 software (Life Technologies/Thermo Fisher Scientific) and with full processing for shotgun analysis. This file was further quality trimmed to remove poor sequence at the end of the reads with QVs of less than 20. The trimmed sequence reads were used for mapping of the sequence reads and the reference genome sequence (Accession number: NC_006594) using the CLC Genomics Workbench 8.5.1 software (QIAGEN, Hilden, Germany) with default settings for alignments of 3 mismatch cost, 3 insertion cost, 3 deletion cost, 0.9 length fraction and 0.9 similarity fraction parameters. Remaining gaps or ambiguous nucleotides were determined by the direct sequencing of PCR products obtained with appropriate primers. The completed and annotated genomic sequence was submitted to DDBJ (DNA databank) with the accession number LC271133.

Dot-matrix analysis

Dot-matrix analysis of the reference *DLA* genomic sequence NC_006594 and the novel *DLA* genomic sequence LC271133

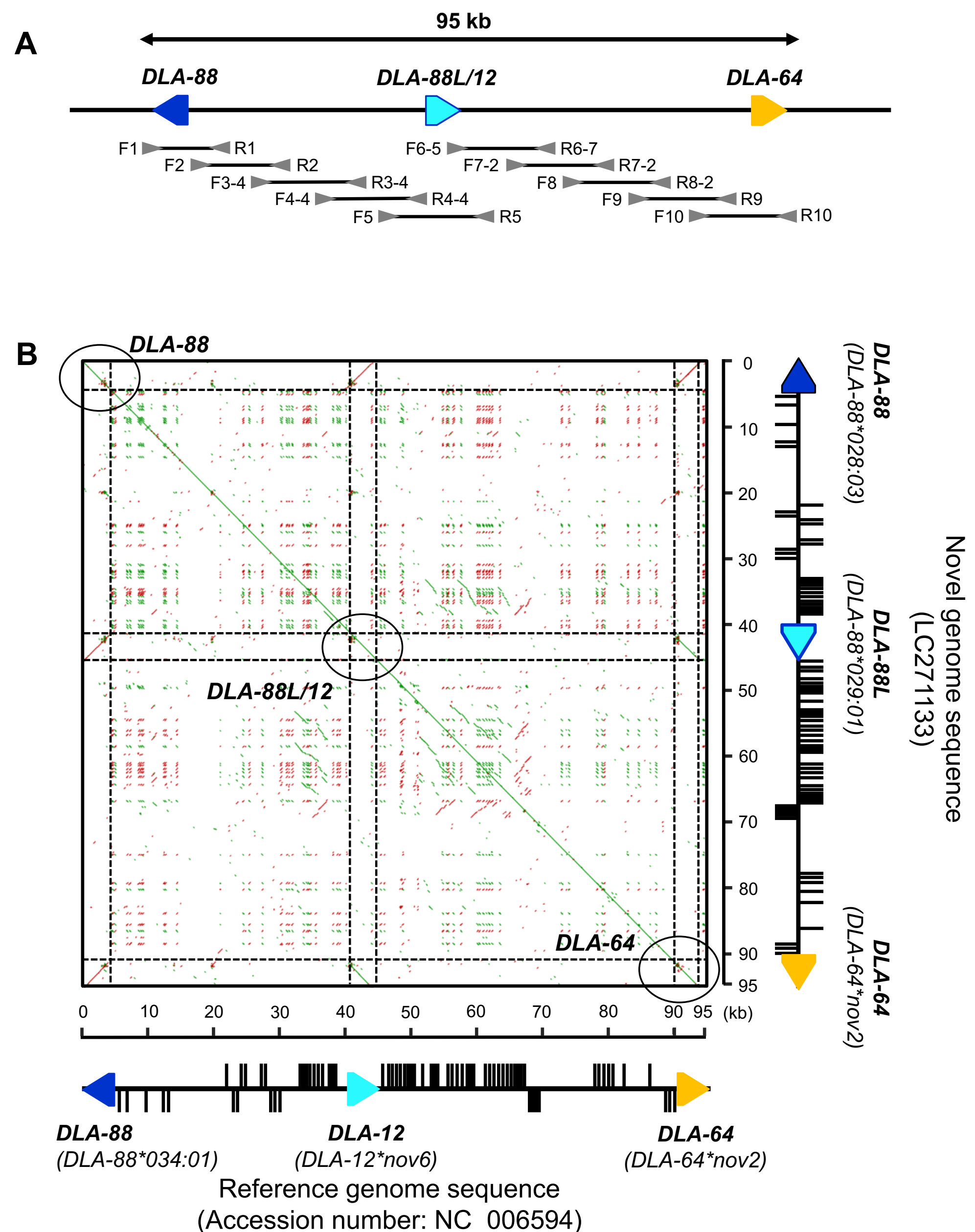

Fig. 2 Genomic comparison of *DLA-class I* segments by dot-matrix analysis. **a** Sequence-ready map of the 95-kb genomic segment, ranging from *DLA-88* to *DLA-64*, for the genomic comparison between the *DLA-88–DLA-12–DLA-64* and the *DLA-88–DLA-88L–DLA-64* segments. The exact primer names and primer sequences are shown in Supplementary table 1B1. **b** Dot-matrix of the 95-kb gene segments between *DLA-88–DLA-12–DLA-64* and the *DLA-88–DLA-88L–DLA-64* segments. Horizontal and vertical axes show the reference *DLA-88–DLA-12* genome sequence (accession number: NC_006594) and the novel *DLA-88–DLA-88L* genome sequence (LC271133). Green dots represent alignments of forward reads and red dots correspond to alignments between the reverse complement of one sequence and the forward read of the other. Dark blue, light blue, and orange triangles indicate positions of *DLA-88*, *DLA-88L/12*, and *DLA-64* loci, respectively, and the black vertical bars between these triangles (*DLA-class I* genes) indicate the position of LINE interspersed sequences. Parenthesis indicates *DLA-class I* allele name. The three large circles in the dot-matrix indicate the location of the orthologous *DLA-class I* genes in the two genomic sequences

was performed by using a genomic similarity search tool (YASS) (Noe and Kucherov 2005). NC_006594 is a part of the dog genome reference sequence derived from the dog genome project *Canis lupus familiaris* breed boxer chromosome 12, CanFam3.1, whole genome shotgun sequence (Lindblad-Toh et al. 2005). LC271133 is the novel *DLA* genomic sequence that we sequenced as described above.

Reclassification of DLA-88 alleles as DLA-12 and DLA-88L alleles

The genomic DNA samples that were used as templates to characterize the *DLA-88* alleles for reclassification as *DLA-12* and *DLA-88L* were extracted from three dogs: (1) a dog with the homozygous haplotype *DLA-88–DLA-12* carrying one *DLA-88* allele and one *DLA-12* allele (*DLA-88*006:01* and tentatively named *DLA-12*1* that was reported by Burnett et al. (1997) as "*DLA-12*"), (2) a dog with a homozygous haplotype *DLA-88–DLA-88* carrying two *DLA-88* alleles (*DLA-88*003:02* and *DLA-88*017:01*) and no *DLA-12* allele, and (3) a dog with the heterozygous haplotypes *DLA-88–DLA-88* and *DLA-88–DLA-12* carrying three *DLA-88* alleles (*DLA-88*016:03, DLA-88*025:01, DLA-88*501:01)* and one *DLA-12* allele (*DLA-12*1*). The 5.6 kb PCR product included the entire DLA-12 (DLA-12 and/or DLA-88L) locus, ranging from the promoter-enhancer region to the 3′-untranslated region, and it was amplified using the long-range primer pair 88L/12-seg-F and 88L/12-seg-R (Fig. 3a and

Supplementary table 1B2). The cycling parameter was as follows: an initial denaturation of 94 °C/2 min followed by 33 cycles of 98 °C/10 s, 58 °C/30 s and 68 °C/5 min. The PCR product was used for the nested PCR with the genotyping primer pairs, *DLA-12* (12-F and 88/88L/12-R) and *DLA-88L* (88/88L/12-F and 88/88L/12-R) (Fig. 3a and Supplementary table 1A). The 5.6 kb genomic sequence of the PCR product from the *DLA-88*003:02–DLA-88*017:01* homozygous dog was determined using the *DLA-88L* primers, the 88L/12-seg-F and 88L/12-seg-R primer pair and six direct-sequencing primers (Supplementary table 1A, 1B2 and 1B3).

In total, 105 genomic DNAs, 102 unrelated dogs with three *DLA-88* alleles, two unrelated dogs with four *DLA-88* alleles and one Beagle in family 2 with three *DLA-88* alleles, were used for the genotyping of *DLA-12* and *DLA-88L* at the *DLA-12* locus.

Phylogenetic analysis among the *DLA-class I* allele sequences

Multiple *DLA-class I* nucleotide sequences were aligned using the ClustalW Sequence Alignment program of the Molecular Evolution Genetics Analysis software 6 (MEGA6) (Tamura et al. 2011). The phylogenetic tree, consisting of 52 *DLA-88* (45 major and seven minor official alleles), 15 major *DLA-12*, six major *DLA-64* sequences determined in this study, and six published *DLA-79* sequences (Venkataraman et al. 2013), was constructed with only synonymous substitutions used to identify differences by the Neighbor-joining (NJ) method in MEGA6 (Saitou and Nei 1987) using exons 1 to 3 (alignment length: 610 bp excluding gap sites) with the modified Nei-Gojobori model. The tree was reconstructed using only synonymous substitution sites to identify differences in order to remove the influence of positive selection. We used mouse *H2-D1* (DNA accession numbers: NM_010380) and *H2-K1* (NM_001001892) as the outgroup sequences.

Measurement of gene expression level in the DLA-class I genes by quantitative real-time PCR

Nine allele-specific primer pairs (Supplementary figure 1 and Supplementary table 1C) were used by real-time PCR for gene expression analysis of *DLA-88, DLA-12, DLA-88L* and *DLA-64* in 22 (ten *88-12-64/88-12-64* and 12 *88-88L-64/88-88L-64*) homozygous dogs. The allele-specific primer pairs were designed in exons 3 and 4 (amplified length: 124 to 131 bp) (Supplementary table 1C). A pair of primers designed from the dog glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene sequence was used for the analysis as an

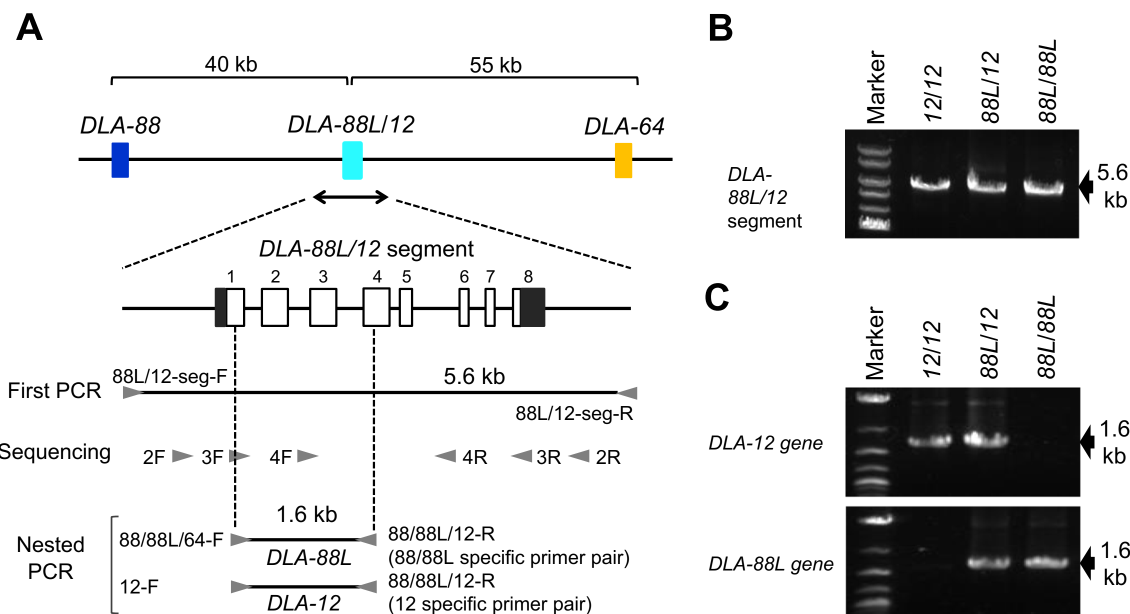

Fig. 3 Development of a PCR nested method for detection of polymorphism at *DLA-12* and *DLA-88L* using genomic DNA samples. **a** Schematic diagram shows the location of the *DLA-88L/12* gene, the exon/intron structure of *DLA-88L/12*, and the primer sites and operational map for the genomic analysis of the haplotype structural segments. Numbers around the gene structure indicate exon numbers. White and black boxes show the coding exons and the 5′ and 3′ untranslated regions, respectively. Primer sequences (sequencing primers 2F, 3F, 4F, 2R, 3R and 4R, 88L/12-seg-F and 88L/12-seg-R, 12-F, 88/88L/12-R, and 88/88L/64-F) are shown in Supplementary table 1A, 1B2 and 1B3. Of them, the primer specificity of 12-F, 88/88L/12-R and 88/88L/64-F are shown in Supplementary figure 1. **b**, **c** Electrophoresis images of the PCR products that were amplified using the 88L/12-seg-F and 88L/12-seg-R primer pair (5.6 kb) and the *DLA-12* or *DLA-88L* specific primer pairs (1.6 kb), respectively. Two types of DNA ladder markers, Quick-Load 1 Kb DNA Ladder (New England BioLabs) and Quick-Load 2-Log DNA Ladder (New England BioLabs), were used as DNA size markers for the image B and image C, respectively

internal control (Livak and Schmittgen 2001). Expression levels were measured by real-time PCR using the Thermal Cycler Dice Real Time System II (TaKaRa Bio) with SYBR Premix Ex Taq II (TaKaRa Bio). The 25 μl amplification reaction volume contained 50 ng of cDNA, 12.5 ul SYBR Premix Ex *Taq* II and 0.2 μM of each primer. The cycling parameter was as follows: 40 cycles of 95 °C/10 s, 60 °C/20 s. Melting curve analysis showed that there was no primer dimer formation. The relative quantitative values in each sample were normalized and calibrated by the 2 (-DeltaDeltaC(T)) method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). The paired *t* test program with two-samples assuming an unequal variances model in Excel determined the statistical differences of expression levels between *DLA-88* and *DLA-12* and between *DLA-88* and *DLA-88L*.

Estimation of the DLA-class I allelic haplotypes

Of 404 unrelated dogs, 355 dogs with more than three heterozygous dogs, or at least two homozygous dogs per dog breed were used for allelic haplotype estimation. The *DLA-class I* haplotype (*88-88L/12-64*) was initially characterized by manually sorting the major 38 *DLA-88,* seven *DLA-88L,* 15 *DLA-12* and six *DLA-64* alleles based on the *88-88L/12-64* homozygous dogs. Estimations of the *88-88L/12-64* allelic haplotype frequencies were performed by using the PHASE 2.1.1 program (Stephens et al. 2001) and the *88-88L/12-64* homozygous haplotype data.

Results

Evaluation of the DLA-class I polymorphism detection method using four Beagle families

To evaluate our newly designed *DLA-class I* gene-specific primers and PCR conditions for amplifying and sequencing of *DLA-88, DLA-12* and *DLA-64*, we initially performed genotyping and haplotyping using 38 related Beagles from four families that had well defined familial relationships (Supplementary figure 2). Although no polymorphism was detected in *DLA-64,* seven *DLA-88* and three *DLA-12* alleles were identified in these families. Of the 11 alleles, six of them, *DLA-64*nov2, DLA-12*nov1-2, DLA-12*nov1-3, DLA-88*nov2, DLA-88*nov9* and *DLA-88*nov19,* were novel. A total of six *DLA-class I* allelic haplotypes was estimated without any pedigree discrepancies. One *DLA-class I* haplotype that was composed of two *DLA-88* alleles (*DLA-88*nov2* and *DLA-88*nov19*) was observed in one Beagle in family 2 (Supplementary figure 2).

Polymorphism analysis of the DLA-class I genes using cDNA samples of 404 unrelated dogs

In our initial characterization of the *DLA-88, DLA-12* and *DLA-64* allele sequences (exons 1 to 4 in *DLA-88, DLA-12* and *DLA-64*) in 404 unrelated dogs including 49 breeds and mongrels by RT-PCR based sub-cloning and Sanger sequencing methods, we identified 76 alleles for *DLA-88*, 21 for *DLA-12* and seven for *DLA-64*. Of them, 45 *DLA-88,* 15 *DLA-12* and six *DLA-64* were "major" alleles identified in more than three heterozygous dogs, or at least two homozygous dogs. On the other hand, 31 *DLA-88,* six *DLA-12* and one *DLA-64* were "minor" alleles identified in three or less heterozygous dogs, or a single homozygous dog. All the major and minor alleles were identified by matching our sequences with all of the known *DLA-class I* allele sequences released in the GenBank and the IPD-MHC Canines databases using Sequencher and Assign ATF software (Table 2 and Supplementary table 2A and 2B). Of all the *DLA* class I alleles detected in this study, 25 *DLA-88* alleles and one *DLA-12* allele were previously reported in the IPD-MHC Canines database and NCBI database (Burnett et al. 1997; Graumann et al. 1998; Ross et al. 2012; Venkataraman et al. 2007) and the remaining 20 *DLA-88,* 14 *DLA-12* and six *DLA-64* were newly identified in this study (DDBJ/EMBL/GenBank accession numbers LOC130502-LC130511, LC130513-LC130547, LC171419-LC171437 and LC171439-LC171445, Table 2 and Supplementary table 2A). The most frequent alleles for each gene were *DLA-88*006:01* (85 of the 404 dogs), *DLA-12*1* (275 dogs) and *DLA-64*nov2* (380 dogs) that were observed in 15, 40, 48 and 20 breeds and/or mongrels, respectively (Supplementary table 2A).

Genomic organization of the DLA-88 and DLA-12 gene segment

Two previous reports described three *DLA-88* alleles in one dog that inferred that there were duplicated *DLA-88* genes, *DLA-88*028:03* and *DLA-88*029:01*, located at a single chromosome as a *DLA-88* haplotype (*DLA-88*028:03–DLA-88*029:01*) (Kennedy et al. 2012; Ross et al. 2012). In our RT-PCR analysis, we identified three types of *DLA-88* and one type of *DLA-12* allele per dog in 102 unrelated dogs and one Beagle derived from the familial analysis, and four types of *DLA-88* per dog were detected in two unrelated dogs. In total, we found that 105 dogs had unusual *DLA-88* allele numbers. On the basis of our *DLA-88–DLA-88* haplotype estimations using *DLA-88* genotypes in the 105 dogs, we identified the following eight *DLA-88* allelic haplotypes: *DLA-88*003:02–DLA-88*017:01*, *DLA-88*025:01–DLA-88*016:03*, *DLA-88*028:01–DLA-88*029:01*, *DLA-88*028:03–DLA-88*029:01*, *DLA-88*nov2–DLA-88*nov19*, *DLA-88*nov10–DLA-88*L*, *DLA-88*nov15–*

Table 2 Summary of the number of alleles identified by *DLA-class I* cDNA analysis

Locus	*DLA-88*	*DLA-12*	*DLA-64*	*DLA-79**
Number of nucleotide sequences	76	21	7	_
Novelty				
Previously published sequences	32	1	0	_
Newly identified sequences	44	20	7	_
Frequency				
Major sequences (*N* > 3)	45	15	6	_
Minor sequences (*N* < 4)	31	6	1	_
Official alleles released from IPD-MHC and NCBI database				
Number of alleles	73	1	1	6
Identified in this study	32	1	0	0
Not identified in this study	41	0	1	0
Number of amino acid sequences	74	16	4	_
Frequency				
Major sequences (*N* > 3)	45	11	2	_
Minor sequences (*N* < 4)	29	5	2	_

*Although genotyping of *DLA-79* was not performed in this study, the *DLA-79* allele information was added in this table and used for comparison of different allelic nucleotide and amino acid sequences

*DLA-88*029:01* and *DLA-88*nov23–DLA-88*nov12*. There were 25 dogs in the 404 unrelated dogs that were homozygous for any one of those *DLA-88–DLA-88* haplotypes. Furthermore, we could not detect the *DLA-12* gene by PCR in 27 dogs including two dogs with four types of *DLA-88* alleles and 25 homozygous dogs with the *DLA-88–DLA-88L* haplotype. Hence, we hypothesized that a genomic arrangement event had occurred that involved the *DLA-88* and *DLA-12* genes and that had generated two distinct *DLA-class I* haplotype structures, such as *DLA-88–DLA-12* and *DLA-88–DLA-88,* in some of the *DLA-class I* haplotypes.

To elucidate the *DLA-88* and *DLA-12* genomic structures in more detail, we sequenced the complete 95-kb-nucleotide sequence of a *DLA-88–DLA-88* haplotype in a homozygous dog with the allelic haplotype *DLA-88*028:03–DLA-88*029:01*, ranging from *DLA-88* to *DLA-64*, by long-range PCR and next generation sequencing (NGS) methods using ten primer pairs (Fig. 2a and Supplementary table 1B1). Sequence read information was obtained after sequencing of the pooled PCR products using the Ion S5 system in a single sequencing run. Draft read numbers in total were 1,794,204 sequence reads that were high quality sequence reads with more than 20 quality values (QV). The average read length of 304 bases and an overall mode read length of 416 bases. Therefore, the sequence reads had high quality and sufficient sequence volume for further genomic analysis. After the NGS run, gap filling and validation of the assembled sequences, we determined a 94,790 bp complete genomic sequence and deposited it in a DNA databank with the accession ID of LC271133. We then compared our LC271133 sequence with the reference genome sequence (NC_006594, 96,115 bp) that has the *DLA-88–DLA-12–DLA-64* genomic structure.

In the genomic sequence the two *DLA-88* genes (*DLA-88*028:03* and *DLA-88*029:01*) were identified to be located in the *DLA-88* and *DLA-12* orthologous regions, respectively, when compared to the dog genome RefSeq (NC_006594) (Fig. 2b). However, we tentatively named the *DLA-88* gene locating in *DLA-12* orthologous region as *DLA-88L* because of its stronger sequence similarity to *DLA-88* than to *DLA-12*. Nevertheless, the *DLA-12* genotyping primer pair (12-F and 88/88L/12-R) and the *DLA-88* genotyping primer pair (88/88L/64-F and 88/88L/12-R) amplify *DLA-12* and *DLA-88L,* respectively.

Figure 2b shows the dot-matrix analysis of the genomic comparison between the *DLA-88–DLA-12–DLA-64* segment and the *DLA-88–DLA-88L–DLA-64* segment. The dot-matrix plot reveals that the two genomic sequences have similar structural features, proportions and lengths such as the dense insertions of long interspersed elements (LINE) in approximately 30 kb around the *DLA-88L/DLA-12* gene region, suggesting that the genomic position of *DLA-88L* is at the same location as *DLA-12*. Dot-matrix analysis using the previously released 5726 bp of *DLA-88* (*DLA-88*034:01*, NC_006594, position: 891280-897005), 5649 bp of *DLA-12* (identified and tentatively named in this study as *DLA-12*nov6* corresponds to RefSeq NC_006594, position: 932034-937682) and 5585 bp of the newly determined *DLA-88L* (LC189199) sequences showed that the *DLA-12* vs *DLA-88L* comparison with 93.5% similarity was more conserved than either the *DLA-88* vs *DLA-88L* comparison with 71.0% similarity or the *DLA-88* vs *DLA-12* comparison with 69.3% similarity (Supplementary Table 3). These comparisons incorporated both the gene regions (approximately 3.4 kb) and the upstream and downstream regions of the gene. In contrast, comparisons

of the nucleotide similarities of *DLA-class I* gene regions using 3347 bp of *DLA-88*, 3451 bp of *DLA-12* and 3401 bp of *DLA-88L* nucleotide sequences showed 93.8% similarity between *DLA-88* and *DLA-88L,* 92.2% similarity between *DLA-12* and *DLA-88L,* and 88.7% similarity between *DLA-88* and *DLA-12*. The greatest gene divergence and sequence dissimilarity occurred in the 1.1 kb of the gene regions between exon 1 and exon 3 including introns with 83.4% similarity between *DLA-12* and *DLA-88L* and 82.7% similarity between *DLA-88* and *DLA-12,* compared to the 95.1% similarity between *DLA-88* and *DLA-88 L* for the same region. Therefore, it is evident that the *DLA-88L* gene has higher sequence similarity with *DLA-88* in the region of exons 1 to 3*,* whereas *DLA-88L* has higher sequence similarity with *DLA-12* in the genomic region outside of *DLA class I* gene.

Reclassification of DLA-88 alleles as DLA-88L and estimated frequency of the DLA-88-DLA-88L haplotypes

The dot-matrix analysis suggests that the some of the alleles initially classified as *DLA-88* alleles are part of the *DLA-12* locus that is composed of one of two distinct gene types, *DLA-12* or *DLA-88L* (Fig. 2b). In order to differentiate between the true *DLA-88* alleles that originated from *DLA-88* locus and the *DLA-88L* alleles, initially classified as *DLA-88,* but that were generated by the *DLA-12* locus, we investigated the 5.6 kb genomic segment that included the *DLA-12* and/or *DLA-88L* genes using one *DLA-88–DLA-12* haplotype in a homozygous dog, one *DLA-88–DLA-88* haplotype in a homozygous dog and a *DLA-88–DLA-12* and *DLA-88–DLA-88* haplotype in one heterozygous dog as structural templates. Although the 5.6 kb bands obtained using the long-range primer pair for *DLA-12* and *DLA-88L* genes (88L/12-seg-F and 88L/12-seg-R) (Fig. 3a and Supplementary table 1B2) were observed in all samples without non-specific products (Fig. 3b), the 1.6 kb PCR product obtained by nested PCR using the *DLA-12* primer pair (12-F and 88/88L/12-R) was amplified from the *DLA-88–DLA-12* haplotype of a homozygous dog, but not from the *DLA-88–DLA-88* haplotype of a homozygous dog that has the allelic haplotype *DLA-88*003:02* and *DLA-88*017:01*. On the other hand, the 1.6 kb PCR product obtained by nested PCR using the *DLA-88L* primer pair (88/88L/64-F and 88/88L/12-R) was amplified from the *DLA-88–DLA-88* haplotype of a homozygous dog, but not from the *DLA-88–DLA-12* haplotype of a homozygous dog that has *DLA-88*006:01* and *DLA-12*1* (Fig. 3c). Therefore, this experiment showed that the *DLA-12* and *DLA-88L* primer pairs were specific for the detection of the *DLA-12* and *DLA-88L* genotypes, respectively, in a nested PCR reaction. These results strongly point to two distinct *DLA-class I* haplotype structures in domestic dogs, the *DLA-88–DLA-12* haplotype and the *DLA-88–DLA-88L* haplotype.

To distinguish between the true *DLA-88* and *DLA-88L* alleles from the 45 major *DLA-88* alleles that had been previously classified in this study, we performed sequence-based genotyping of 105 DNA samples that have three or four *DLA-88* alleles by using the long-range primer pair for *DLA-12* and *DLA-88L* genes (88L/12-seg-F and 88L/12-seg-R), and then the nested PCR was performed using the primer pairs for the exons 1–4 segments of *DLA-88L* genes (88/88L/64-F and 88/88L/12-R) (Fig. 3a and Supplementary table 1A and 1B2). Of the 45 major *DLA-88* alleles, seven (15.6%) were identified to be from the *DLA-88L* locus (Table 3).

To summarize the reclassification of the *DLA-class I* alleles, 38 *DLA-88*, seven *DLA-88L,* 15 *DLA-12* and six *DLA-64* were identified as the major alleles. Consequently, eight *DLA-88–DLA-88L* major allelic haplotypes (16 haplotypes in total) were inferred in 105 dogs, and the most frequent allelic haplotype observed in nine breeds was *DLA-88*003:02–DLA-88*017:01* with an estimated 12.4% frequency (44 of the 355 dogs) (Table 4 and Supplementary table 4). Only one of the previously reported *DLA-88–DLA-88L* haplotypes (*DLA-88*028:03–DLA-88*029:01*) (Ross et al. 2012) was detected in our study.

Phylogenetic relationship among the DLA-class I alleles

We constructed a phylogenetic tree using the neighbor-joining method to examine the inter- and intra-relationships of the *DLA-88, DLA-12* and *DLA-64* allele sequences along with six of the previously detected *DLA-79* allele sequences (Venkataraman et al. 2013). The phylogenetic tree of the 79 aligned *DLA-class I* allele sequences supported a gene-specific evolution of the *DLA-class I* genes. Namely, the *DLA-class I* allele sequences could be separated into four lineages (*DLA-88, DLA-12, DLA-64* and *DLA-79*) after the divergence of the mouse MHC class I (*H2-D1* and *H2-K1*) sequences (Fig. 4). Both the *DLA-88L* and the *DLA-88* alleles were widely dispersed in the *DLA-88* lineage.

Table 3 Major *DLA-88L* alleles identified by *DLA-class I* cDNA and genomic analyses

*DLA-88*016:03*
*DLA-88*017:01*
*DLA-88*029:01*
*DLA-88*L*
*DLA-88*nov12*
*DLA-88*nov13*
*DLA-88*nov19*

Table 4 Eight major *DLA-88 - DLA-88L* allelic haplotypes based on *DLA-class I* cDNA and genomic analyses

DLA-88	*DLA-88 L*	Count of haplotypes	Number of dogs	Breed
**003:02*	**017:01*	54	44	Chihuahua, Collie, Maltese, Pomeranian, Toy Poodle, Shetland Sheepdog, Jack Russell Terrier, Yorkshire Terrier, Welsh Corgi
**025:01*	**016:03*	5	5	Beagle, Chihuahua
**028:01*	**029:01*	32	27	French Bulldog, Pomeranian, Toy Poodle, Pug, Yorkshire Terrier, Mongrel
**028:03*	**029:01*	7	5	Collie, Jack Russell Terrier
**nov2*	**nov19*	9	9	Beagle, Miniature Dachshund, Husky, Mongrel
**nov10*	**L*	11	8	Husky, Shih Tzu
**nov15*	**029:01*	11	11	Miniature Dachshund, Yorkshire Terrier
**nov23*	**nov12*	4	2	Pug
Eight minor haplotypes		10	10	Miniature Schnauzer, Pomeranian, Toy Poodle, Shiba, Jack Russell Terrier, Welsh Corgi

Eight major *DLA-88–DLA-88L* allelic haplotypes observed in 355 dogs, and eight minor haplotypes observed in three or less heterozygous dogs, or a single homozygous dog per dog breed

Relative gene expression level of the DLA class I genes in PWBCs

To compare the gene expression levels among the *DLA-class I* genes in PWBCs, we performed a relative quantification assay of the *DLA-class I* genes by the real-time PCR method using newly designed gene-specific primer pairs (Supplementary figure 1 and Supplementary table 1C). Figure 5 and Supplementary figure 3 show the mean differences in the relative gene expression levels of 22 RNA samples isolated from

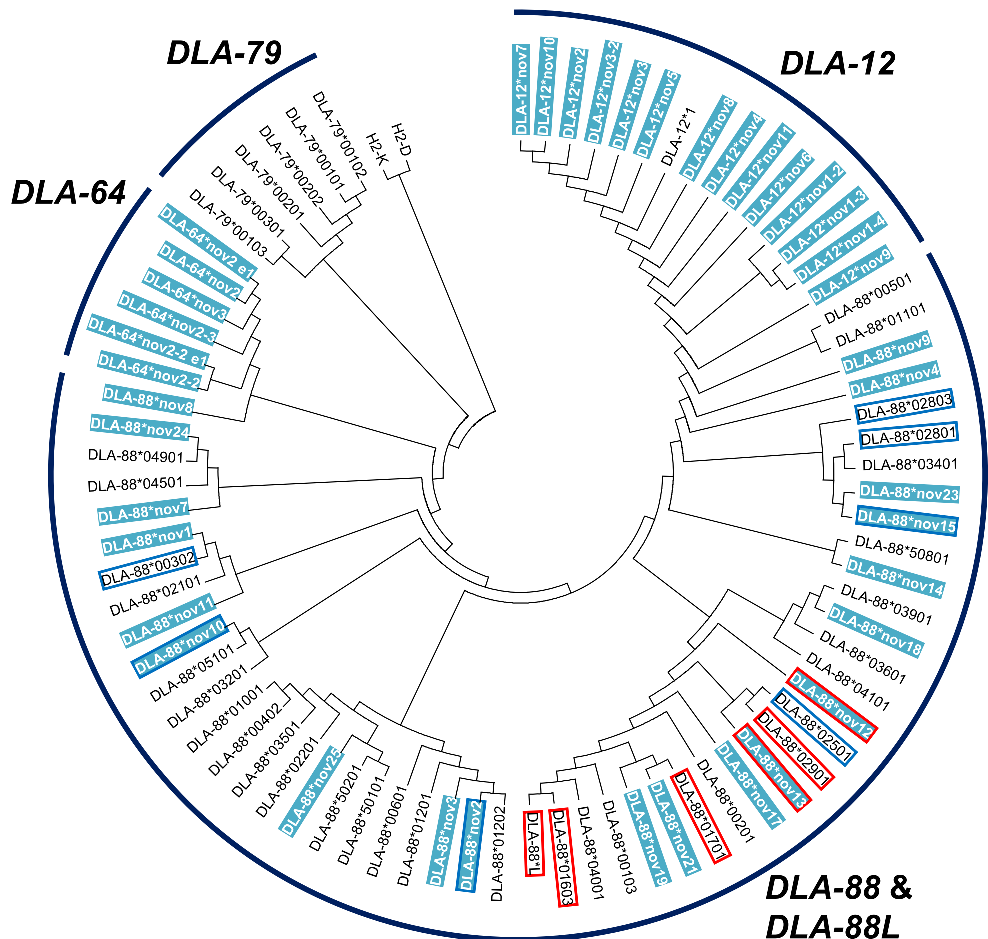

Fig. 4 Nucleotide sequence-based phylogenetic tree of *DLA-class I* alleles constructed by the Neighbor joining method. 66 major and seven minor official *DLA-class I* cDNA sequences that were identified in this study and eight released sequences (six *DLA-79* and two mouse *H-2D* and *H-2K*) were used for constructing the tree. Light blue background and white letters indicate the newly identified *DLA-class I* alleles. Of the *DLA-class I* alleles that are composed of *DLA-88–DLA-88L* haplotypes detected in this study, the *DLA-88* and *DLA-88L* allele names are framed by dark blue and red rectangular lines, respectively. *DLA-64*nov2* is identical with *DLA-64* sequence of the dog genome reference sequence (NC_006594). *DLA-88L* alleles are shown in Table 3

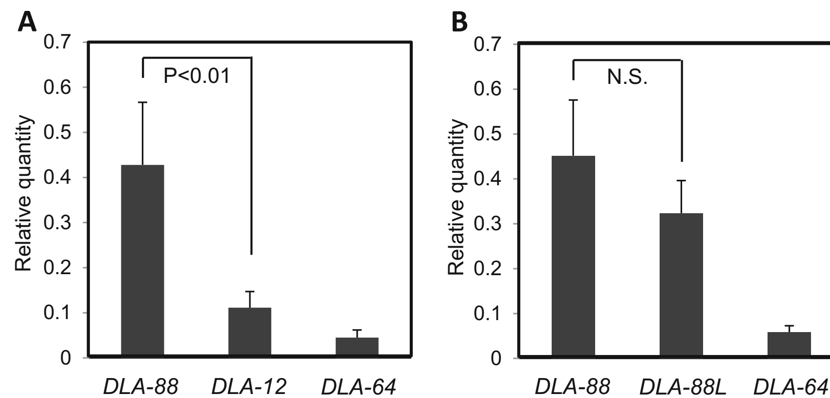

Fig. 5 Relative gene expression levels of *DLA-class I* genes. **a**, **b** Summary of the mean differences between the relative gene expression levels of *DLA-88*, *DLA-88L/12* and *DLA-64* genes of *DLA-88–DLA-12–DLA-64* and *DLA-88–DLA-88L–DLA-64* haplotypes using ten and 12 dogs for each haplotype, respectively. Vertical axis shows the relative quantitative values by the real-time PCR method. Primer sequences and specificity are shown in Supplementary table 1C and Supplementary fig 1, respectively. Thin bars show standard errors, and *P* values indicate statistical significant difference between *DLA-88* and *DLA-12* and *DLA-88* and *DLA-88L* genes. The detail relative gene expression levels of each *DLA* haplotype are shown in Supplementary figure 3

ten *DLA-88–DLA-12–DLA-64* and 12 *DLA-88–DLA-88L–DLA-64* haplotype homozygous dogs. In the case of both haplotype structures, the relative expression levels were observed as follows: *DLA-88* > *DLA-88L*> *DLA-12* > *DLA-64*, with a significant difference ($P < 0.01$) in the mean expression level between *DLA-88* and *DLA-12,* and no significant difference between *DLA-88* and *DLA-88L* (Fig. 5). Taken together, these results show that the gene expression levels of *DLA-88* and *DLA-88L* were significantly higher than those of *DLA-12* and *DLA-64* in the PWBCs.

Comparison of amino acid sequences among the DLA-class I genes

A total of 45 *DLA-88*, seven *DLA-88L*, 11 *DLA-12* and two *DLA-64* nucleotide sequences that were classified as major and minor official alleles in this study were translated as amino acid sequences that yielded 45 *DLA-88,* seven *DLA-88L,* 15 *DLA-12* and six *DLA-64* different allele sequences (Supplementary table 2A and 2B). Of the *DLA-88* alleles, three signature nucleotides coding for the amino acid residue leucine, L, at position 155 were inserted in eight of the known *DLA-88* alleles (*DLA-88*501:01, DLA-88*502:01, DLA-88*503:01*, *DLA-88*504:01, DLA-88*505:01, DLA-88*506:01*, *DLA-88*507:01* and *DLA-88*508:01*) in the exon 3 region (Kennedy et al. 1999). The same location for the three signature nucleotide insertions was observed in five of the newly identified *DLA-88* alleles, *DLA-88*nov25, DLA-88*nov26, DLA-88*nov27, DLA-88*nov37* and *DLA-88*nov41*. Therefore, we defined these 13 *DLA-88* alleles as a *DLA-88*50X* group of alleles.

Supplementary figure 4 shows that amino acid sequence logos of the T cell recognition sites (TRSs) and the peptide binding regions (PBRs) that were deduced from the structural analyses of the HLA-class I and DLA-88 molecules using the newly identified major alleles in this study and six previously published *DLA-79* alleles (Bjorkman et al. 1987; Parham et al. 1988; Xiao et al. 2016; Venkataraman et al. 2013). Amino acid sequences of the TRSs translated from each *DLA-class I* gene were well-conserved with only one (DLA-79) to six (DLA-88) amino acid differences, and of them, three amino acid differences (positions 62, 72 and 169 or 170) were commonly observed in the DLA-88*,* DLA-12 and DLA-88L sequences. In contrast, amino acid sequences of the PBRs on DLA-88 and DLA-88L were more variable with 19 (DLA-88) and nine (DLA-88 L) amino acid differences, although the amino acid sequences of DLA-12 were relatively more conserved than those of DLA-88 and DLA-88 L (Supplementary figure 4). On the other hand, amino acid sequences were well conserved for DLA-64 and DLA-79 with one and four amino acid differences for the DLA-64 and DLA-79 antigens, respectively. The results of this amino acid sequence analysis show that DLA-88, DLA-12 and DLA-88L have relatively high amino acid variability at TRSs and PBSs when compared to DLA-64 and DLA-79 (Supplementary figure 4). Moreover, one TRS residue at position 154 and four PBR residues at positions 97, 99, 114 and 147 were different amino acids between the DLA-12 and DLA-88L protein sequences.

Estimation of DLA-class I allelic haplotypes and their detection in homozygous dogs

A total of 45 different *DLA-class I* major allelic haplotypes (Hp. 01 to Hp. 45) were composed of *DLA-88-DLA-88L/12-DLA-64* (*88-88L/12-64*); 37 were *88-12-64* and eight were *88-88L-64* (Supplementary table 4). Some *DLA-88* alleles such as *DLA-88*501:01* were observed in several different haplotypes and *DLA-88L* alleles were observed in mostly different

haplotypes. The combinations of the two major structural haplotypes, *88-12-64/88-12-64*, *88-12-64/88-88L-64* and *88-88L-64/88-88L-64*, were observed in 239, 90 and 26 unrelated dogs, respectively. The haplotype frequencies of the *88-12-64* and *88-88L-64* were 80.0 and 20.0%, respectively. Of the major 45 *DLA-class I* allelic haplotypes, Hp.04, Hp.07, Hp.18, Hp.21 and Hp.38 collectively showed high frequencies with 36 to 76 dogs in the 355 unrelated dogs, and the *DLA-class I* haplotypes were also widely observed in seven to ten breeds (Supplementary table 4).

Table 5 shows the *DLA-class I* allelic haplotype information in five representative breeds, 48 dogs of Miniature Dachshund, 42 of Toy Poodle, 41 of Yorkshire Terrier, 37 of Shiba and 36 of Chihuahua. Six to 14 *DLA-class I* haplotypes were identified in each breed. A comparison of the inferred *DLA* haplotypes among the five breeds revealed the presence of breed-specificity with high frequency haplotypes such as Hp.15 (haplotype frequency of 0.107) and Hp.32 (0.131) in the Toy Poodle, Hp.04 (0.268) and Hp.05 (0.183) in the Yorkshire Terrier, and Hp.33 (0.162) and Hp.37 (0.189) in the Shiba (Table 5 and Supplementary table 5).

An examination of the *DLA-class I* allelic haplotypes in homozygous dogs of the 355 unrelated dogs and 38 related dogs in four Beagle families revealed that 86 homozygous dogs in 18 breeds had 22 different *DLA-class I* allelic haplotypes by *DLA-class I* gene genotyping data and *DLA-88–DLA-88L* allelic haplotype information. In comparison, these 22 *DLA-class I* allelic haplotypes were also detected in 275 heterozygous dogs (70% of the 393 dogs) from 20 breeds and mongrels (Table 6).

Inferred evolutionary model for the genomic structures of the DLA-88–DLA-12 and DLA-88–DLA-88L haplotypes

Figure 6 shows the inferred model for the generation of the genomic structures of the *DLA-88–DLA-12* and *DLA-88–DLA-88L* haplotypes. Essentially, a gene transfer (unequal crossing over) or gene conversion has replaced the *DLA-12* gene locus with a *DLA-88* gene that we have unofficially designated here to be *DLA-88L* thereby resulting in a *DLA-88–DLA-88L* structural haplotype. The original *DLA-88–DLA-12* haplotype is retained in various dog populations at a frequency of 80%, whereas the newer haplotype has emerged at a lower frequency of 20%. This haplotype frequency was similar to the previously reported frequency (18%) that has duplicated *DLA-88* alleles in at least one haplotype (Kennedy et al. 2012). Also, while the *DLA-12* allelic sequences have diverged over time to form their own single lineage separate from the *DLA-88* lineage, the more recent *DLA-88L* allelic sequences have not diverged sufficiently from the *DLA-88* allelic sequences to establish their own unique lineage. Consequently, the *DLA-88* and *DLA-88L* alleles are intermixed in the same *DLA-88* lineage as seen in the phylogenetic tree in Fig. 4. A hypothetical *DLA-12–DLA-12* structural haplotype that might have arisen from the same gene crossover event that generated the *DLA-88–DLA-88L* haplotype (Fig. 6) has not been identified in any dog populations and it is therefore extremely rare, extinct or it was never generated.

Discussion

Recent genomic studies suggest that the dog species was first domesticated in Asia (Pang et al. 2009; Vonholdt et al. 2010), but with a possible dual origin of domestic dogs in Europe and Asia (Frantz et al. 2016). Domestic dogs that originated from Asia have a much greater genetic diversity of the *DLA-DRB1* gene than those that originated from Europe (Niskanen et al. 2013). From this viewpoint, we identified 20 *DLA-88,* 14 *DLA-12* and six *DLA-64* novel and major alleles in 38 related and 404 unrelated dogs (Table 2) with a greater focus on small-sized dogs such as the Chihuahua, Miniature Dachshund and Toy Poodle, and Asian dogs such as the Japanese Spitz, Shiba and Shih Tzu than had been previously studied. Namely, 61% of them were novel alleles (Table 2 and Supplementary table 2A). Whereas previous studies reported numerous polymorphisms within the *DLA-88* locus and few or none at *DLA-12* and *DLA-64*, we found that there are also

Table 5 *DLA-class I* haplotype information identified in five dog breeds based on *DLA-class I* cDNA and genomic analyses

Breed	Number of dogs	Count of haplotypes	The most frequent haplotype	
			Haplotype ID	Frequency (%)
Miniature Dachshund	48	12	Hp.18	21.9
Toy Poodle	42	14	Hp.07	16.7
Yorkshire Terrier	41	10	Hp.04	26.8
Shiba	37	6	Hp.07	52.7
Chihuahua	36	14	Hp.21	15.3

The detailed information is shown in Supplementary table 5

Table 6 Numbers of homozygous and heterozygous dogs for each *DLA-class I* allelic haplotype based on *DLA-class I* cDNA and genomic analyses

Haplotype ID	Number of homozygous dog by breed	Number of homozygous dogs	Heterozygous	
			Number of breeds	Number of dogs
Hp. 01	French Bulldog (1), Papillon (2)	3	3 breeds + mongrel	8
Hp. 02	Chihuahua (1)	1	2 breeds + mongrels	7
Hp. 04	Labrador Retriever (1), Miniature Pinscher (1), Papillon (1), Pomeranian (2), Yorkshire Terrier (3)	8	7 breeds + mongrels	31
Hp. 07	Miniature Dachshund (1), Toy Poodle (2), Shiba (10)	13	9 breeds + mongrels	63
Hp. 09	Beagle (5), Miniature Dachshund (1), Maltese (1), Welsh Corgi (1)	8	7 breeds + mongrels	37
Hp. 11	Shetland Sheepdog (3)	3	3 breeds + mongrels	11
Hp. 15	Toy Poodle (1)	1	1 breed	7
Hp. 18	Beagle (1), Miniature Dachshund (1), Yorkshire Terrier (1), Mongrel (2)	5	10 breeds + mongrels	62
Hp. 20	Beagle (7)	7	1 breed + mongrels	21
Hp. 21	Chihuahua (1), Labrador Retriever (2)	3	7 breeds + mongrel	39
Hp. 22	Maltese (1)	1	1 breed + mongrels	5
Hp. 27	Shih Tzu (2)	2	1 breed + mongrels	7
Hp. 31	French Bulldog (1), Welsh Corgi (1)	2	4 breeds + mongrels	16
Hp. 32	Toy Poodle (2)	2	1 breed + mongrels	10
Hp. 33	Shiba (3)	3	1 breed + mongrel	7
Hp. 36	Labrador Retriever (1)	1	1 breed	4
Hp. 37	Shiba (3)	1	1 breeds + mongrels	14
Hp. 38	Shetland Sheepdog (10)	10	9 breeds	34
Hp. 40	French Bulldog (4), Pug (1)	5	5 breeds + mongrel	22
Hp. 41	Collie (2)	2	2 breeds	3
Hp. 43	Husky (2), Shih Tzu (1)	3	2 breeds	5
Hp. 45	Pug (1)	2	1 breed	2

The detailed information of "Haplotype ID" is shown in Supplementary table 4

many polymorphisms within the *DLA-12* and *DLA-64* gene loci. This suggests that future polymorphism analysis of the *DLA-class I* genes using a greater number of dogs and different breeds is likely to result in the discovery of many more *DLA-class I* alleles at the four *DLA* loci, *DLA-88*, *DLA-88L, DLA-12* and *DLA-64*.

A serious problem for performing a polymorphism analysis by Sanger sequencing of the *DLA-class I* genes is the difficulty of obtaining clear genotyping and haplotyping results because 1–4 types of *DLA-88* alleles per dog can be identified within two types of *DLA-class I* structural haplotypes, *DLA-88–DLA-12* and *DLA-88–DLA-88L* (Fig. 2). The existence of two types of *DLA-class I* haplotype structures was first indicated by the observation of three *DLA-88* alleles in one dog in a previous report (Ross et al. 2012). Although the *DLA-12* locus was identified to express a protein that classified as the probable class Ib gene (Wagner et al. 2005), our study is the first to highlight that the *DLA-12* locus is moderately to highly polymorphic with the identification of at least 22 major *DLA-12* alleles (seven *DLA-88L* and 15 *DLA-12* alleles) whereby some of them had been previously misclassified as *DLA-88* alleles at the *DLA-88* locus. As our genomic and phylogenetic analyses suggests, the possible reason for this misclassification is that the alleles at the *DLA-88L* locus have very high sequence similarities with the *DLA-88* alleles at the *DLA-88* locus (Figs. 2 and 4 and Supplementary table 3), and that it is difficult to separate the *DLA-88* and *DLA-88L* alleles by simple PCR amplification of sequences shared between the two loci. For example, the intron 2 sequence of *DLA-88*016:03* (Venkataraman et al. 2017) showed 100% match with *DLA-88*034:01* and 99.5% match with the novel *DLA-88 L* (*DLA-88*029:01*), although the allele showed 62.6% with *DLA-12*nov6*. Consequently, we developed a nested method to specifically detect the *DLA-88 L* alleles by performing the PCR amplification using one primer pair for the exons 1–4 segment of the *DLA-88L* gene (88/88L/64-F and 88/88L/12-R) after PCR amplification of the 5.6 kb segment using the long-range primer for the *DLA-12* and *DLA-88L* genes (88L/12-seg-F and 88 L/12-seg-R) (Fig. 3). This new PCR nested genotyping method will be most helpful for differentiating

between the *DLA-12* and *DLA-88L* alleles. This method can be modified and applied to genotyping *DLA-88* alleles by simply changing the primer sequences of the 88L/12-seg-F and 88L/12-seg-R primer sites and instead use *DLA-88* specific primers such as the DLA88-12-64_F1 and DLA88-12-64_R1 pair (Fig. 2a and Supplementary table 1B). Although, at least seven *DLA-88* alleles were classified as *DLA-88L* major alleles in our study (Table 3), a large-scale haplotype analysis will be required to elucidate the diversity and frequency of the *DLA-88–DLA-88L* haplotype structures among various dog breeds and mongrels, which was beyond the scope of our current study.

The *DLA-88–DLA-88L* haplotype appears to have been generated by (1) duplication of the *DLA-88* gene with the production of *DLA-12* after divergence from the Feliformia species (52.9 Mya) (Hedges and Kumar 2009), and (2) a gene conversion or an unequal crossing over between *DLA-88* and *DLA-12,* resulting in the production of the *DLA-88–DLA-88L* and the *DLA-88–DLA-12* haplotypes (Fig. 6). Although the *DLA-12* allele lineage has a close evolutionary relationship with the *DLA-88* allele lineage, all of the ten *DLA-88L* allele sequences (exons 1 to 3) were included in the *DLA-88* lineage (Fig. 4). If mutations were evolving at the same rate between *DLA-12* and *DLA-88L*, then the allele numbers for *DLA-12* (15 alleles) would suggest that they have evolved and diverged over a longer time period than those for *DLA-88L* (seven alleles) (Table 3 and Supplementary table 2A). Although the overall allele number for *DLA-88L* was less than that for *DLA-12*, the amino acids on PBRs and TRSs in *DLA-88L* were more polymorphic than those in *DLA-12* (Supplementary figure 4). This suggests that these two genes were derived from different *DLA-class I* ancestral genes and/or that they were subjected to different natural selection pressures during their evolution (Fig. 6).

The *DLA-88* and *DLA-88L* showed slightly higher nucleotide sequence similarity with 93.6% than those of *DLA-12* and *DLA-88L* (92.2%) and *DLA-88* and *DLA-12* (88.7%) at the 3.3–3.4 kb of the *DLA-class I* gene region. However, the sequence similarity comparisons show that the 5.6 kb of genomic structures between the *DLA-12* and *DLA-88L* segments are more conserved than those between the *DLA-88* and *DLA-12* segments and between the *DLA-88* and *DLA-88L* segments (Supplementary table 3). This suggests that the *DLA-12* and *DLA-88L* genes were exchanged with each other from the upstream region to at least the 3'untranslated region. However, it is noteworthy from the *DLA-12* and the *DLA-88L* primers and the PCR experiment shown in Fig. 3 that there are nucleotide sequence differences between the *DLA-12* and *DLA-88L* in the regions of exon 1 and exon 4. Furthermore, the amino acid variability analysis supports the view that *DLA-12* and *DLA-88L* have a classical MHC class I function because the amino acid variations at PBRs and at the TRSs of both genes are similar to those of *DLA-88* that is known to have a classical MHC class I function (Supplementary figure 4). Although new MHC genes have been inferred to have been generated by duplication,

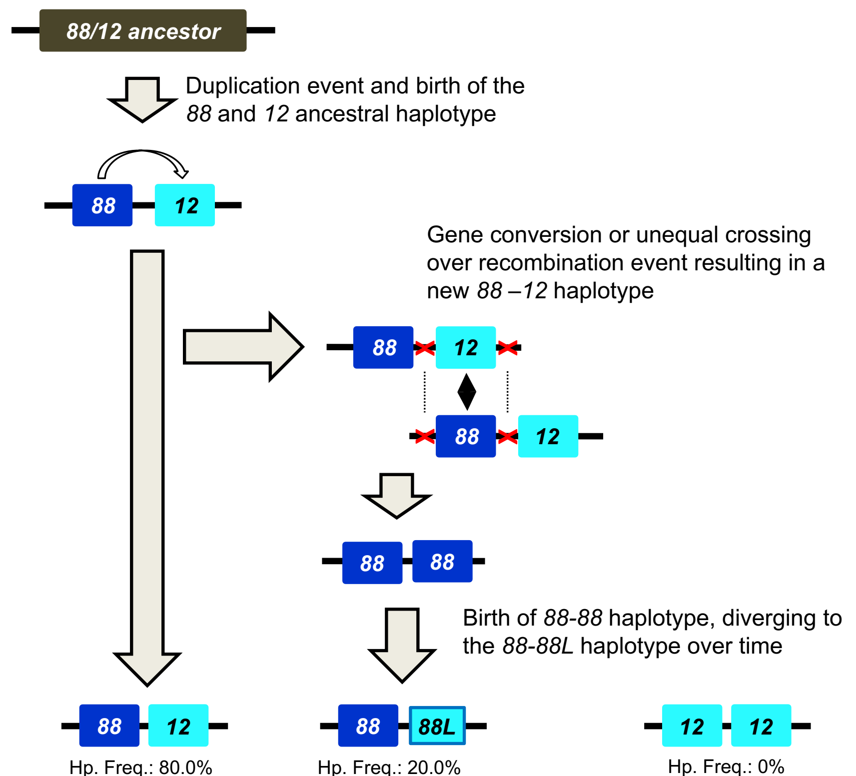

Fig. 6 Inferred evolutionary model for the genomic structures of *DLA-88–DLA-12* and *DLA-88–DLA-88L* haplotypes. Dark and light blue boxes show the *DLA-88* and *DLA-12* genes, respectively, and the light blue box with a dark blue rectangular line shows *DLA-88L*. "Hp. Freq." indicates the haplotype frequency. Because the *DLA-88* gene in a gene conversion or crossing over event replaced the *DLA-12* locus, we have unofficially named the replacement gene *DLA-88L* despite its location

crossing over and gene conversion events in the MHC class I genomic structure of some vertebrate species (Holmes et al. 2003; Hosomichi et al. 2008), this is the first report attributing unequal crossing over or gene conversion at a single working *DLA* locus contributing to the MHC class I diversity of the domestic dog species.

The *DLA-88–DLA-88L* haplotype was observed in 17 Asian and European breeds and mongrels (Table 4). On the other hand, although the *DLA-12–DLA-12* haplotype may have been generated immediately after the first unequal crossing over event in domestic dogs, the detection of the *DLA-88* alleles in all of the dogs supports the premise that the *DLA-12–DLA-12* haplotype without *DLA-88* and *DLA-88L* alleles was negatively selected against by birth and death evolution (Fig. 6) (Nei et al. 1997).

The level of *HLA* gene expression activity such as the association of the highly expressed *HLA-C*14:02* allele and progression after infection of HIV (Apps et al. 2013), and the association of *HLA-C*14:02* and *HLA-DP5* alleles and acute graft versus host disease (GVHD) (Petersdorf et al. 2014; Petersdorf et al. 2015) has become an important topic in human MHC genetics in recent years. In regard to the dog MHC, one of the main differences between *DLA-12* and *DLA-88L* is that the gene expression level of *DLA-88L* is significantly higher than that of *DLA-12,* and almost the same as *DLA-88* (Fig. 5 and Supplementary figure 3). The *DLA-88L* positive dogs are thought to have a higher peptide presentation ability and an higher gene expression level than the *DLA-88L* negative dogs. Therefore, the functional difference in gene expression levels may have originated from the unequal crossing over event and this difference may influence the resistance and susceptibility of dogs to various viral infections such as canine distemper virus (CDV), canine adenovirus (CAV) and canine parvovirus (CPV) (Laurenson et al. 1998), and various inherited diseases and those that may be associated with transplantation outcome in dogs.

Of the *DLA-class I* genes, *DLA-64* was confirmed to have a non-classical function with low polymorphisms and gene expression levels in the PWBCs, and limited amino acid variations in the PBRs and TRSs domains compared to *DLA-88* and *DLA-88L* that have a classical class I function as described in Supplementary figure 4. In contrast, although *DLA-79* was reportedly expressed at high levels in muscle (Burnett and Geraghty 1995), the detailed expression patterns are unknown. Therefore, gene expression analysis of the *DLA-class I* genes including *DLA-88*, *DLA-12, DLA-88L, DLA-64* and *DLA-79* are necessary for future biomedical studies.

In humans, *HLA-G* is a non-classical class I gene that is highly expressed in the trophoblasts of the placenta and it has acquired a maternal immunity function by inhibiting cytotoxic activity of maternal natural killer (NK) cells (Kovats et al. 1990; Rouas-Freiss et al. 1997). Moreover, the human and mouse non-classical MHC class I genes *HLA-E* and *Qa-1^b* respectively, are recognized by NK cells and result in the inhibition of the cytotoxic activity (Braud et al. 1998; Vance et al. 1998). As inhibitory receptors for NK cells, the killer immunoglobulin receptors (KIRs) in human and the Ly49 receptor in mouse play an especially important role for the recognition of the MHC molecules and their genomic regions are extremely diverged and polymorphic (Anfossi et al. 2006; Rahim and Makrigiannis 2015). However, the dog genome surprisingly does not have any KIR genes and it has only one Ly49 gene (Gagnier et al. 2003; Hammond et al. 2009). Therefore, domestic dogs may have a different mechanism to inhibit NK activity that is not seen in the human and mouse. In this regard, the elucidation of the essential functions of *DLA-64* and *DLA-79* and their functional relationships with NK cells is necessary for a better understanding of the mechanisms of acquired immunity and transplantation rejection in domestic dogs.

Our study of *DLA-class I* allelic haplotypes among five representative breeds (Table 5 and Supplementary table 5) supports previous observations that there are breed-specific *DLA* haplotypes, although genetic bias of *DLA* allelic haplotype numbers was observed in some breeds such that only three haplotypes (Hp. 07, Hp. 33 and Hp. 37) occupied 87.9% of all haplotypes (six haplotypes) in the Shiba breed used in this study (Supplementary table 5). The English Bulldog breed was reported recently to have low genetic diversity because of inbreeding and a small founder population (Pedersen et al. 2016). This low genetic diversity may endanger the health and well-being of the breed by increasing the number and prevalence of inherited diseases. On the other hand, a small number of polymorphisms and low diversity are thought to be one of the advantages for the allo-transplantation model by allowing the easier detection and graft acceptance of *DLA* matched donor and recipient dogs. Namely, grafts from *DLA* haplotype homozygous dogs as donors are theoretically more likely to be accepted in homozygous dogs with the same haplotype and in heterozygous dogs with the recipient having one of the donor's haplotypes. In this study, we identified 86 dogs with *DLA* homozygous allelic haplotypes and 275 dogs with *DLA* heterozygous allelic haplotypes (Table 6). If we can stock stem cells such as dedifferentiated fat (DFAT) cells and induced pluripotent stem (iPS) cells representing the 22 major *DLA-class I* allelic haplotypes from homozygous dogs, then 91.9% of all dogs might be useful as animal models for studying transplantation mechanisms and benefit regenerative medicine for dogs and humans (Matsumoto et al. 2008; Yamanaka 2009).

In conclusion, we have identified and presented a large number of novel alleles for the *DLA-class I* genes and provided some further insights into the demographic and selection factors on the genomic structure, gene expression level, nucleotide diversity and phylogenetic relationships of *DLA-class I* alleles and haplotypes in 49 domestic dog breeds. This *DLA*

polymorphism information and genetic differences among the dog breeds could be used as a standard internal control of the MHC genetic background for the benefit of biomedical research into regeneration medicine using the most common *DLA* allelic haplotypes as models for human MHC-related diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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