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Evidence for extensive DLA polymorphism in different dog populations

Key words:
DLA-DQA1; DLA-DQB1; DLA-DRB1; Dog; MHC

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Abstract: Many of the genes within the Canine Major Histocompatibility Complex are highly polymorphic. Most of the alleles defined to date for DLA-DRB1, DQA1 and DQB1 come from the analysis of European or North American pure bred dogs. Little is known about DLA gene polymorphisms in other dog populations. We have studied Alaskan Husky dogs and Brazilian mongrel dogs and compared them with a panel of 568 European dogs and 40 Alaskan gray wolves. DNA sequence based typing was used to characterize a series of 12 Alaskan Huskies and 115 Brazilian mongrels for their DLA-DRB1, DQA1 and DQB1 alleles. Within these dogs, 22 previously undescribed DLA class II alleles were identified: 10 DRB1, 5 DQA1 and 7 DQB1 alleles. All these alleles were found in more than one animal, and, in some cases, as a homozygote. Several alleles initially observed in Alaskan gray wolves were found in these dogs. Each new allele was found in specific haplotypic combinations. Many new DLA class II haplotypes were identified. Several of the new alleles and haplotypes were also identified in the European dogs used for comparison. One new haplotype, containing a previously unknown DLA-DRB1 allele together with DQA1 and DQB1 alleles only seen before in gray wolves, was found in 20 Brazilian dogs, including three homozygous animals. It appears likely that the extent of polymorphism of the DLA genes will increase substantially as dogs from a wider geographic distribution are studied. This has major implications for the study of disease susceptibility and immune responsiveness in dogs.

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Many of the genes within the canine Major Histocompatibility Complex (DLA) appear to be highly polymorphic. The latest DLA nomenclature report (1) lists 52 DLA-DRB1 alleles, 16 DLA-DQA1 alleles and 41 DLA-DQB1 alleles. However, most of the DLA-DRB1, DQA1 and DQB1 alleles defined to date originate from studies of European or North American purebred dogs. Considerable data have been generated regarding allele frequencies for a variety of dog breeds (2, 3). These data were also based exclusively on European and North American dog breeds. Other data (LJ Kennedy, unpublished) generated from Alaskan gray wolves (*Canis lupus*) have

revealed further DLA alleles at all three class II loci studied, suggesting that the full extent of the polymorphism of these genes has not yet been revealed. Little is known about polymorphisms in the DLA class II genes in other dog populations, and to address this issue a population of mongrel dogs from Brazil and Husky dogs from Alaska were characterized for their class II alleles.

As more dog breeds are characterized for three-locus DLA haplotypes, it may be possible to clarify any interbreed genetic relationships and also the relationship of the domestic dog with other canid species. Analysis of MHC haplotypes may reveal patterns of infection, as the distribution of MHC alleles and haplotypes varies between breeds, with some breeds showing a limited MHC distribution, depending on founder effects and their exposure to different infective agents.

Materials and methods

Samples

Blood samples were collected from 12 Alaskan Husky dogs living near Fairbanks, Alaska, and DNA was extracted using a DNeasy extraction kit (Qiagen, Crawley, UK). Bone marrow biopsies were collected from 115 mongrel dogs from the city of Belèm or Marajó Island, Pará state, Brazil, as part of a study of the immuno-epidemiology of visceral leishmaniasis (4). Biopsies were digested with Proteinase K and DNA was extracted using phenol-chloroform.

DNA typing

DNA sequence based typing (SBT) was used to characterize the Alaskan Husky and Brazilian mongrel dogs for their DLA-DRB1, DQA1 and DQB1 alleles. For comparison, DLA data from a panel of 40 Alaskan gray wolves plus 568 European dogs (including animals from over 70 different breeds) were available from previous studies (3).

All the locus specific primers were intronic, and are shown in Table 1. The DLA-DRB1 and DQA1 primers were M13 tailed and these portions are underlined in Table 1. The DRB1 primers were from Wagner (5). The DQA1 forward primer was from Wagner (6), and the reverse from Kennedy (7). The DQB1 forward primer was designed by Wagner (personal communication) and the reverse by Kennedy (previously unpublished).

For SBT, all PCR reactions were performed with 100 ng DNA in a 25-μL reaction containing 1× KCl based PCR buffer and final concentrations of 0.1–0.25 μM for each primer, 200 μM each dNTP, 1.5–2.0 mM $MgCl_2$ and 2 units of Taq polymerase (Bioline, London, UK; Qiagen, Crawley, UK; ABgene, Epsom, UK) (5, 7, 8).

A standard PCR programme was used for all amplifications, which consisted of an initial 3 min at 95°C, then 30 cycles of 95°C for 30–40 s, followed by the annealing temperature for 1 min and extension at 72°C for 1 min, plus a final 10 min extension at 72°C. Annealing temperatures used were 61°C, 48°C and 66°C for DLA-DRB1, DQA1 and DQB1, respectively. Annealing temperatures were adjusted for use in a 96 well plate, using adhesive plate sealers, with a heated lid on the thermal cycler, avoiding the need for an oil overlay. All PCR reactions were performed on a DNA Tetrad Engine (MJ Research, Braintree, UK) thermal cycler. A negative control containing no DNA template was included in each run of amplifications to identify any contamination

DNA Sequencing

PCR samples were purified with Qiaquick PCR purification columns (Qiagen) before cycle sequencing. Standard M13 forward and re-

Primers used in this study

Primer sequences: 5′-3′	
DRB1 forward:	5′-TgT AAA ACg ACg gCC AgT CCg TCC CCA CAg CAC ATT TC-3′
DRB1 reverse:	5′-CAg gAA ACA gCT ATg ACC TgT gTC ACA CAC CTC AgC ACC A-3′
DQA1 forward:	5′-TgT AAA ACg ACg gCC AgT CTC AgC TgA CCA TgT TgC-3′
DQA1 reverse:	5′-CAg gAA ACA gCT ATg ACC ggA CAg ATT CAg TgA AgA gA-3′
DQB1 forward:	5′-CTC ACT ggC CCg gCT gTC TC-3′
DQB1 reverse:	5′-CAC CTC gCC gCT gCA Acg Tg-3′
M13 Sequencing primers	
-20 M13 forward:	5′-TgT AAA ACg ACg gCC AgT-3′
M13 reverse:	5′-CAg gAA ACA gCT ATg ACC-3′

Table 1

verse primers were used for the BigDye cycle sequencing of DLA-DRB1 and DQA1. The initial PCR primers were used for the DLA-DQB1 BigDye cycle sequencing. The sequencing products were run on an ABI 377 DNA sequencer.

Data analysis

Analysis of the sequence data was performed with ABI software ('Sequencing Analysis', 'Matchtools' and 'MatchtoolsNavigator'). A reference library of sequences was created, and 'MatchTools' facilitated the comparison of the data generated from published DLA sequences. Heterozygous samples gave clear heterozygous peaks, and the programme 'MatchTools' helped resolve these into known and unknown sequences.

DNA sequencing data produced directly from PCR products were analyzed in the following way. After the data had been inspected visually to assess the quality using the 'Sequencing Analysis' programme, the sequence data were read into 'MatchToolsNavigator'. In this programme the reverse sequence was complemented and aligned with the forward sequence. Each pair of sequences was then inspected together and each site of potential polymorphism was viewed. Any ambiguous calls made by the original basecaller programme were also corrected. Consensus sequences had previously been generated for each locus (DLA-DRB1, DQA1 and DQB1) to provide a reference for the polymorphic positions by comparing the test sequences with the appropriate consensus sequence. Once the test sequences had been corrected, and any heterozygous positions assigned the appropriate International Union of Biochemistry (IUB) code (R = A + G, Y = C + T, K = G + T, M = A + C, S = C + G, W = A + T), the sequences were read into 'MatchTools'. This programme compares the data with the appropriate locus specific allele reference library, and produces a report of the closest allele matches for the input data.

When the programme could not find any matches, the data was analyzed by hand. New alleles were confirmed if or when the same sequence was found in other dogs.

New DLA alleles found in 695 dogs (568 European + 115 Brazilian + 12 Alaskan)

DLA allele	Comment	Number of homozygotes	Number of heterozygotes
DRB1*01302	4 bp different from DRB1*01301	–	1
DRB1*01504	1 bp different from DRB1*01501	–	3
DRB1*01702	4 bp different from DRB1*01701	–	4
DRB1*04001	Most similar to DRB1*02001	1	3
DRB1*04601	Most similar to DRB1*04501	1	5
DRB1*04701	Most similar to DRB1*03101	–	3
DRB1*04801	1 bp different from DRB1*02601	–	4
DRB1*05001	1 bp different from DRB1*01502	–	2
DRB1*05101	1 bp different from DRB1*02701	–	3
DRB1*05201		3	17
DQA1*00402	Most similar to DQA1*00401 with two new polymorphic positions	–	3
DQA1*012011	Also found in gray wolf	4	19
DQA1*012012	1 bp synonymous change, but new polymorphic position	–	6
DQA1*01401	Also found in gray wolf	2	7
DQA1*01501		1	5
DQB1*00802	1 bp different from DQB1*008011	4	10
DQB1*01304	1 bp different from DQB1*01303	–	4
DQB1*02302	3 bp different from DQB1*02301	–	2
DQB1*02801	Most similar to DQB1*00701	1	2
DQB1*03501	Also found in gray wolf	4	25
DQB1*02901	2 bp different from DQB1*02401	–	8
DQB1*03601	2 bp different from DQB1*00201	–	8

Table 2

Definition of haplotypes

Three-locus, DLA-DRB1/DQA1/DQB1, haplotypes were identified by following a sequential analytical process. Firstly, all dogs that were homozygous at all three loci were selected, and from these, several different DLA-DRB1-DQA1-DQB1 haplotype combinations were identified. Dogs that were homozygous at only two loci were then selected. From these dogs many of the previous haplotypes were confirmed and also several further haplotypes were identified. The remaining dogs were examined using the haplotype data al-

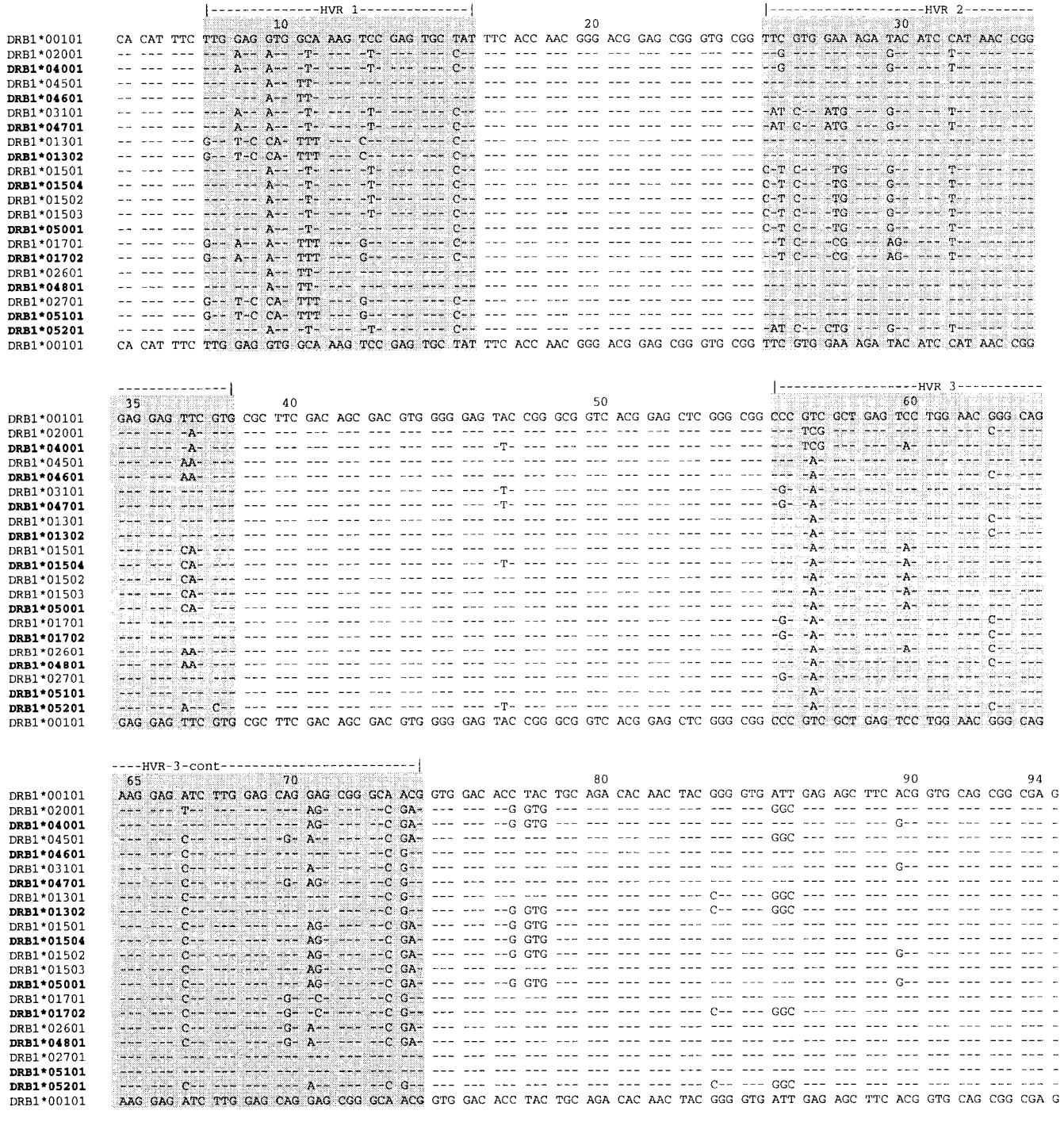

Fig. 1. DLA-DRB1 nucleotide alignment.

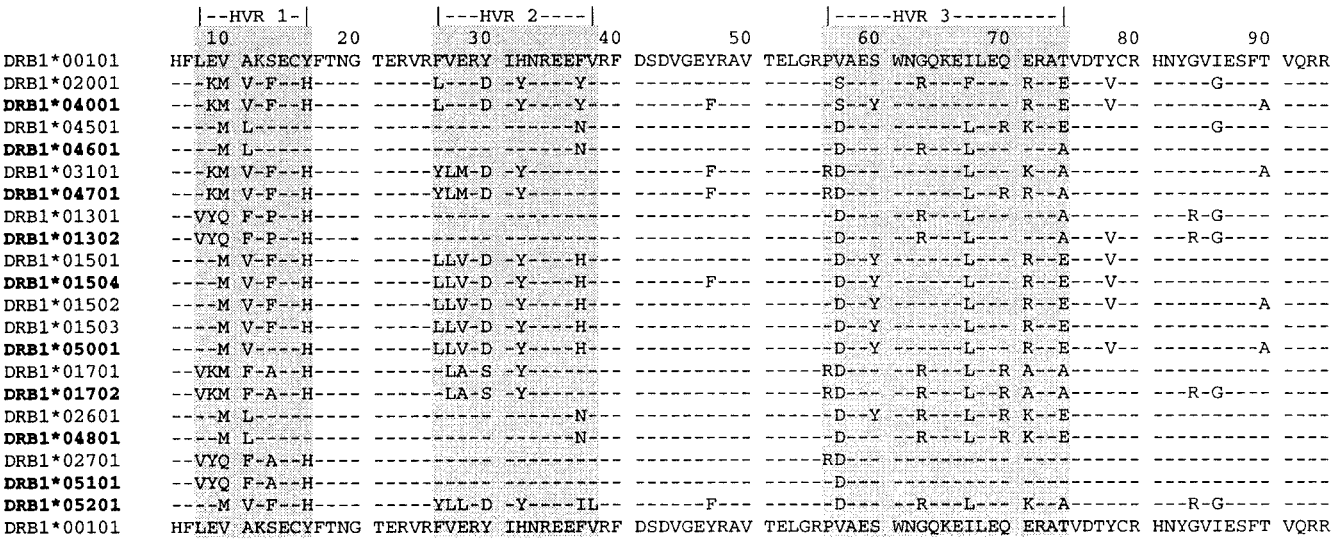

Fig. 2. DLA-DRB1 amino acid alignment.

ready identified and haplotypes were assigned to each of these dogs. More possible haplotypes were identified from these dogs.

Results

Within these dogs, 22 new DLA class II alleles were identified: 10 DRB1, 5 DQA1 and 7 DQB1 alleles*. All of these alleles were found in more than one animal, and, in some cases, as homozygous genotypes. Table 2 lists all the new alleles, with their most similar known alleles. It also lists the number of homozygous and heterozygous animals in which each allele was found. Figures 1 and 2 summarize the DLA-DRB1 nucleotide and amino acid alignments for these alleles plus closely related alleles. Similarly, Figs 3 and 4, and Figs 5 and 6 summarize the nucleotide and amino acid alignments for DLA-DQA1 and DLA-DQB1 alleles, respectively, and closely related alleles. In Figs 1–6, the new allele names are in bold, and the hypervariable regions (HVR) are highlighted. The new alleles have been submitted to the DLA Nomenclature Committee and were all assigned official names, as they fulfilled the criteria laid out in the committees reports* (1, 9)

Table 3 describes the DLA haplotypes that can be assigned to each new allele. Some alleles occured in more than one haplotype, but most were found in one combination only. Many of these haplotypes have been observed as a homozygous genotype. Those haplotypse which have only been found in heterozygotes animals either occurred in animals carrying another common established haplotype or could be seen segregating in families.

Of the 10 new DLA-DRB1 alleles found in this study, seven were seen in Brazilian mongrels only, two in Alaskan Huskies only and one in several different breeds (see Table 2). Two of the new alleles observed in the mongrels were variants of DLA-DRB1*01501, while most of the others were only 1 or 2 base pairs different from alleles already identified. Only one new allele, DLA-DRB1*05201, was unlike any other previously described allele.

One of the two new DLA-DRB1 alleles found in Husky dogs was similar to DLA-DRB1*03101, a common allele found in wolves from the area where these Huskies were collected. DLA-DRB1*03101 and DRB1*04701 are compared in Figs. 1 and 2.

Several alleles, namely DLA-DQA1*012011, DQA1*01401 and DQB1*03501, found in the Brazilian mongrel dogs have also been identified in Alaskan gray wolves. DLA-DQA1*012011 and DQB1*03501 usually occur together in the gray wolf in haplotypic association with DRB1*03601. The same DLA-DQ combination also occurs with a previously unknown DRB1 allele, now called DRB1*05201, in the Brazilian mongrels. This haplotype was found in 20 dogs, including three homozygous animals.

*The accession numbers for the new alleles are:
DRB*01302: AJ311090; DRB1*01504: AJ311091; DRB1*01702: AJ311092; DRB1*04001: AF343741; DRB1*04601: AF343747; DRB1*04701: AF343748; DRB1*04801: AJ311093; DRB1*05001: AJ311094; DRB1*05101: AJ311095; DRB1*05201: AJ311096; DQA1*00402: AJ311099; DQA1*012011: AJ311097; DQA1*012012: AJ311098; DQA1*01401: AF336107; DQA1*01501: AF343736; DQB1*00802: AF343731; DQB1*01304: AJ311101; DQB1*02302: AJ311103; DQB1*02801: AF343730; DQB1*03501: AJ311107; DQB1*02901: AJ311102; DQB1*03601: AJ311100.

Another DLA-DQA1 allele, DQA1*012012, is seen in combination with DLA-DQB1*03501 in a haplotype containing DRB1*02501. This haplotype (DLA-DRB1*02501/DQA1*012012/DQB1*03501) was found in several of the Brazilian mongrels and also in several Shih Tzu.

DLA-DQA1*012012 has one synonymous base change, in codon 34, compared with DQA1*012011, at a position which was previously constant. The other two bases in the same codon are altered in DLA-DQA1*00402; a new allele which was identified in three huskies.

DLA-DQA1*01401 was originally defined in a wolf population (unpublished data), but has since been observed in several dogs also. It appears to occur in at least two different haplotypes in dogs (see Table 3), which are both different from the haplotype found in the gray wolf.

Two of the new haplotypes have only been found in Husky dogs to date. Some overlap of gray wolf and Husky alleles or haplotypes was expected because of close geographic proximity and possible interbreeding. However, only one Husky had an allele previously

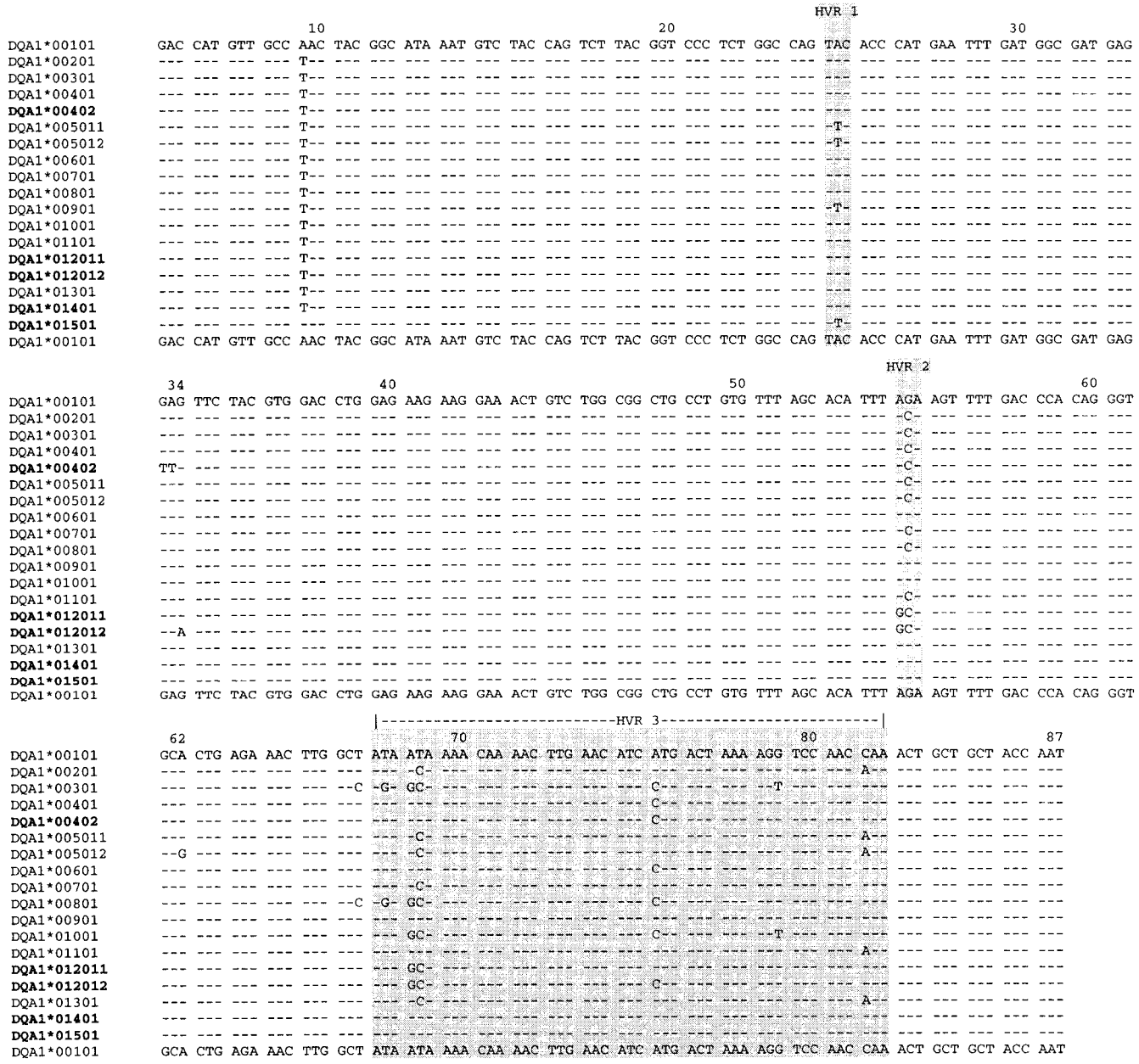

Fig. 3. DLA-DQA1 nucleotide alignment.

observed in wolves, namely DLA-DRB1*03901, and this did not occur in the same haplotypic combination as in the wolf.

One haplotype, DLA-DRB1*04601/DQA1*01501/DQB1*00802, was originally found in a Papillon dog which was homozygous for all three loci. This haplotype was found in at least three different Brazilian mongrels, and two other Papillon dogs.

As with the new DLA-DQA1 alleles, six of the new DQB1 alleles appear to be found in several different haplotypes, see Table 3.

The seventh new allele, DLA-DQB1*02801 was found in only one haplotypic combination: DRB1*00601/DQA1*005011/DQB1*02801. This is interesting, as DQB1*02801 has only three base pair differences from DQB1*00701, which is the allele commonly found in association with DRB1*00601/DQA1*005011.

Discussion

Apart from cattle (10) and some primate populations (11), little is known about the extent of MHC polymorphism in non-human mammals. Many studies have been geographically restricted, while others involve the study of captive breeding colonies, with the associated loss of MHC variation. For example, much is known about the MHC haplotypes in strains of inbred mice, but little data relating to MHC alleles or haplotypes are available for wild populations of mice. In general, the established view of the mammalian MHC is homocentric and inbred strain oriented, with little information on open populations in other species.

The domestic dog offers an opportunity to study the MHC in another species, and although each breed may be inbred, interbreed comparisons where phenotypes are vastly different can be informative, as can the study of mongrel dogs.

The data on the dog MHC collected to date indicate that DLA genes are highly polymorphic. If we include the alleles from this paper, there are now 61 published DLA-DRB1, 18 DLA-DQA1 and 47 DLA-DQB1 alleles. The numbers of dogs tested for these loci vary. To date, we have tested 800 dogs from over 110 different breeds for DLA-DRB1 and DQA1, and 200 dogs from 50 different breeds for DLA-DQB1 (3). This would suggest that DLA-DQB1 will be at least as polymorphic as DLA-DRB1, with DLA-DQA1 somewhat less polymorphic.

In this study, we have characterized DLA-DRB1, DQA1 and DQB1 alleles from dog populations sampled outside Europe, and we have been able to identify a number of new DLA class II alleles. Although some of these alleles appear to be limited to the populations studied, there are some cases where DLA class II haplotypes have already been identified in particular breeds collected in the UK or in gray wolf populations from Alaska (Kennedy, unpublished data). The lack of overlap of alleles between Alaskan Huskies and gray wolves may be due to the small numbers studied. However, Alaskan sled dog breeders are anxious to avoid interbreeding of sled dogs with wolves, as it can add some undesirable behavioral traits to the sled dogs (Happ, personal communication).

DLA-DRB1 alleles tend to occur in only one combination with DQA1 and DQB1 alleles. DLA-DQ alleles, especially DQA1 alleles, appear to occur in combination with multiple DLA-DRB1 types. This is similar to the situation found for HLA class II in human populations (12). This provides compelling evidence that the new DLA alleles we have found represent new polymorphisms, even

```
                                       HVR 1                         HVR 2          |-----HVR 3-----|
                   10         20         30         40         50         60         70         80       87
DQA1*00101      DHVAN YGINVYQSYG PSGQYTHEFD GDEEFYVDLE KKETVWRLPV FSTFRSFDPQ GALRNLAIIK QNLNIMTKRS NQTAATN
DQA1*00201      ----Y ---------- ---------- ---------- ---------- ----T----- --------T- ---------- -K-----
DQA1*00301      ----Y ---------- ---------- ---------- ---------- ----T----- -------RA- -----L--S- -------
DQA1*00401      ----Y ---------- ---------- ---------- ---------- ----T----- ---------- -----L---- -------
DQA1*00402      ----Y ---------- ---------- ---L------ ---------- ----T----- ---------- -----L---- -------
DQA1*005011     ----Y ---------- ----F----- ---------- ---------- ----T----- --------T- ---------- -K-----
DQA1*005012     ----Y ---------- ----F----- ---------- ---------- ----T----- --------T- ---------- -K-----
DQA1*00601      ----Y ---------- ---------- ---------- ---------- ---------- ---------- -----L---- -------
DQA1*00701      ----Y ---------- ---------- ---------- ---------- ----T----- --------T- ---------- -------
DQA1*00801      ----Y ---------- ---------- ---------- ---------- ----T----- -------RA- -----L---- -------
DQA1*00901      ----Y ---------- ----F----- ---------- ---------- ---------- ---------- ---------- -------
DQA1*01001      ----Y ---------- ---------- ---------- ---------- ---------- --------A- -----L--S- -------
DQA1*01101      ----Y ---------- ---------- ---------- ---------- ----T----- ---------- ---------- -K-----
DQA1*012011     ----Y ---------- ---------- ---------- ---------- ----A----- --------A- ---------- -------
DQA1*012012     ----Y ---------- ---------- ---------- ---------- ----A----- --------A- -----L---- -------
DQA1*01301      ----Y ---------- ---------- ---------- ---------- ---------- --------T- ---------- -K-----
DQA1*01401      ----Y ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
DQA1*01501      ----- ---------- ----F----- ---------- ---------- ---------- ---------- ---------- -------
DQA1*00101      DHVAN YGINVYQSYG PSGQYTHEFD GDEEFYVDLE KKETVWRLPV FSTFRSFDPQ GALRNLAIIK QNLNIMTKRS NQTAATN
```

Fig. 4. DLA-DQA1 amino acid alignment.

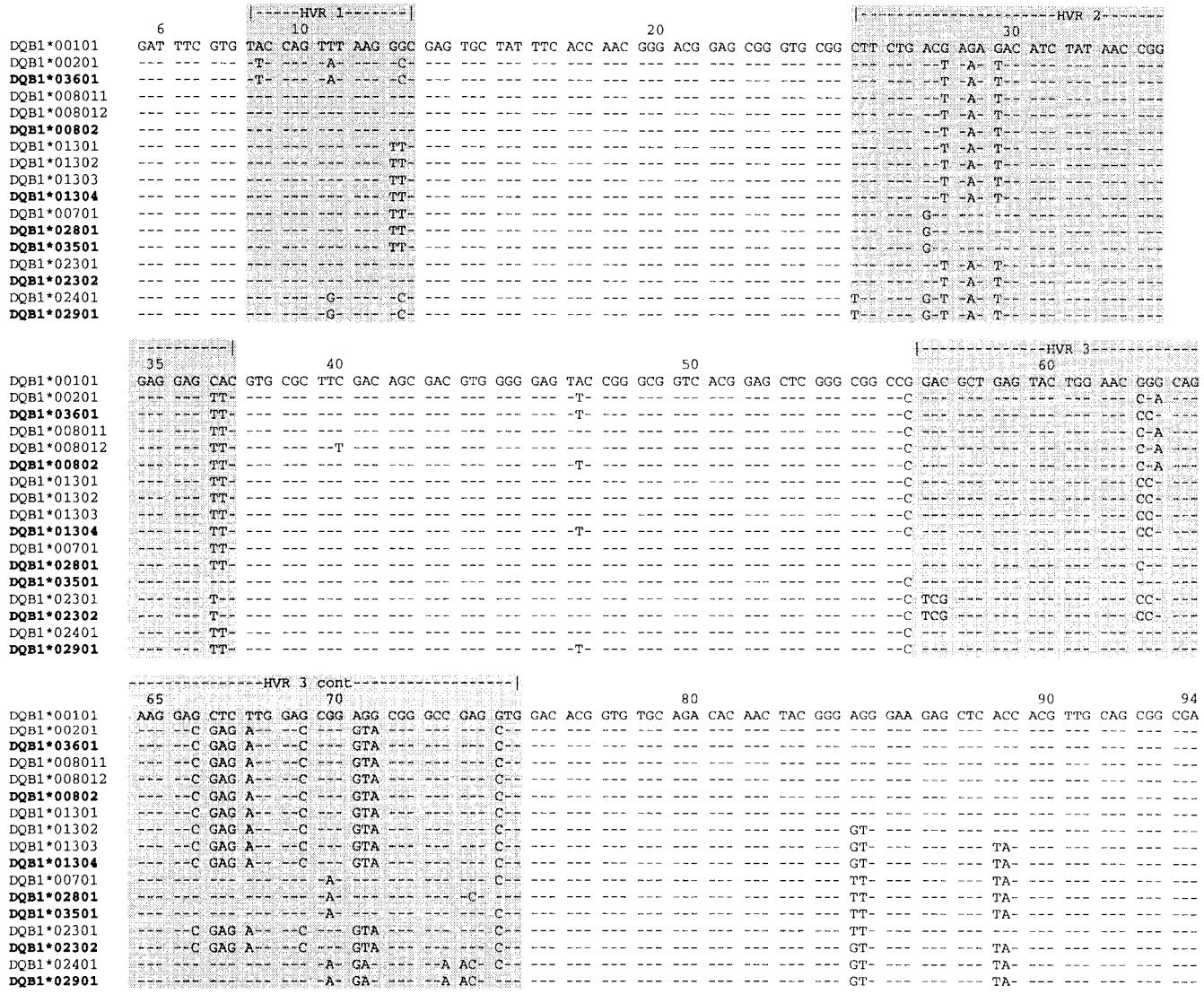

Fig. 5. DLA-DQB1 nucleotide alignment.

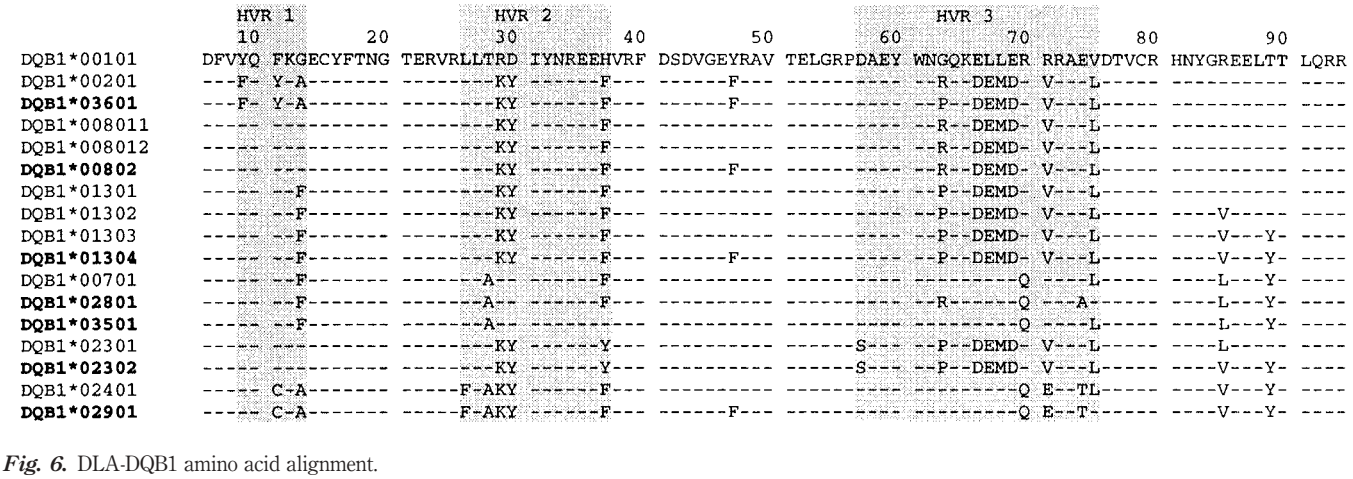

Fig. 6. DLA-DQB1 amino acid alignment.

New DLA alleles and haplotypes

DLA-DRB1*	DLA-DQA1*	DLA-DQB1*	Breed, numbers of homozygotes and heterozygotes found (*n*;*n*)
04001†	01001	–	Alaskan husky (1;3),
04601†	01501†	00802†	Papillon (1;2), Brazil mongrel (0;3)
04701†	00402†	–	Alaskan husky (0;3)
01504†	00601	–	Brazil mongrel (0;3)
01302†	00101	00201	Brazil mongrel (0;1)
01702†	00201	01304†	Brazil mongrel (0;4)
04801†	00101	00802†	Brazil mongrel (0;4)
05001†	00601	02302†	Brazil mongrel (0;2)
05101†	00201	02901†	Brazil mongrel (0;3)
05201†	012011†	03501†	Brazil mongrel (3;17)
03601	012011†	03501†	Alaskan gray wolf (1;2)
02501	012012†	03501†	Shih Tzu (0;3), Brazil mongrel (0;3)
01501	01401†	02601	Crossbreed (1;2), Brazil mongrel (0;3)
04501	01401†	03401	Alaskan gray wolf (0;2)
02401	01401†	00801	Akita (1;0)
01801	00101	00802†	Greyhound (2;0), Bearded Collie (1;0), other (0;5), Brazil mongrel (0;5)
00601	005011	02801†	Airedale Terrier (1;0), Brazil mongrel (0;1)
00101	00101	03601†	Brazil mongrel (0;2)

†new allele; - = DQB1 allele not yet identified

Table 3

when there is only one base pair difference from previously identified alleles.

It appears that the extent of polymorphism of the DLA genes will increase substantially as dogs from a wider geographic distribution are studied. Some DLA alleles and previously identified haplotypes appear to be breed specific, but as more dogs are studied from different geographic locations, these distinctions may not remain. Two of the DLA haplotypes observed in the Brazilian mongrel dogs were previously thought to be limited to single breeds (Papillon and Shih Tzu). These rare breeds may be subject to founder effects, and only by studying large groups of mongrels may we be able to dissect the origin of some haplotypes. It may be possible to use three locus haplotypes to start to identify the genetic relationship between breeds.

The observation that distinct three-locus DLA class II haplotypes exist in very different dog breeds from widely different geographic locations and origins, suggests that it is biologically advantageous for certain allele combinations to be inherited together and conserved as preferential allelic associations. There is already evidence that this occurs in different human populations (13), and it seems likely that similar effects exist in the dog.

The large interbreed variation of DLA class II allele frequencies has obvious implications for any disease association studies, where the control dogs will have to be breed matched to the cases.

The majority of DLA-DRB1, DQA1 and DQB1 alleles identified to date represent non-synonymous coding differences resulting in different proteins, with the majority of differences being in the three hypervariable regions (HVR) of the molecules. The HVR are biologically important regions of MHC molecules influencing peptide binding and presentation to the T cell receptors, suggesting that DLA class II polymorphisms must be driven and selected for by evolutionary fitness, survival and resistance to infection.

The domestic dog represents a very interesting situation where there has been massive selection by man for particular phenotypic breed characteristics, which will have resulted in founder effects and genetic bottlenecks within the different breeds. This interference by man will have resulted in the concentration of certain alleles and haplotypes in particular breeds. Some breeds show a much wider range of DLA alleles than other breeds (2, 3).

Many human diseases do not have good animal models, but it is becoming apparent that there are many canine diseases which may be true homologs of the equivalent human disorder (14). There are several factors that make the dog one of the best models to use for investigating genetic diseases. The relative phenotypic uniformity

within breeds and concomitant genetic homogeneity, coupled with the high level of interbreed diversity provide an ideal background for dissecting complex genetic traits.

In a recent report from the American Kennel Club on the major health issues in dogs, four out of the top 11 conditions had an immune component. These were hypothyroidism, autoimmune diseases (e.g., canine rheumatoid arthritis, SLE, diabetes), cancer and allergic dermatitis. These diseases represent important veterinary problems and also serve as interesting naturally occurring models for human disease. Immune diseases often have a genetic component which may be particularly important in purebred dogs with a restricted gene pool. This restriction is important because: (i) most infectious diseases have an immune component, and (ii) immune diseases can be triggered by exogenous influences such as drugs or vaccines. As in man, variation in the immune response to infection and vaccination is expected to be dependent on genetic background.

If these major canine health issues are to be addressed together with the role of the immune response, it is important to fully understand the genetic influence on the immune response in dogs. Central to this is the characterization of the MHC and the delineation of the extent of polymorphism of the genes in this region. In dogs, these regions are poorly characterized despite the fact that a greater understanding of these genes and how they operate is important for dissecting disease immunopathology. Characterization of the genes within the canine MHC will assist in the study of canine autoimmune diseases and variations in immune response to infection and vaccination. This could ultimately lead to a better understanding of the causes of these conditions and hence their treatment or even prevention via selective breeding programmes.

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