Institutionen för husdjursgenetik

A candidate gene approach to identify genes predisposing for the autoimmune disease canine lymphocytic thyroiditis (CLT)

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Supervisor and
Examiner:
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Examensarbete 276
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Examensarbete ingår som en obligatorisk del i utbildningen och syftar till att under handledning ge de studerande träning i att självständigt och på ett vetenskapligt sätt lösa en uppgift. Föreliggande uppsats är således ett elevarbete och dess innehåll, resultat och slutsatser bör bedömas mot denna bakgrund. Examensarbete på D-nivå i ämnet husdjursgenetik, 20 p (30 ECTS).

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Agrovoc: Canis familliaris, autoimmune disease, thyroiditis, genes
Övrigt: CTLA-4, DLA-DRB1

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Contents

Abbreviations

3’	3 prime
5’	5 prime
APC	Antigen presenting cell
B7	CD80/CD86 Cluster determinant
Bp	base pair
CD28	Cluster determinant
CLT	Canine lymphocytic thyroiditis
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DNA	deoxy-ribonucleic acid
DLA	Dog leukocyte antigen
HT	Hashimoto’s thyroiditis
IgG	immunoglobulin G
MHC	Major Histocompatibility complex
NCBI	National Center for bioinformatics
QTL	quantitative trait loci
SKC	Swedish Kennel Club
SLE	Systemic Lupus erythomatosus
SNP	Single nucleotide polymorphism
T4	Thyroxine
TCR	T cell receptor
TNF-α	tumour necrosis factor alpha
TPO	Thyroid peroxidase
TSH	Thyroid stimulating hormone
TgAA	Thyroglobulin auto-antibody
UCSC	University of California at Santa Cruz
UTR	Untranslated region
WICGR	Whitehead Institute/MIT Center for Genome Research

Abstract

The overall aim with this project is to apply a candidate gene approach to identify genes predisposing for the autoimmune disease, canine lymphocytic thyroiditis (CLT). Individuals clinically diagnosed either as CLT-affected or as healthy non-affected controls were analyzed from two birth cohorts of the breeds Giant Schnauzer and Hovawart, breeds that both has high incidence of CLT.

Two different genes were evaluated for their potential involvement in CLT disease aetiology. Selection of the candidate genes was based on their confirmed role in both human and mouse as genetic risk factors in thyroid autoimmune disease. Firstly, to evaluate whether certain *DLA-DRB1* Major Histocompatibility complex (*MHC*) class II exon 2 genotypes predispose for the development of CLT, cloning and sequencing of *DLA-DRB1* exon 2 PCR products were performed. Secondly, studies of the gene encoding cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) were performed using single nucleotide polymorphism (SNP) analysis and microsatellites. Nucleotide sequence analysis of the cloned *DLA-DRB1* alleles will allow us to evaluate whether certain *DRB1* alleles are predisposing for CLT [4]. A microsatellite analysis of *CTLA-4* strongly suggested that *CTLA-4* may be excluded as a gene predisposing for CLT in Giant schnauzer, with a chi-square value of 1.96 with three degrees of freedom. For a Chi square to be significant for three degrees of freedom it should be greater than or equal to 7.82. The P-value was 0.58, which indicates no statistical significance in the results. However, additional CLT-affected individuals and healthy controls must be analysed to obtain conclusive results.

Introduction

Autoimmune disease is common in the dog (*Canis familiaris*) resulting in substantial suffering for the affected dog and high veterinary costs. Some autoimmune diseases such as LT are common in both human and dogs, autoimmune diseases therefore constitute excellent comparative models for the corresponding human disease, in particular since human and dogs often share many environmental factors that affect multifactorial disorders [24].

Autoimmune disease

The results of defects in one or more components of the complex immune cascade that operates to establish tolerance to self-antigens and that is required for the defence against pathogenic foreign antigens from the body may lead to autoimmune diseases. Autoimmunity is characterized by immune reactions directed towards self-antigens and may be associated with failure to establish tolerance during thymic education of T lymphocytes or by breaking already established tolerance. The outcome of this process is an immune response directed towards self antigens and a resulting attack of one or more organs by the immune system [5]. The immune system recognizes self-antigens during the development of the immune system in the thymus. This thymic education is a complex process that is only partly understood. It involves the removal of T cells by negative selection through programmed cell death of T cells that express T cell receptors with specificity for self antigens. T cells with intermediate and low affinity to self antigens are positively selected to survive to ensure a sufficient capacity of the individual to mount immune responses to foreign antigens. This process establishes tolerance. Disturbances in these complex pathways could result in the presence of auto-reactive T cells with affinity for self-antigens in the periphery. The establishment of central tolerance occurs in the thymus and thus, involves both positive and negative selection of T cells [18].

Autoimmune disease is common in many species and cells from many different organs can be affected in some of these diseases i.e. systemic autoimmune disease such as systemic lupus erythematosus (SLE) or a specific cell type in diseases such as autoimmune thyroiditis or autoimmune type 1 diabetes [18]. The aetiology of autoimmune disorders is largely unknown but is thought to be the result from complex interactions between genetic and environmental factors. Some environmental factors that trigger these diseases have been defined and include body stress such as diet and infections. The contribution of environmental factors is only poorly understood and little evidence for their involvement in disease is found. The genetic factors in autoimmune disease have instead strong evidence with increased concordance rates seen in monozygotic twins in comparison with dizygotic twins, also individual diseases cluster within families. Another indication of genetic influence is that multiple autoimmune diseases also cluster within families which strongly suggest that individuals predisposed to a particular autoimmune disease share a common genetic background. The predisposal to disease may be the outcome of a combination of both general and specific genes [5, 18].

Genetic association studies in human populations have convincingly shown two gene regions to be strongly associated with autoimmunity in general and those are particular haplotypes of the Major Histocompatibility complex (*MHC*) class II, in humans called human leukocyte antigen (*HLA*), and the gene encoding cytotoxic T-lymphocyte antigen 4 (*CTLA-4*), which is a regulatory gene in the immune system. These loci encode molecules that are important in the immune system and they are actively involved in antigen presentation and T-cell activation [5]. Other immune and immune regulatory genes are also probably involved.

Hashimoto's thyroiditis

Hashimoto's thyroiditis (HT) is one of the most common human autoimmune diseases. It is an organ-specific T cell-mediated disease that affects the thyroid gland and genetics play a contributory role in its complexity. The disease HT is defined by the dramatic loss of thyroid follicular cells, hypothyroidism, goiter and circulation of autoantibodies against two primary antigens, thyroglobulin (Tg) and thyroid peroxidase (TPO).

A central phase of HT is characterized by the recognition of presented auto-antigens by T lymphocytes, followed by a consistent uncontrolled production of auto-reactive T cells and immunoglobulin G (IgG) autoantibodies. Autoimmune responses against thyroid-specific antigens are primary determinants in thyroid autoimmunity. Thyroglobulin (Tg) is the main protein synthesized in the thyroid gland and serves both in the synthesis and in the storage of thyroid hormones. Tg is one of the major auto-antigens in the thyroid and Tg-antibodies are detected in almost all patients with HT [7]. Thyroid peroxidase (TPO) is another significant autoantigen in patients affected with HT. TPO is an enzyme that catalyses the oxidation of iodine that forms iodotyrosines in a Tg molecule. [4] As any disease of a regulatory system the problem could lie in the signalling pathway, signal production, signal transmission, signal reception or the effectors response downstream. Some examples are genes involved in apoptosis such as "death ligands", Fas and tumour necrosis factor alpha (TNF-α), important immune-regulatory proteins, cytokines, and many other genes crucial for a functional immune response [7].

Canine Lymphocytic thyroiditis

Autoimmune canine lymphocytic thyroiditis (CLT) is a complex disease caused by unknown predisposing genetic and environmental factors. CLT is considered by veterinary clinicians to be analogous to the human disease, Hashimoto's disease, and has a common aetiology with hypothyroidism. However, future clinical, physiological and immunological studies are required to formally establish that these diseases are homologous. Most likely, HT has a more diverse aetiology compared with CLT. The CLT disease is characterized by insufficient production of thyroxine (T4) in the thyroid gland. Thyroid stimulating hormone (TSH) stimulates the secretion of T4. The T4 then work with a negative feedback on the pituitary gland and down-regulate TSH production. If T4 is not produced because of hypothyroidism, the TSH levels do not decrease as in a normal healthy individual.

CLT is one of the most common endochrinopathies in dogs affecting several purebred breeds [14]. The disease is most common in dogs between the age of 4 and 10 years and certain breeds seem to be predisposed for the disease, such as the breeds Hovawart and Giant schnauzer both having approximately 13% incidence proportion in the Swedish populations [11].

Autoimmune thyroid diseases are characterized by circulating auto-antibodies to antigens expressed by the thyroid gland, activated auto-reactive T cells and lymphocytic infiltration of the thyroid gland. In canine lymphocytic thyroiditis there are high concentrations of TSH and detectable amounts of circulating autoantibodies. In dog the principal circulating autoantibodies are directed against thyroglobulin and are denoted TgAA [25].

Dog as an model organism for autoimmune disease

The complete dog genome nucleotide sequence is now available [37]. The sequencing effort was performed by the Broad Institute at MIT and Harvard University; formerly the Whitehead Institute/MIT Center for Genome Research (WICGR), a female boxer called Tasha was selected as the individual for sequencing because Boxer is one of the breeds with the least variation in its genome. Boxer has very low haplotype polymorphism and the nucleotide sequences generated are more efficiently annotated. This makes it easier to produce a correct genomic nucleotide sequence.

The unique breeding history of the domestic dog provides an unparalleled opportunity to explore the genetic basis of disease susceptibility, morphological variation and behavioral traits. The position of the dog within the mammalian evolutionary tree also makes the dog genome sequence an important resource for comparative analysis of the human genome. Dogs evolved through a mutually beneficial relationship with humans, sharing living space and food sources [21]. Canine population genetics will be used as a tool to identify quantitative trait loci (QTL) and identify the genes underlying important complex traits. Purebred dogs are providing information about morphology, behavior and complex diseases, both of themselves and humans, by supplying tractable populations in which responsible genes can be mapped. The diversification of dog breeds has led to the development of breeds enriched for particular genetic disorders. Nearly half of genetic diseases reported in dogs occur predominantly or exclusively in one or a few breeds [29]. The high prevalence of specific diseases within certain breeds suggests that a limited number of loci underlie each disease, making their genetic dissection potentially more tractable in dogs than in humans. This offers an enormous advantage in the search for genes associated with complex diseases, which, in theory, can be more easily mapped using dog families than human families. [29]

Cytotoxic T-lymphocyte antigen 4 (*CTLA-4*)

The cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) gene encodes a protein that negatively regulates T cells [5]. Mutations in the *CTLA-4* gene have been documented as contributing to the development of several autoimmune diseases. Thus, the *CTLA-4* gene is a major autoimmune disease risk factor in general and variations in the gene also play a significant role in determining susceptibility to autoimmune thyroid disease in various species [1-3, 5-10, 15, 21- 23, 27, 30- 31].

The canine *CTLA-4* gene is located on dog Chromosome 37 (Cfa 37) and spans approximately 6.1 kbp and contains four short exons.

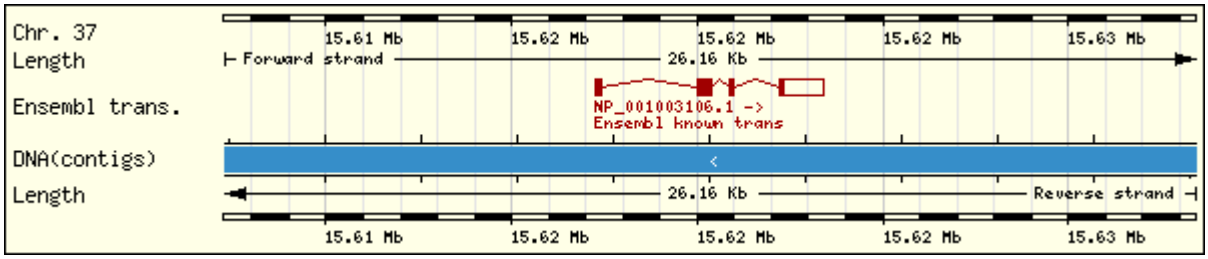

Figure 1. The *CTLA-4* gene in dog. 4 exons. Picture adapted from reference [38].

T cells are activated when the first signal is provided by the interaction of the T cell receptor (TCR) on the lymphocyte with major histocompatibility class (MHC) antigens on the antigen-presenting cell (APC). The second, co-stimulatory, signal is required to avoid an apoptotic or anergic response by the lymphocyte. The interaction of CD28 on the lymphocyte with B7 proteins on the APC provides this necessary co-stimulatory second activation signal.

The co-stimulatory molecule that the *CTLA-4* gene encodes suppresses T cell-mediated immune response and is crucial in the maintenance of self-tolerance. The gene was therefore early recognized as a good candidate gene for autoimmune thyroid disease because of its importance in T cell regulation. CTLA-4 is a receptor, homologous to CD28, but with opposite inhibitory function. The gene belongs to the same family of cell-surface molecules as CD28 and like CD28 it binds to B7. The CTLA-4/ B7 complex competes with the CD28/ B7 complex and delivers negative signals to the T cell and effects T cell expansion, cytokine production and immune response [1].

The B7–CD28/CTLA-4 pathway consists of two B7 family members, B7.1 and B7.2, which bind to the same two receptors, CD28 and CTLA-4. These two receptors have different affinities for B7.1 and B7.2; CD28 is constitutively expressed on the surface of T cells whereas CTLA-4 expression is rapidly up-regulated following T cell activation and has higher affinity for the B7 receptors. The outcome of an immune response involves a balance between CD28-mediated T cell activation and CTLA-4-mediated inhibition [12].

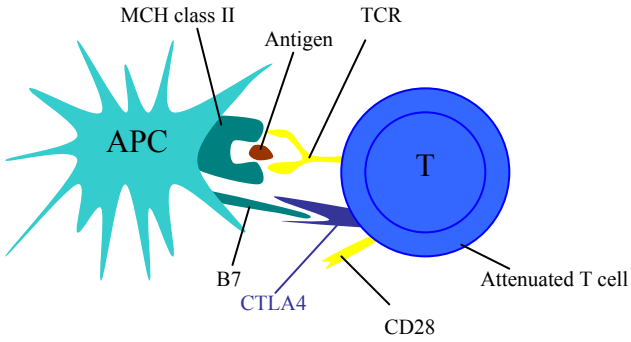

Figure 2. CTLA-4 binds to the B7 receptor and down regulates T cell response.

The importance of CTLA-4 in the down regulation of T-cell response and the induction of anergy and tolerance to alloantigens, tumour antigens and pathogens, has been clearly demonstrated in experiments with *CTLA*-4-deficient mice [33]. The exact mechanism how CTLA-4 maintains breakdown of self-tolerance that subsequently leads to the initiation of autoimmune responses has also been demonstrated in murine models of autoimmune diabetes and thyroiditis [7]. The mechanism by which *CTLA*-4 down-modulates T cell responses is not yet clearly defined but several mechanisms have been suggested. CTLA-4 might successfully compete with CD28 for its B7 ligands and thereby inhibit the co-stimulatory effect of CD28. Alternatively, CTLA-4 might apply its inhibitory effect by acting on downstream signaling pathways at activation [5]. Any or all of these hypothesized mechanisms involving *CTLA*-4 could contribute to the development of autoimmunity.

The human *CTLA-4* gene is known to contain genetic polymorphism in three regions; a single base substitution in the promoter, a dimorphism in exon 1 and an multi-allelic di-nucleotide repeat in the 3'-UTR of exon 4 [23]. After studies of *CTLA-4* it was suggested that polymorphisms within the gene are associated with the development in Hashimoto's disease [8]. Further functional studies of CTLA-*4* are required to obtain definitive answers as to how it affects the autoimmune disease process and if other molecules with similar function as negative regulators of T-cell activation might also play a role [5].

DLA-DRB1

The canine *MHC* class II region is located on dog chromosome 12 (Cfa 12). The *MHC* is as mentioned above a genetic region that encodes several class II molecules that are strongly associated with multiple autoimmune disorders in both dog, human and mouse. The *MHC* genotype is thus far the strongest genetic risk factor for the development of autoimmune disease in both human and mouse.

All higher animal species examined so far have within their genome a *MHC*, a region of tightly linked genes largely responsible for the presentation of self and non-self antigens T cells of the immune system. The dog *MHC* is referred to as the dog leukocyte antigen (DLA) system [17]. In the present study we have analyzed one class II gene denoted *DLA-DRB1*. In the dog, unlike the human class II region, which exhibits haplotype polymorphism in addition to allelic polymorphism, a single *DRB* locus with extensive allelic polymorphism is present in most if not all haplotypes [32]. The fact that the *DRB1* locus in all mammals are in strong linkage disequilibrium with other polymorphic class II genes e.g. *DQB1*, allows investigators to deduce that individuals expressing a particular *DRB1* allele also carries a particular *DQB1* allele on the same chromosome.

The association of *MHC* genotype with autoimmune disease is quite expected because autoimmune responses involve T cells and the ability of T cells to react with specific antigens that are presented in the context of *MHC* class II molecules. Allelic variants of *MHC* class II molecules determine differences in the ability to present auto-antigens to auto-reactive T cells. [17].

Alleles and haplotypes of the *MHC* class II regions have been consistently shown to confer either predisposition or protection to many autoimmune diseases. In particular, the human *HLA-DQB1* locus but also the *DRB1* locus has been shown to predispose to autoimmune disease. Both *DRB1* and *DQB1* loci are highly polymorphic with more than 400 human *DRB1* alleles and some 60 *DQB1* alleles reported [40]. Also in dogs, both these loci are polymorphic and currently 52 *DLA-DRB1* alleles have been identified [40]. (See **Appendix 1** with all the 52 different alleles of *DLA-DRB1* Figure adapted from reference [40]. The region shown is exon 2 which is highly polymorphic and is known to encode a domain that is responsible for antigen presentation. There are three hypervariable regions that fold into the peptide-binding groove).

The DRB1 peptide-binding domain appears to play a key role in susceptibility to various autoimmune diseases. However, MHC class II molecules alone are insufficient to initiate a T-cell response to antigen, and the presence of co-stimulatory molecules such as CD28 and CTLA-4 (see discussion above) is a requirement for positive and negative regulation of T-cell proliferation, respectively [5].

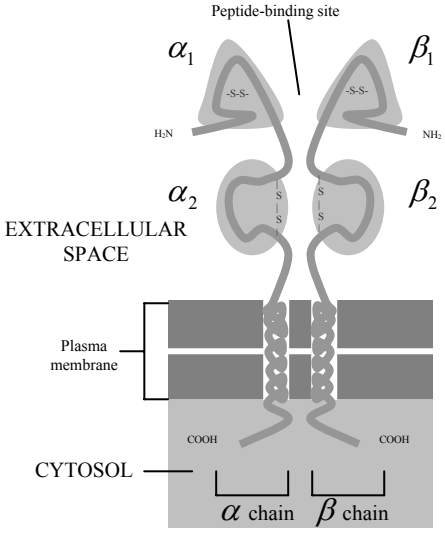

Figure 3. Schematic structure of a *MHC* class II molecule.

Microsatellites

Microsatellites are defined as simple sequence repeats with repeat length up to five bases. The most common classes used in genotyping are di-, tri- and tetra nucleotide repeats, witch occurs at a rate of about one every 10 kb in eukaryotic genomes [11]. Microsatellites may arise by different mechanisms and probably the most common is replication slippage. Once established, unequal replication between repeats can generate stepwise changes in repeat number. Microsatellites have a much higher mutation rate than non-repetitive sequences and they are therefore extremely useful in estimating evolutionary relationship between populations within species, but generally evolve too rapidly to be phylogenetically informative between species. The simple sequence repeats are important in genetic studies both as markers and for pedigree analysis. The repeats usually occur in non-coding part of the genome, and their number is highly variable [11].

Characterization of PCR product

The PCR products obtained was ligated into the TOPO TA cloning vector (TOPO TA cloning kit, Invitrogen^{TM}). The cloning strategy is for direct insertion of Taq Polymerase-amplified PCR products into a plasmid vector. The vector is supplied linear with a single 3´-Thymidin (T) overhang and topoisomerase covalently bound to the vector, which makes an "active vector". Taq polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´-ends of PCR products. The linearized vector supplied with in the kits has a single, overhanging 3´-deoxythymidine (T) residue. This allows PCR inserts to ligate efficiently with the vector. The recombinant vector is then transformed chemically into competent cells of *E.coli*.

The TOPO TA vector contains the gene *LacZα* gene which, on plates containing X-gal, produces a blue substrate. When vectors contain PCR fragments the *LacZα* gene is destroyed and no blue substrate is produced [15].

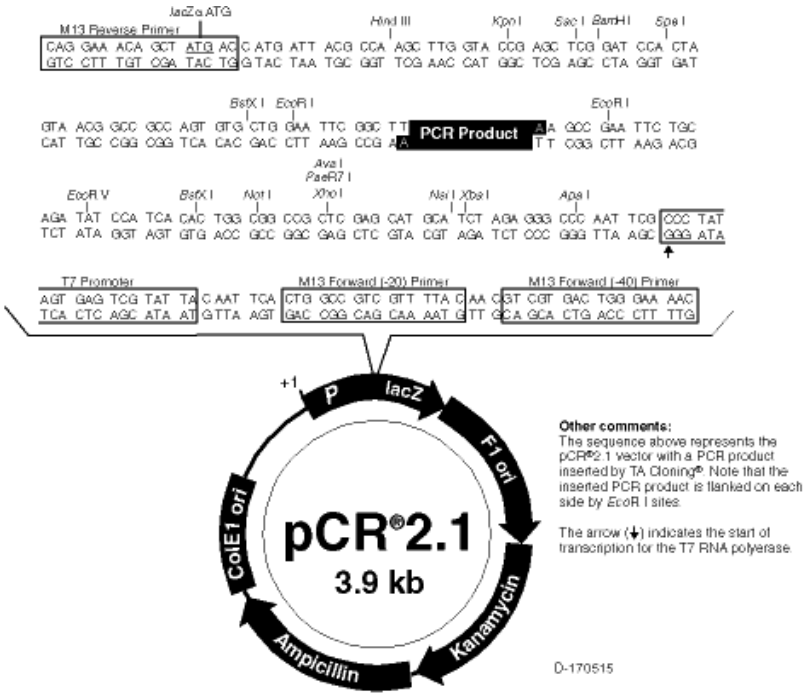

Figure 4. Map of pCR®2.1-TOPO® Vector 3.9 kb. *LacZα* gene, restriction- and Primer sites. Picture adapted from reference [15].

Material and methods

Bioinformatics studies

The sequence from *CTLA-4* gene was retrieved from NCBI and Ensembl genome browser [41, 38]. To ensure that the promoter sequence of *CTLA-4* was included in the analysis a region spanning 1 kb 5' of the defined transcriptional start site was chosen to design of the PCR primers. Promoters can be analyzed in the program found on rVista 2.0 [43]. To find SNPs a database called Dog SNPs from Broad Institute was used [37].

The microsatellite used was a tetra nucleotide repeat found on the dog UCSC genome browser [45] and verified manually in the genome sequence found on Ensembl genome browser [38].

Study material

Samples from the two high-risk breeds defined above are available (Giant Schnauzer and Hovawart) indicated by epidemiological data accessed and evaluated from databases maintained by Agria Insurance company. The clinical diagnostic procedures involve measurement of thyroxine (T4), thyroid stimulating hormone (TSH) and autoantibodies against thyroglobulin (TgAA) in dogs suspected of suffering from CLT. TSH levels above 40mlU/L and TgAA antibody concentration are used as inclusion criteria. The concentration of TgAA is determined by arbitrary ELISA units and dogs with TgAA above 200% of the negative control are considered to be CLT-positive [25]. Case selection is made through cooperation with veterinary clinics by screening for inclusion criteria mentioned before. Control selection is done by access of the study populations recorded in registries at the Swedish Kennel Club (SKC) and Agria insurance company using same criteria for exclusion. The validation of control status is done by veterinary examination and owner questionnaires. The *DLA-DRB1* sequence is investigated in samples from animals in the two birth cohorts [4]. The *CTLA-4* gene is examined in cases and controls from Giant schnauzer.

Extraction of genomic DNA
Genomic DNA was extracted from 200µl of heparinised blood with modified standard procedures. Concentration and quality of the DNA was measured in NanoDrop® ND-1000 spectophotometer 3.1.0 (Saveen Werner).

PCR amplification
All primers for *CTLA-4* were designed using the program Primer 3 [42]. The primers used to amplify and sequence the gene and their location can be viewed in **Appendix 2**. Primers were ordered from the company TAG Copenhagen A/S [44] and all PCR reactions were done using Applied Biosystem 2720 Thermal Cycler. PCR program for *CTLA-4* sequences and the microsatellite amplification was optimized using different PCR programs, annealing temperatures and Elongation time. All PCR products were separated on 2% agarose gel to verify correctly sized amplified products.

***CTLA-4* Promoter and exon 1 PCR amplification**
All PCR fragments containing the *CTLA-4* promoter sequence and exon 1 were amplified with the forward and reverse primer, called primer-pair 1, shown in Table 1 and the product size from the Primers is 1943 base pairs long.

Table 1. Primers for amplification of *CTLA-4* promoter region and exon1.

Forward	5'-GCC CGT ATT CCA CAG AGT GT-3'
Reverse	5'-TCT GAA ACC TGG GGA ATC TG-3'

The PCR reaction included, for one 50µl reaction, 1* AmpliTaq Gold buffer containing 1.5 mM $MgCl_2$. 0.2µM of forward and reverse primer, 0.2 mM dNTP and 2U AmpliTaq Gold™ Taq polymerase and 100ng of genomic DNA. The PCR program used included an initial denaturation step at 94ºC for 5 min followed by 40 cycles of amplification, denaturation at 94 ºC for 40 seconds, annealing at 55 º in 40 seconds and extension at 72 ºC in 3 min, the program the contains a final extension step at 72 ºC in 5 min.

To be able to sequence this product internal sequencing primers were designed (Table 2). The sequence product from these primers is 888 bp.

Table 2. Sequencing primers for the *CTLA-4* promoter and exon 1 region.

Forward	5'- AAA GCT GTC ATG GGT CAA GG-3'
Reverse	5'- TTG GCT TCT GGC TTG GTT AT -3'

***CTLA-4* exon 2 PCR amplification**
Primers used to amplify exon 2 were forward and reverse primer, called primer-par 2, found in Table 3. These primers give a product of 2315 bp.

Table 3. Forward and reverse primer for amplification of *CTLA-4* exon 2.

Forward	5'-AGG CAT TGA CGA GGA GCT TA-3'
Reverse	5'- CCA GGC TCA AGC AAA ATC TC-3'

PCR amplification of *CTLA-4* Microsatellites
On the forward primer an M13 tail was added to be able to fluorescently mark the PCR product. The microsatellites were amplified with the forward and the reverse primer found in Table 4. Each PCR reaction was 12.5µl and contained 1* AmpliTaq Gold buffer containing 1.5 mM $MgCl_2$. 0.02 µM of forward primer, 0.2 µM TET and 0.2µM of reverse primer, 0.2mM dNTP, 0.5U AmpliTaq Gold™ Taq polymerase and 12.5ng of genomic DNA.

To amplify the DNA fragment a touch down program was used that included an initial denaturation step at 94ºC for 5 min followed by 14 cycles of 94º C for 30s. Annealing with touchdown from 61ºC- 47ºC 30s dropping one degree ºC each cycle, and finallyan extension step in 72ºC for 30s. After the 14 cycles a second cycle starts with 35 cycles of 94º C for 30s, the annealing with 52ºC 30s and then a extension step in 72ºC. The program is terminated with a final extension step at 72 ºC for 15 min.

Table 4. The primers for amplification of the *CTLA-4* microsatellite. On the forward primer an M13-tail of 19 nucleotides has been added.

Forward	5 '- CAC GAC GTT GTA AAA CGA CAA TAA TGC CTG GGA ATG TGG-3'
Reverse	5 '- ATG GTA ACA GGG TGC CTT CC-3 '

The length of the microsatellite found in the dog genome database was 257 bp.

gtgaagaagggagagtggcggaagaggtggtggcggtggcaggggaagcccacagaagtt
agcagcagggttgcctcagcctacagaggaaacgacctggtacccccctgctctgtggctt
ccttcatttatcagcatccctcccccctgataataatgcctgggaatgtggcagctggcac
aatgatccagggttcagccactgtggctgataggggtacagggccaaggaaaatgtaggc
agaggtttgtgaggtatgcattgaggggtaaggcaagattctatacttcagcctctaaaa
tttcccttactactctttttaaattttatttatttatttatttatttatttattta
tttattttttcccctactactctttatagttcctgtaattctatgataaccctagaatac
cagaggtggaaggcaccctgttaccatcaaaccctccttactgtgtgtgagggaaaatga
aggttacaggggatgcatgacttgccccccaaatcacatatttcatgggagggtcaggcct
tcagtttgtcacatcagtgttctttctgctataggaaactcttgtccaataagaaaacgc
cttttggagtttagctggaagatagccaagagattgaggggacagatgcggggagagga

Figure 5. Microsatellite TTTA and location of primers.

M13-tail
M13-tail is added 5' on the forward primer witch is used for labelling of the PCR products. The fluorescently labelled universal M13 primer technique was developed to decrease the cost of labelling. M13-tails are available in different colours; the different dyes may be 6-carboxy-fluorescine (FAM) or tetrachloro-6-carboxy-fluorescine TET, blue respectively green. [28].

MegaBace
M13 labelled PCR products are then analyzed in a MegaBACE 1000 (Amersham Biosciences). The MegaBACE is a capillary instrument which separates the products in capillaries containing poly-acrylamide-gel. Samples are electro-kinetically injected and separated. Standard curve spans from 60bp to 400bp the PCR products containing microsatellites may therefore have a size between 80 bp and 350bp. The results are then viewed using a program called Genetic Profiler 2.2 (Amersham Biosciences).

DLA-DRB1 exon 2 PCR amplification

PCR primers used, to amplify the *DLA-DRB1* exon 2 sequences, were already designed [4]. Forward and reverse primers used are found in table 5. All PCR reactions to amplify *DLA-DRB1* exon 2 was 50µl reactions each containing 1* AmpliTaq Gold buffer, 1.5 mM $MgCl_2$. 0.1µM of forward and reverse primer, 0.1 mM dNTP, 1U AmpliTaq Gold ^{TM} Taq polymerase and 100ng of genomic DNA.

The PCR program for amplification contained an initial denaturation step at 94º C in 5 min followed by 40 cycles of 94 ºC in 40 s 64 º in 40 s, 72 º C in 1 min, and then a conclusive extension step in 72 º in 5 min.

Table 5. PCR primers used to amplify *DLA-DRB1* sequence. The product from these primers is 269 bp.

Forward	5' –GAT CCC CCC GTC CCC ACA G-3'
Reverse	5'-TGT GTC ACA CAC CTC AGC ACC A-3'

PCR purification

PCR purification was performed before sequencing and cloning using commercial kits. Kits for gel purification (E.Z.N.A.) and PCR purification kit (Quiagen) was used.

Cloning of *DLA-DRB1* PCR fragments

All cloning was performed with modified commercial kit. (TOPO TA cloning kit, Invitrogen^{TM}). Samples were cloned into TOPO TA plasmid vector and made on plates with ampicillin and X-gal following a modified protocol from the TOPO TA Cloning Kit [14]. The clones were purified with a QIAprep Spin Miniprep Kit (Quiagen). DNA sequencing was performed from at least three separate plasmid clones from each cloning experiment with the T7 forward primer and the M13 reverse primer.

Plasmid PCR

To control if the clones contained the *DLA-DRB1* sequence some were tested in a plasmid PCR. Using colonies cultured in water and the same PCR program for the amplification of the *DLA-DRB1* segment only with 20µl reactions instead of 50µl. PCR products was the analyzed on a 2% agarose gel. Positive clones were then sequenced.

DNA Sequencing

For PCR products a concentration of about 10ng/100bp is needed for sequencing.

For plasmid DNA a concentration of 150ng/reaction was used.

The purified PCR products were run in a 10µl sequence reaction together with 0.5µM of primer and 4µl of Sequencing Reagent Premix chemistry (DYEnamic TM ET Dye Terminator Cycle Sequencing Kit (MegaBACE, Amersham Biosciences)). The sequencing profile included 40 cycles of amplification (denaturation for 20s at 96°C, annealing at 50C° for 15s and extension for 1 min and 30s at 60°C). After the sequencing reaction the samples were precipitated using a modified Ethanol Precipitation Protocol (Amersham Biosciences) and finally diluted in 10µl loading Solution for MegaBACE ^{TM} 1000.

Analyzing sequences
Sequences from *CTLA-4*, promoter region and exon 1, and *DLA-DRB1*, exon 2, were analyzed using Sequencher 3.1.1.

Results & Discussion

The primers denoted *CTLA-4* primer-pair 1 amplified the sequence in the optimized PCR program. Because of the length of the amplified sequence; high PCR product concentrations were needed. I was unable to obtain this concentration; even after 40 cycles of amplification the concentrations was to low. Sequencing using the MegaBACE requires approximate concentrations of 10ng/100bp. Therefore, sequencing primers were designed for this matter but not even this resolved the problem. To avoid these problems, PCR products can be purified using the kit MiniElute™ (Qiagen) which is supposed to give higher concentrations. If this doesn't give sufficient concentrations new primers need to be designed to amplify shorter sequences. When the sequence is available, cases and controls can be compared to find SNPs. The promoter sequence can be analyzed in the database rVista 2.0 [43] to determine whether conserved regions have any mutations that could affect the gene expression or function.

The second primer pair used to amplify CTLA-4 exon 2 did not amplify the sequence. I performed different approaches to solve this problem without obtaining successful results. I tried different annealing temperatures in the PCR, touch-down PCR, betaine PCR and genomic DNA from different breeds; Giant Schnauzer, Hovawart, Drever and Boxer, the latter is the breed from which the genomic nucleotide sequence present in the database was derived and thus used for primer design. The lack of successful PCR amplification may therefore be caused by sequencing artefacts of the sequence found in the database. The difficulties in amplification could also depend on the size of the sequence selected for amplification. To solve the problem, additional primers should be designed.

Because of the sequencing problems with both primer pairs, I decided to perform a microsatellite approach. A microsatellite placed immediately upstream of exon 2 was examined with MegaBACE and genetic profiler. The results from Giant Schnauzer gave four different alleles for this marker. I also investigated cases and controls of Hovawart with this microsatellite but because of the close relationship between individuals in this breed the marker was not informative. There were four alleles present with one of these alleles represented in almost all the Hovawart individuals tested. Therefore, I preceded the study with only Giant Schnauzer because of their high allelic variability.

Cases and controls from Giant Schnauzer were analyzed in a program called Conting version 2.71. [35]

Table 6. List of the cases and the alleles analyzed with microsatellites and the program Conting 2.71 [35].

Cases	Allele 1	Allele 2
2.45.48	312	312
2.45.80	316	316
2.45.99	312	312
2.45.293	296	296
2.45.289	312	312
2.45.292	296	296
2.45.164	296	296
2.45.39	296	296
2.45.250	312	316
2.45.218	296	296
2.45.298	312	312
2.45.133	312	312
2.45.32	296	296
2.45.225	312	312
2.45.26	296	296
2.45.150	312	316
2.45.206	296	296
2.45.232	296	312

Table 7. List of the controls and the alleles that were analyzed with microsatellites and the program Contig 2.71 [35].

Controls	Allele 1	Allele 2
2.45.49	312	312
2.45.68	296	296
2.45.30	296	296
2.45.297	312	316
2.45.296	312	316
2.45.285	296	296
2.45.60	312	312
2.45.295	312	312
2.45.51	312	312
2.45.290	312	324
2.45.95	312	312
2.45.288	296	296
2.45.286	296	296
2.45.23	296	312
2.45.43	316	316
2.45.17	312	312
2.45.18	316	316
2.45.27	296	296
2.45.30	296	312
2.45.54	312	312

After the program calculated a chi square for three degrees of freedom and the analysis gave a result of 1.96. For a Chi square to be significant for three degrees of freedom it should be greater than or equal to 7.82. The P-value produced was 0.58 and the distribution is statistically significant when the P-value is less than 0.05. The definition of P-value is the probability of obtaining a result as extreme as the observed one, if there is truly no effect [11]. The Chi square and P-values obtained in this analysis show no significance between the alleles. To obtain conclusive results additional animals needs to be analyzed.

Future studies of the potential involvement of the *CTLA-4* gene are to investigate whether the 3'-UTR has mutations associated with CLT. In Hashimoto's thyroiditis a mutation in the 3'-UTR has been associated with the disease [5, 12, and 23].

The cloning of exon 2 PCR products of *DLA-DRB1* was performed using different approaches due to difficulties to exclusively isolate the correct exon 2 PCR product. In several cases, additional fragments were obtained. The nature of these fragments has not been evaluated. The PCR conditions were stringent according to already published procedures. However, the obtained results suggest that the PCR primers have the capacity to amplify additional products from other templates in the dog genome. Characterization by PCR of the isolated recombinant plasmids, however, allowed us to exclude clones that contained other sequences than *DRB1*. Another complication associated with cloning of the PCR products are artefacts due to errors introduced by Taq polymerase in the PCR product used in cloning. In order to avoid these artefacts, characterization of PCR products from multiple PCR reactions and at least three different clones are required to obtain conclusive results. The initial problems associated with false-positive clones and problems with the T7 forward primer used for nucleotide sequencing were solved after extensive experimentation. Allele investigation of the obtained *DLA-DRB1* sequences was performed in collaboration with Susanne Björnerfeldt. The complement of my clones to previous result was satisfying. My cloning results made it possible to remove eight wrongly characterized alleles and addition of one new allele to the study. All alleles currently identified in this study from Giant Schnauzer and Hovawart can be viewed in **Appendix 3**.

The current characterization of the complexity of *DLA-DRB1* allelic polymorphism in Giant Schnauzer and Hovawart has allowed us to conclude that these populations have at least 13 and 6 different *DRB1* alleles, respectively. An allele of *DRB1* denoted #5 is suggested as a potential risk factor predisposing for CLT. Ongoing studies will evaluate this hypothesis with further sequencing of *DRB1* alleles and a variety of statistical analysis. A study where susceptibility epitopes will be evaluated will also be performed based on the *DRB1* sequences in these breeds. Certain *DRB1* epitopes are known to be associated with the autoimmune disease, Rheumatoid arthritis [26]. Such alleles can be found in Hovawart and Giant Schnauzer. Their potential involvement as risk factors in CLT will be assessed.

In future *DRB1* studies of these populations aimed at defining individual *MHC* class II genotypes we will employ a high-throughput system developed by Kennedy and co-workers [19]. This new method has been developed to efficiently deduce the *DLA-DRB1* genotype. This article describes a method that uses reference strand-mediated conformational analysis for a high-resolution characterization of the locus [19]. In addition to defining the *MHC* class II genotype in these CLT-affected populations, a genome-wide association mapping study will be performed using a SNP-based array platform with 20.000 SNPs. To perform this genome-wide association analysis, more CLT cases and healthy controls are required. A genome scan requires at least samples from 100 cases and 100 controls to obtain significant power in the study. Currently, we have collected a total of 157 samples, among these samples, 36 are CLT-positive and 74 are CLT-negative [11].

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Appendix 1.

```
DLA-DRB1*00101    CA CAT TTC TTG GAG GTG GCA AAG TCC GAG TGC TAT TTC ACC AAC GGG ACG GAG CGG GTG CGG TTC GTG GAA AGA
DLA-DRB1*00102    -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00201    -- --- --- --- --- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- -CG ---
DLA-DRB1*00202    -- --- --- --- --- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- -CG ---
DLA-DRB1*00301    -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00401    -- --- --- G-- T-C CA- TTT --- C-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00501    -- --- --- --- --- A-- TT- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00601    -- --- --- --- --- --- --- --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00701    -- --- --- --- --- --- --- --- --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00801    -- --- --- G-- A-- A-- TAT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- ATG ---
DLA-DRB1*00802    -- --- --- G-- A-- A-- TAT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- ATG ---
DLA-DRB1*00901    -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*010011    ** *** *** G-- T-C CA- TTT --- C-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*010012    ** *** *** G-- T-C CA- TTT --- C-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01101    -- --- --- G-- A-- A-- TTT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- C-T C-- -CG ---
DLA-DRB1*01201    -- --- --- G-- AG- A-- TAT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --T C-- -CG ---
DLA-DRB1*01301    -- --- --- G-- T-C CA- TTT --- C-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01401    -- --- --- --- --- A-- TT- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01501    -- --- --- --- --- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- C-T C-- -TG ---
DLA-DRB1*01502    -- --- --- --- --- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- C-T C-- -TG ---
DLA-DRB1*01503    -- --- --- --- --- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- C-T C-- -TG ---
DLA-DRB1*01601    -- --- --- --- --- --- --- --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01701    -- --- --- G-- A-- A-- TTT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --T C-- -CG ---
DLA-DRB1*01801    -- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01901    -- --- --- G-- AG- A-- TAT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --T C-- -CG ---
DLA-DRB1*02001    -- --- --- --- A-- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- --G --- --- ---
DLA-DRB1*02101    -- --- --- G-- A-- A-- TTT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- C-T C-- -CG ---
DLA-DRB1*02201    -- --- --- G-- T-C CA- TTT --- C-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02301    -- --- --- --- --- A-- TT- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02401    -- --- --- --- --- --- TT- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02501    -- --- --- --- --- --- TT- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- --G --- --- ---
DLA-DRB1*02601    -- --- --- --- --- --- A-- TT- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02701    -- --- --- G-- T-C CA- TTT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02801    -- --- --- --- --- --- --- --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02901    -- --- --- G-- A-- A-- TAT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- ATG ---
DLA-DRB1*03001    ** *** *** --- --- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- C-T C-- -TG ---
DLA-DRB1*03101    -- --- --- --- A-- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- ATG ---
DLA-DRB1*03201    -- --- --- G-- T-C CA- TTT --- C-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*03301    -- --- --- --- --- A-- TT- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- -TG ---
DLA-DRB1*03501    -- --- --- G-- A-- A-- TTT --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- C-T C-- -CG ---
DLA-DRB1*03601    -- --- --- --- --- A-- TT- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*03701    -- --- --- --- --- --- --- --- G-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*03801    -- --- --- --- --- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- C-T C-- -TG ---
DLA-DRB1*03901    -- --- --- G-- T-C CA- TTT --- C-- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*04001    -- --- --- --- A-- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- --G --- --- ---
DLA-DRB1*04101    -- --- --- --- --- A-- TT- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- ATG ---
DLA-DRB1*04201    -- --- --- --- --- A-- TT- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- -TG ---
DLA-DRB1*04301    -- --- --- --- --- A-- TT- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- -TG ---
DLA-DRB1*04401    -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- --- ---
DLA-DRB1*04501    -- --- --- --- --- A-- TT- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*04601    -- --- --- --- --- A-- TT- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*04701    -- --- --- --- A-- A-- -T- --- -T- --- --- C-- --- --- --- --- --- --- --- --- --- -AT C-- ATG ---
```

DLA-DRB1*00101 TAC ATC CAT AAC CGG GAG GAG TTC GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG GCG GTC ACG GAG CTC GGG
DLA-DRB1*00102 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*00201 G-- --- T-- --- --- --- --- A-- C-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00202 G-- --- T-- --- --- --- --- A-- C-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00301 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*00401 C-- ---
DLA-DRB1*00501 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00601 --- --- T-- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00701 --- --- T-- ---
DLA-DRB1*00801 G-- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*00802 G-- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*00901 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*010011 ---
DLA-DRB1*010012 --- --- --C ---
DLA-DRB1*01101 AG- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*01201 AG- --- T-- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01301 ---
DLA-DRB1*01401 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01501 G-- --- T-- --- --- --- --- CA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01502 G-- --- T-- --- --- --- --- CA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01503 G-- --- T-- --- --- --- --- CA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01601 ---
DLA-DRB1*01701 AG- --- T-- ---
DLA-DRB1*01801 ---
DLA-DRB1*01901 AG- --- T-- ---
DLA-DRB1*02001 G-- --- T-- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02101 AG- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*02201 ---
DLA-DRB1*02301 ---
DLA-DRB1*02401 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02501 --- --- T-- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02601 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02701 ---
DLA-DRB1*02801 --- --- T-- ---
DLA-DRB1*02901 G-- --- T-- --- --- --- --- AA- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*03001 G-- --- T-- --- --- --- --- CA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*03101 G-- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*03201 ---
DLA-DRB1*03301 G-- --- T-- --- --- --- --- CA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*03501 AG- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*03601 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*03701 --- --- T-- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*03801 G-- --- T-- --- --- --- --- CA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*03901 ---
DLA-DRB1*04001 G-- --- T-- --- --- --- --- -A- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*04101 G-- --- T-- --- --- --- --- AA- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*04201 G-- --- T-- --- --- --- --- AA- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*04301 G-- --- T-- --- --- --- --- CA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*04401 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
DLA-DRB1*04501 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*04601 --- --- --- --- --- --- --- AA- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*04701 G-- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---

```
DLA-DRB1*00101    CGG CCC GTC GCT GAG TCC TGG AAC GGG CAG AAG GAG ATC TTG GAG CAG GAG CGG GCA ACG GTG GAC ACC TAC TGC
DLA-DRB1*00102    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00201    --- --- A-- --- --- --- --- --- C-- --- --- --- --- --- --- --- AG- --- --C G-- --- --- --- --- ---
DLA-DRB1*00202    --- --- A-- --- --- --- --- --- C-- --- --- --- --- --- --- --- AG- --- --C G-- --- --- --- --- ---
DLA-DRB1*00301    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- -G- A-- --- --C G-- --- --- --- --- ---
DLA-DRB1*00401    --- --- -A- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*00501    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- --- --- --C G-- --- --- --- --- ---
DLA-DRB1*00601    --- --- -A- --- --- -A- --- --- CC- --- --- --- C-- --- --- -G- -C- --- --C G-- --- --- --- --- ---
DLA-DRB1*00701    --- --- -A- --- --- -A- --- --- CC- --- --- --- C-- --- --- GG- -GC --- --C G-- --- --- --- --- ---
DLA-DRB1*00801    --- -G- -A- --- --- -A- --- --- --- --- --- --- --- --- --- -G- A-- --- --C G-- --- --- --- --- ---
DLA-DRB1*00802    --- -G- -A- --- --- -A- --- --- --- --- --- --- --- --- --- -G- A-- --- --C G-- --- --- --- --- ---
DLA-DRB1*00901    --- --- -A- --- --- --- --- --- C-- --- --- --- T-- --- --- -G- A-- --- --C G-- --- --- --- --- ---
DLA-DRB1*010011   --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*010012   --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*01101    --- -G- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- AG- --- --C G-- --- --- --- --- ---
DLA-DRB1*01201    --- -G- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- -G- AG- --- --C GA- --- --- --- --- ---
DLA-DRB1*01301    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- --- --- --C G-- --- --- --- --- ---
DLA-DRB1*01401    --- --- -A- --- --- -A- --- --- CC- --- --- --- C-- --- --- -G- -C- --- --C G-- --- --- --- --- ---
DLA-DRB1*01501    --- --- -A- --- --- -A- --- --- --- --- --- --- C-- --- --- --- AG- --- --C GA- --- --- --G GTG ---
DLA-DRB1*01502    --- --- -A- --- --- -A- --- --- --- --- --- --- C-- --- --- --- AG- --- --C GA- --- --- --G GTG ---
DLA-DRB1*01503    --- --- -A- --- --- -A- --- --- --- --- --- --- C-- --- --- --- AG- --- --C GA- --- --- --- --- ---
DLA-DRB1*01601    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- -G- A-- --- --C GA- --- --- --- --- ---
DLA-DRB1*01701    --- -G- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- -G- -C- --- --C G-- --- --- --- --- ---
DLA-DRB1*01801    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- -G- A-- --- --C GA- --- --- --- --- ---
DLA-DRB1*01901    --- -G- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- -G- AG- --- --C GA- --- --- --- --- ---
DLA-DRB1*02001    --- --- TCG --- --- --- --- --- C-- --- --- --- T-- --- --- --- AG- --- --C GA- --- --- --G GTG ---
DLA-DRB1*02101    --- -G- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- AG- --- C-C G-- --- --- --- --- ---
DLA-DRB1*02201    --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --C GA- --- --- --- --- ---
DLA-DRB1*02301    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- --- --- --C G-- --- --- --- --- ---
DLA-DRB1*02401    --- -G- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- -G- A-- --- --C GA- --- --- --- --- ---
DLA-DRB1*02501    --- -G- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- AG- --- --C G-- --- --- --- --- ---
DLA-DRB1*02601    --- --- -A- --- --- -A- --- --- C-- --- --- --- C-- --- --- -G- A-- --- --C GA- --- --- --- --- ---
DLA-DRB1*02701    --- -G- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*02801    --- --- -A- --- --- -A- --- --- CC- --- --- --- C-- --- --- -G- -C- --- --C G-- --- --- --- --- ---
DLA-DRB1*02901    --- -G- -A- --- --- --- --- --- --- --- --- --- --- --- --- -G- A-- --- --C G-- --- --- --- --- ---
DLA-DRB1*03001    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- AG- --- --C GA- --- --- --G GTG ---
DLA-DRB1*03101    --- -G- -A- --- --- --- --- --- --- --- --- --- C-- --- --- --- A-- --- --C G-- --- --- --- --- ---
DLA-DRB1*03201    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G GTG ---
DLA-DRB1*03301    --- --- -A- --- --- -A- --- --- C-- --- --- --- C-- --- --- -G- AG- --- --C GA- --- --- --- --- ---
DLA-DRB1*03501    --- -G- -A- --- --- --- --- --- C-- --- --- --- T-- --- --- --- AG- --- --C G-- --- --- --- --- ---
DLA-DRB1*03601    --- -G- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- -G- A-- --- --C GA- --- --- --- --- ---
DLA-DRB1*03701    --- --- -A- --- --- --- --- --- CC- --- --- --- C-- --- --- -G- -C- --- --C G-- --- --- --- --- ---
DLA-DRB1*03801    --- --- -A- --- --- -A- --- --- --- --- --- --- C-- --- --- -G- AG- --- --C GA- --- --- --G GTG ---
DLA-DRB1*03901    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DLA-DRB1*04001    --- --- TCG --- --- -A- --- --- --- --- --- --- --- --- --- AG- --- --C GA- --- --- --G GTG ---
DLA-DRB1*04101    --- -G- -A- --- --- --- --- --- --- --- --- --- C-- --- --- --- A-- --- --C G-- --- --- --- --- ---
DLA-DRB1*04201    --- --- -A- --- --- -A- --- --- --- --- --- --- C-- --- --- --- A-- --- --C G-- --- --- --- --- ---
DLA-DRB1*04301    --- --- -A- --- --- -A- --- --- C-- --- --- --- C-- --- --- -G- AG- --- --C GA- --- --- --G GTG ---
DLA-DRB1*04401    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- AG- --- --C G-- --- --- --- --- ---
DLA-DRB1*04501    --- --- -A- --- --- --- --- --- --- --- --- --- C-- --- --- -G- A-- --- --C GA- --- --- --- --- ---
DLA-DRB1*04601    --- --- -A- --- --- --- --- --- C-- --- --- --- C-- --- --- --- --- --- --C G-- --- --- --- --- ---
```

DLA-DRB1*04701 --- -G- -A- --- --- --- --- --- --- --- --- --- C-- --- --- -G- AG- --- --C G-- --- --- --- --- ---

DLA-DRB1*00101 AGA CAC AAC TAC GGG GTG ATT GAG AGC TTC ACG GTG CAG CGG CGA G
DLA-DRB1*00102 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*00201 --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- -
DLA-DRB1*00202 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*00301 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*00401 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*00501 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*00601 --- --- --- --- --- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*00701 --- --- --- --- --- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*00801 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*00802 --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- -
DLA-DRB1*00901 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*010011 --- --- --- --- --- --- --- *** *** *** *** *** *** *** *** *
DLA-DRB1*010012 --- --- --- --- --- --- --- *** *** *** *** *** *** *** *** *
DLA-DRB1*01101 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*01201 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*01301 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*01401 --- --- --- --- --- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*01501 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*01502 --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- -
DLA-DRB1*01503 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*01601 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*01701 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*01801 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*01901 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*02001 --- --- --- --- --- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*02101 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*02201 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*02301 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*02401 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*02501 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*02601 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*02701 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*02801 --- --- --- --- --- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*02901 --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- -
DLA-DRB1*03001 --- --- --- --- --- --- --- *** *** *** *** *** *** *** *** *
DLA-DRB1*03101 --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- -
DLA-DRB1*03201 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*03301 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*03501 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*03601 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*03701 --- --- --- --- --- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*03801 --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*03901 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*04001 --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- -
DLA-DRB1*04101 --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- -
DLA-DRB1*04201 --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- -
DLA-DRB1*04301 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*04401 --- --- --- --- C-- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*04501 --- --- --- --- --- --- GGC --- --- --- --- --- --- --- --- -
DLA-DRB1*04601 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DLA-DRB1*04701 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -

Appendix 2.

The *CTLA-4* gene and primers, the highlighted sequences mark the primers used in this study.

Promoter sequence and exon 1

Forward	5'-GCC CGT ATT CCA CAG AGT GT-3'
Reverse	5'-TCT GAA ACC TGG GGA ATC TG-3'

Sequencing primers

Forward	5'- AAA GCT GTC ATG GGT CAA GG-3'
Reverse	5'- TTG GCT TCT GGC TTG GTT AT -3'

Exon 2 primers

Forward	5'-AGG CAT TGA CGA GGA GCT TA-3'
Reverse	5'- CCA GGC TCA AGC AAA ATC TC-3'

gcctgaacatacattttccagttttgtatcttcagtgccctctctgggatctggccctta
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Appendix 3.

```
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#Allel_1A  ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_2   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3   ... ... ... ... ... ... ... ..G ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3A  ... ... ... ... ..A ... ... ..G ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3c  ... ... ... ..A ..A ..T ... ..G ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3D  ... ... ... ... ..A ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3F  ... ... ... ... ..C ... ... ..G ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_4   ... ... ... ... ..A ..T T.. ... T.. ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_5   ... ... ..G ..A G.A ..T AT. ..G ... ... ..C ... ... ... ... ... ... ... ... ... ... .TC ... CG. ..A G..
#Allel_5A  ... ... ..G ..A ..A ..T AT. ..G ... ... ..C ... ... ... ... ... ... ... ... ... ..C .TC ... CG. ..A G..
#Allel_6   ... ... ..G ..T .CC A.T TT. ..C ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_6A  ... ... ..G ..T .CC A.T AT. ..C ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_7   ... ... ... ... ..A ... T.. ... T.. ... ..C ... ... ... ... ... ... ... ... ... ..C .TC ... TG. ..G ...
#Allel_8   ... ... ... ... ..A ..T TT. ... T.. ... ..C ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ...
#Allel_10C ... ... ... ... ..A ... T.. ... T.. ... ..C ... ... ... ... ... ... ... ... ... ... ATC ... CG. ..G ...

#Allel_1   TCC ATA ACC GGG AGG AGT TCG TGC GCT TCG ACA GCG ACG TGG GGG AGT ACC GGG CGG TCA CGG AGC TCG GGC GGC CCG
#Allel_1A  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_2   ... ... ... ... ... ..A A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3   ..T ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3A  ..T ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3c  ..T ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3D  ..T ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3F  ..T ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_4   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_5   ..T ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... G..
#Allel_5A  ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ... G..
#Allel_6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_6A  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_7   ..T ... ... ... ... ..C A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_8   ..T ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_10C ..T ... ... ... ... ..A ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A

#Allel_1   TCG CTG AGT CCT GGA ACG GGC AGA AGG AGA TCT TGG AGC AGG AGC GGG CAA CGG TGG ACA CCT ACT GCA GAC ACA ACT
#Allel_1A  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_2   A.. ... ... ... ... ..C ... ... ... ..T ... ... ... G.A ... ... .CG ... ... ... ... ... ... ... ...
#Allel_3   A.. ... ... A.. ... ..C C.. ... ... ..C ... ... ... G.. C.. ... .CG ... ... ... ... ... ... ... ...
#Allel_3A  A.. ... ... A.. ... ..C C.. ... ... ..C ... ... ... G.. C.. ... .CG ... ... ... ... ... ... ... ...
#Allel_3c  A.. ... ... A.. ... ..C C.. ... ... ..C ... ... ... G.. C.. ... .CG ... ... ... ... ... ... ... ...
#Allel_3D  ... ... ... A.. ... ..C C.. ... ... ... ... ... ... G.. C.. ... .CG ... ... ... ... ... ... ... ...
#Allel_3F  A.. ... ... A.. ... ..C C.. ... ... ..C ... ... ... G.. C.. ... .CG ... ... ... ... ... ... ... ...
#Allel_4   A.. ... ... ... ... ..C ... ... ... ..C ... ... ... ... ... ... .CG ... ... ... ... ... ... ... ...
#Allel_5   A.. ... ... ... ... ..C ... ... ... ..C ... ... ... G.A G.. ... .CG A.. ... ... ... ... ... ... ...
#Allel_5A  A.. ... ... ... ... ..C ... ... ... ..C ... ... ... ..A G.. ... .CG ... ... ... ... ... ... ... ...
#Allel_6   A.. ... ... ... ... ..C ... ... ... ..C ... ... ... ... ... ... .CG ... ... ... ... ... ... ... ...
#Allel_6A  A.. ... ... ... ... ..C ... ... ... ..C ... ... ... ... ... ... .CG ... ... ... ... ... ... ... ...
#Allel_7   A.. ... ... A.. ... ... ... ... ... ..C ... ... ... ..A G.. ... .CG A.. ... ... .GG TG. ... ... ... ...
#Allel_8   A.. ... ... ... ... ..C ... ... ... ..C ... ... ... G.A ... ... .CG ... ... ... ... ... ... ... ...
#Allel_10C ... ... ... ... ... ..C ... ... ... ... ... ... ... ..A G.. ... .CG ... ... ... ... ... ... ... ...

#Allel_1   ACG GGG TGA TTG AGA GCT TCA CGG TGC AGC GGC GAG
#Allel_1A  ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_2   ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3   ... ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_3A  ..C ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_3c  ... ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_3D  ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_3F  ... ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_4   ..C ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_5   ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_5A  ..C ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_6   ..C ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_6A  ..C ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_7   ... ... ... ... ... ... ... ... ... ... ... ...
#Allel_8   ..C ... ..G GC. ... ... ... ... ... ... ... ...
#Allel_10C ... ... ... ... ... ... ..G ... ... ... ... ...
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