universität wien

DIPLOMARBEIT

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Characterization of swine leukocyte antigen polymorphism by sequence-based and low-resolution typing methods

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Summary

The porcine major histocompatibility complex (MHC) harbours the SLA (swine leukocyte antigen) class I and II gene clusters. The SLA genes are highly polymorphic; they encode a series of cell-surface glycoproteins which function mainly in presenting antigenic peptides to T cells, therefore representing one of the most important determinants in swine immune response to infectious disease and vaccination.

In Austria, the majority of commercial pigs are F2 descendants of F1 Large White/Landrace hybrids paired with Pietrain boars. The repertoire of SLA alleles and haplotypes present in Pietrain pigs thus has an important influence on that of their descendants.

In this study, we characterized the SLA class I (SLA1, SLA2, SLA3) and class II (DRB1, DQB1, DQA) genes of 27 purebred Pietrain pigs using a combination of the high-resolution sequence-based typing (SBT) method and a low-resolution PCR-based method using allele-group sequence-specific primers (PCR-SSP).

A total of 15 class I and 12 class II haplotypes were identified in the studied cohort. The most common SLA haplotype Lr-43.14 (SLA1*11XX-SLA3*04XX-SLA2*04XX-DRB1*0901-DQB1*0801-DQA*03XX) was identified in seven animals with a frequency of 13.0%. Three class I and two class II haplotypes appeared to be novel that have never been reported in other pig populations, suggesting a breed-specific constriction of SLA gene diversity in Pietrain pigs.

In conclusion, this study may facilitate a better understanding of the influence of SLA genes on various immunological and pathophysiological conditions. It may also facilitate the design of more effective vaccines aiming to improve the overall swine health in Austrian commercial pigs.

Zusammenfassung

Der Haupthistokompatibilitätskomplex wird im Schwein durch die Klasse I und Klasse II Swine Leukocyte Antigen (SLA) Gencluster kodiert. Die hochpolymorphen SLA Gene kodieren für eine Reihe von Glykoproteinen, deren Aufgabe darin besteht, prozessierte Peptidantigene auf der Oberfläche von Zellen zu präsentieren und anschließend in T-Zellen eine entsprechende Immunantwort auszulösen. Somit stellen SLA Genprodukte eine der wichtigsten Determinanten innerhalb der porcinen Immunantwort auf Infektion und Vakzination dar.

Die Mehrheit der in Österreich für die Fleischerzeugung verwendeten Schweinerassen stellt eine F2 Generation dar. Diese Tiere sind Nachkommen einer F1 Generation der Edelschwein/Landrasse, die mit Pietrain-Ebern gepaart wird. Somit ist das SLA Allel- und Haplotypen-Repertoire in der Nachkommenschaft festgelegt und aufgrund des Repertoires der Elterntiere begrenzt.

Diese Arbeit beschreibt sowohl die Charakterisierung der SLA Klasse I (SLA1, SLA2, SLA3) als auch der Klasse II (DRB1, DQB1, DQA) Gene von 27 reinrassigen Pietrain Schweinen anhand der Sequenz-basierten (SBT) und der PCR-basierten Methode unter der Verwendung von sequenzspezifischen Primern (PCR-SSP).

Insgesamt wurden 15 Klasse I und 12 Klasse II Haplotypen in dieser Gruppe von Schweinen identifiziert. Der häufigste auftretende SLA Haplotyp ist Lr-43.13 (SLA-1*11XX-SLA-3*04XX-SLA-2*04XX-DRB1*0901-DQB1*0801-DQA*03XX) der in sieben Individuen in einer Frequenz von 13,0% detektiert wurde. Zusätzlich wurden drei Klasse I und zwei Klasse II Haplotypen ermittelt, die bisher noch in keiner Schweinepopulation charakterisiert wurden. Dies lässt den Schluss zu, dass die Pietrain Rasse eine zuchtspezifische Einschränkung der SLA Gendiversität aufweist.

Die Ergebnisse dieser Arbeit können zu einem besseren Verständnis der Beeinflussung der SLA Gene auf unterschiedliche immunologische und pathophysiologische Bedingungen führen. Weiters ist es möglich, effektivere Impfstoffe herzustellen, um somit die gesundheitliche Fitness der in Österreich vorkommenden Schweinepopulation zu erhöhen.

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I Introduction

1.1 The Immune System

The immune system is a potent defense mechanism against infectious pathogens and can be divided into the innate immunity and the adaptive immunity. The innate immuntity provides the first lines of defense in responding to microbes and various microorganisms and is composed of physical and chemical barriers, phagocytes, natural killer cells (NK cells), the complement system and various cytokines. The second part of the immune response arise from stimulation with specific antigenic determinants on pathogens, this type is termed adaptive immunity. The major characteristics of the adaptive immunity are the specificity for certain molecules and the ability to remember and respond more effective to repeated exposure to the same antigens. Its cellular components are highly specified lymphocytes (B and T lymphocytes) (Abbas 2003, Chapter 1).

1.2 Major Histocompatibility Complex (MHC)

B and T lymphocytes are the major key players in the response against foreign antigens. In order to activate T lymphocytes, specialized proteins encoded in the so called major histocompatibility complex (MHC) present foreign antigens to T lymphocytes. Depending on which type of T lymphocyte has to be activated, two main types of MHC molecules are distinguished: proteins encoded by the MHC class I genes and proteins encoded by MHC class II genes. MHC class II molecules display peptides derived from proteins which were internalized from the extracellular environment and present these foreign antigens to $CD4^+$ helper T cells (Abbas 2003, Chapter 4, 5). Proteins encoded by the MHC class I genes present endogeneous synthesied peptide antigens to $CD8^+$ cytolytic T cells.

1.2.1 MHC class I Molecules

MHC class I molecules are expressed on nearly all nucleated cells and present peptides derived from cytosolic proteins to $CD8^+$ cytolytic T lymphocytes. Each MHC class I molecule is composed of a single α-chain and a polypeptide termed β_2-microglobulin (**Figure 1**). The polymorphic α-chain can be divided into three different segments (α_1, α_2, α_3) in which only the α_1 and α_2 segments fullfill the task of forming the peptide binding cleft and presenting the peptide to $CD8^+$ cytolytic T-lymphocytes. The carboxy terminus of the α_3 segment contains several hydrophobic amino acids which traverse the lipid bilayer of the plasma membrane. The β_2-microglobulin interacts noncovalently with the α_3 domain and exhibits a muss less degree of polymorphism than the α-chain (Abbas 2003, Chapter 4).

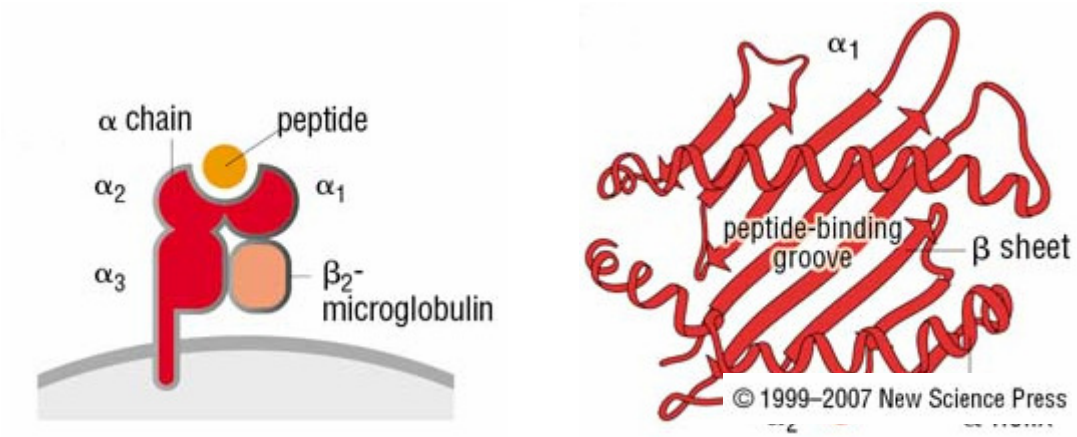

Figure 1. Structure of a MHC class I molecule. On the left the different regions of a MHC class I molecule are illustrated. The α_1 and α_2 domains build the peptide binding cleft in which the peptide is presented to $CD8^+$ cytolytic T lymphocytes. The α_3 segment establishes the connection to the plasma membrane and is noncovalently attached to the β_2-microglobulin. The ribbon diagram on the right displays the locations of α-helices and β sheets within the peptide binding cleft.

Before peptides are presented by MHC class I molecule, proteins are proteolytically degraded in the proteasome. Then peptides are delivered from the cytoplasm to the endoplasmaic reticulum (ER) by an ATP-dependent transporter called TAP (transporter associated with antigen processing). After the assembly of peptide class I complexes is accomplished in the ER, stable MHC class I molecules with bound peptides are transported to cell surface (Abbas 2003, Chapter 5).

1.2.2 MHC class II Molecules

MHC class II molecules are expressed on antigen presenting cells (APC) such as B-lymphocytes, dendritic cells (DC) and macrophages and present peptides derived from internalized proteins to $CD4^+$ T lymphocytes (mainly T-helper cells). Each MHC class II molecule is composed of an α-chain and a β-chain (**Figure 2**). Both chains are divided into two different segments (α_1, α_2, β_1, β_2) in which the α_1 and β_1 segments fullfill the tasks of forming the peptide binding cleft and presenting peptides to $CD4^+$ helper T-cells. The carboxy ends of the α_2 and β_2 segments are composed of several hydrophobic amino acids which traverses the lipid bilayer of the plasma membrane. The α-chain interacts noncovalently with the β-chain. The α_1- and β_1-chain exhibit the highest degree of polymorphism (Abbas 2003, Chapter 4).

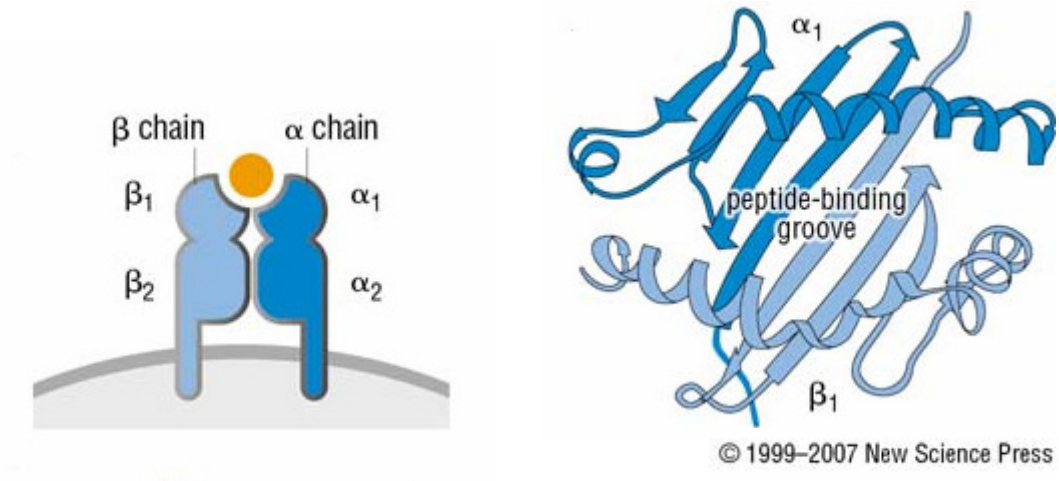

Figure 2. Structure of a MHC class II molecule. On the left the different regions of a MHC class II molecule are illustrated. The α_1 and β_1 domains build the peptide binding cleft in which the peptide is presented to $CD4^+$ helper T-cells. The α_2 and β_2 segments establish the connection to the plasma membrane. The ribbon diagram on the right displays the locations of α-helices and β sheets within the peptide binding cleft.

MHC class II associated antigen presentation begins with the internalization of extracellular proteins into endosomes. These proteins are proteolytically degraded by endosomal enzymes. Within the ER, the peptide binding is inhibited by the binding of invariant chains (I_i) which block the peptide binding cleft of the MHC II molecules. After the cleavage of the I_i the peptide binding cleft is vacant for the foreign peptide. Then stable MHC class II molecules with bound peptides are delivered and displayed at the cell surface (Abbas 2003, Chapter 5).

1.2.3 MHC Polymorphism

In human the MHC contains a multigene family encoding human leukocyte antigen (HLA) class I and class II molecules. The genes encoding HLA class I and class II molecules are known to show an extensive degree of genetic polymorphism, some of which have over 200 allelic variants (Horton et al. 2004).

Generation of new MHC alleles upon mutations may result in different interaction pattern such as altering the T-cell receptor (TCR) contact, binding new spectrum of peptides or binding of the same peptide but in a different conformation. These changes will logically affect the T-cell repertoire selection of the individual and hence the susceptibility and/ or resistance to pathogens (Nikolich-Zugich et al. 2004).

It is further believed that the high extent of polymorphism have arised in respone to the evolutionary pressures generated by contact with pathogens such as intracellular bacteria and viruses. The resistance against certain infectious diseases can be directly as well as indirectly linked to the MHC diversity of an individual or a population (Messaoudi et al. 2002).

1.2.4 The MHC interaction with T-cells

The recognition of peptide MHC complexes on APC by T cells is mainly driven by a distinct set of molecules expressed on the surface of $CD8^+$ cytoloytic T-lymphocytes and $CD4^+$ helper T-cells. The most important components during antigen recognition are the TCR and the accessory molucles. The TCR is a heterodimer of two transmembrane polypeptide chains – an α-chain and a β-chain. The transmembrane regions contain hydrophobic amino acid residues and are followed by a short cytoplasmaic region. Each α and β chain consists of a variable (V) domain and a constant (C) domain (**Figure 3**). The antigen-binding is generated by the Vα and Vβ domains (Abbas 2003, Chapter 6).

In order to ensure a proper interaction between the APC and the T-cell receptor, serveral co-receptors such as CD4/CD8 and CD28 are expressed on the surface of T cells. Hence, three distinct sets of signals are required to induce proliferation and differentation of naive T-cells

into effector cells. Binding of MHC complexes to the TCR provides the first signal, the second signal is provided by the costimulatory signals. In the absence of costimulation, T-cells that recognize antigens either fail to respond and die by apoptosis or enter a state of unresponsiveness called anergy (Abbas 2003, Chapter 8). A third signal delivered by the cytokine milieu generated by the APC is responsible for a differentiation in to distinct effector T cell populations.

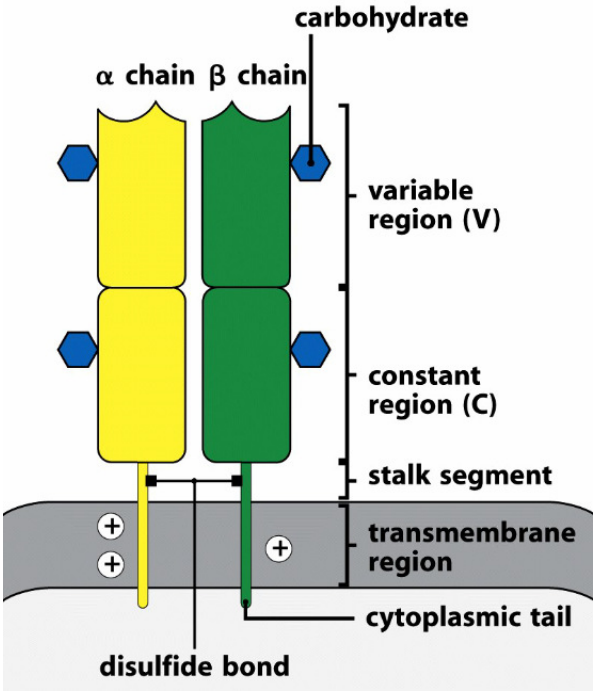

Figure 3. Structure of the T-cell receptor. The TCR is a heterodimer consisting of two transmembrane polypeptide chains (α, β). Both chains are divided into a variable (V) and a constant (C) domain. The domains crucial for the MHC interaction are illustrated as pockets at the end of the variable region, based on Janeway's Immunobiology, 7th ed, 2008, Garland Science, NY.

The set of responses of the T-cell to antigen and costimulators includes synthesis of cytokines and effector molecules, cellular proliferation and differentation into effector and and memory cells.

A major characteristic of T cells is the ability to recognize a foreign peptide antigen only when it is bound to a particular allelic form of an MHC molecule (Abbas 2003).

1.3 Swine Leukocyte Antigen (SLA)

In swine, the MHC is termed as swine leukocyte antigen (SLA) and has been mapped to chromosome 7. It consists of three major gene clusters (class I, III, II) which are located in the 7p1.1 band of the short arm (class I and III) and in the 7q1.1 band of the long arm (class II). The regions of the three classes were found to span approximately 1.1, 0.7, 0.5Mb containing over 150 loci and at least 121 genes are predicted for a specific function (**Figure 4**) (Lunney et al. 2009). On the other hand, 18 pseudogenes, eigeht novel conserved domain sequences (CDS), three novel transcripts and one putative gene were found within the SLA region (Renard et al. 2006).

One of the most striking features of the SLA region is its high degree of polymorphism which may be maintained by selective pressures possibly associated with diseases (Luetkemeier et al. 2009). The high extent of polymorphism of the SLA coding region is also related to reproductive performance and production traits (Renard C., Vaiman M. 1989; Gautschi C., Gaillard C. 1990).

1.3.1 SLA class I Genes

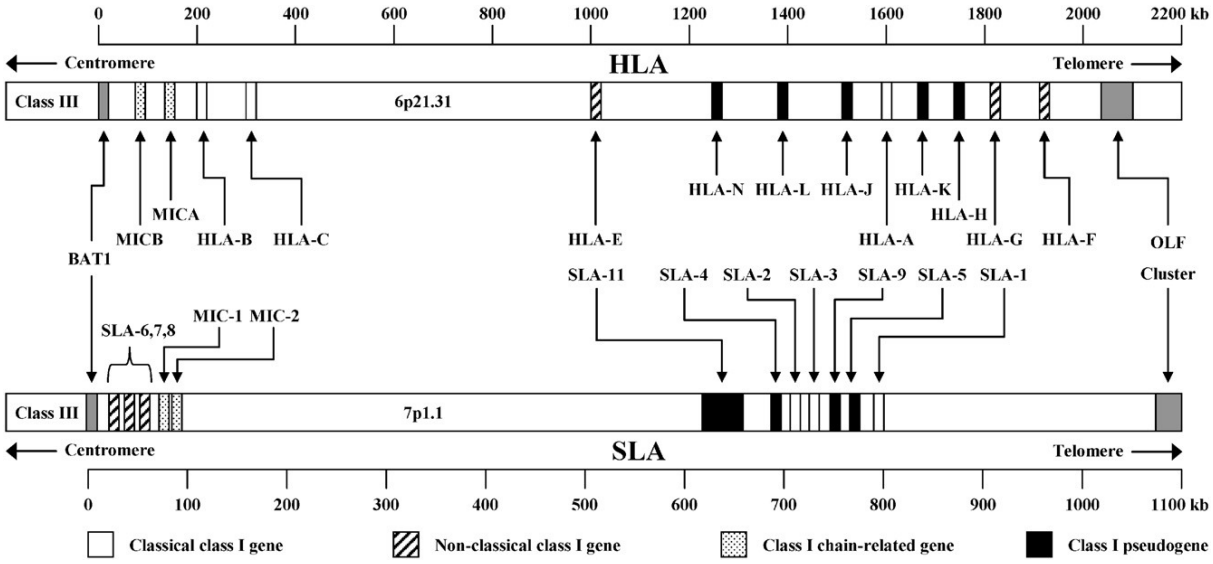

Figure 4. Comparative genomic organization of the human and swine major histocompatibility complex (MHC) class I region, based on Lunney et al. 2009.

The SLA class I region consists of seven classical class I genes (SLA11, SLA4, SLA2, SLA3, SLA9, SLA5, SLA1; indicated as black and white bars) and three non-classical class I genes (SLA6, SLA7, SLA8; indicated as striped bars) (**Figure 4**). The only constitutively expressed classical class I genes are SLA2, SLA3 and SLA1 (white bars) whereas the rest are designated as pseudogenes (black bars) generating no functional proteins. Investigation of the three non-classical class I genes revealed their location in a closed proximity to the centromeric region. Besides the classical and non-classical SLA class I genes there are genes which also arise in the HLA system termed MHC class I chain-related genes (MIC). In swine only MIC-2 is predicted to be functional while the MIC-1 gene appears to be a pseudogene (Lunney et al. 2009).

By observing and comparing the entire genomic organisation of the HLA- and SLA- class I region, only minor similarities between the two organisms can be detected.

The classical SLA1, SLA2 and SLA3

The sequence homology between the SLA1, SLA2 and SLA3 loci is much higher than that between any HLA class I genes (Smith et al. 2004). Smith et al. suggest a high degree of sequence similarity between SLA1 and SLA3 which arose form a gene duplication (Smith et al. 2004), whereas Ando et al. propose a closely relation between SLA1 and SLA2 after an interlocus genetic exchange might have occured (Ando et al. 2003).

SLA 1, SLA2 and SLA3 code for 45 kDa transmembrane glycoproteins as described in 1.2.1. Due to expression levels, SLA1 has the highest and SLA3 has the lowest expression levels among these three genes (Tennant et al. 2007).

The genomic structure of classical SLA class I genes is distributed in eight exons. Exon 1 encodes the leader sequence, exon 2-4 encode extracellular α_1, α_2 and α_3 domains, exon 5 the transmembrane domain and exons 6-8 encode the cytoplasmic domain (Satz et al. 1985). The coding region in all of the expressed classical class I genes exhibit a high degree of similarity (Lunney et al. 2009).

Due to extent polymorphism within the SLA coding region, a total of 116 SLA classical class I genes have been identified to date and their sequences based in the Immuno Polymorphism Database (IPD) MHC database (http://www.ebi.ac.uk/ipd/mhc) (Robinson et al. 2005). There

are 44 SLA1 alleles, 46 SLA2 alleles and 26 SLA3 alleles and 29 SLA class I haplotypes have been identified so far, by using the high resolution typing method (SBT). In contrast, 70 SLA class I haplotypes were characterized by using the low resolution typing method (PCR-SSP). As expected, polymorphism is mainly concentrated in exon 2 and 3 which encode the class I protein peptide-binding cleft (Lunney et al. 2009).

The non-classical SLA6, SLA7 and SLA8

The precise function of the non-classical SLA class I genes is yet not fully understood. Chardon et al. suggest that all three genes might code for membrane anchored glycoproteins with the potential of binding peptides or might be asscociated with β_2-microglobulin (Chardon et al. 2001). Investigation of SLA6, SLA7 and SLA8 mRNA transcripts revealed that SLA6 and SLA8 trancripts appear in plenty of tissues exept for brain, whereas SLA7 transcripts exhibited more limited tissue distribution (Crew et al. 2004).

The genomic structure of the non-classical SLA class I genes is comparable to their classical counterparts, execpt that only two exons encode the cytoplasmic domain. The lack of an interferon regulatory element in the SLA8 gene suggests that this gene might be regulated differently than the SLA6 and SLA7 genes. It is also believed that SLA6 may undergo alternative splicing, similar to the non-classical HLA-G gene (Lunney et al. 2009).

According to the IPD all non-classical SLA class I genes seem to be largely monomorphic. SLA6 is represented by 9 alleles to date whereas only two alleles for the SLA7 and SLA8 genes have been reported so far (Lunney et al. 2009).

1.3.2 SLA class II Genes

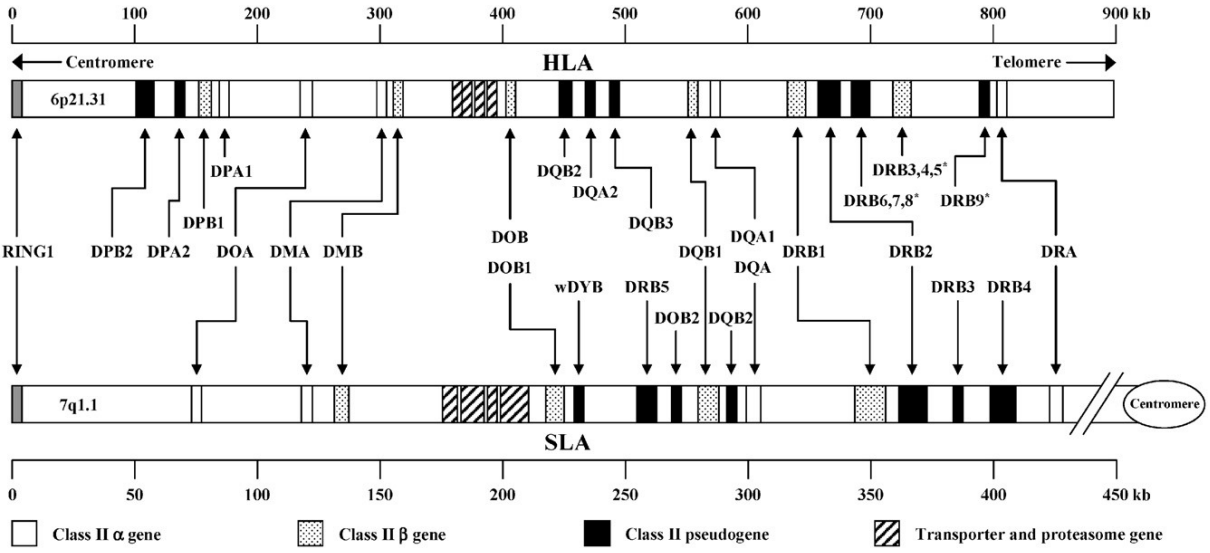

Figure 5. Comparative genomic organization of the human and swine major histocompatibility complex (MHC) class II region, based on Lunney et al. 2009.

The SLA class II region consists of several loci encoding the SLA class II antigens which include the α- and β-chain genes for the SLA-DR, -DQ, -DM and DO proteins. Expressed SLA class II genes are DRA, DRB1, DQA, DQB1, DOB1, DMB, DMA and DOA (**Figure 5**, white bars). Analogous to the class I genes, the SLA class II genomic region harbors genes (DRB2, DRB3, DRB4, DRB5, DQB2, DOB2 and wDYB) wich encode for nonfunctional proteins (pseudogenes, indicated as black bars.) Additionally, there are genes - between the DOB1 and DMB loci - that are involved in the antigen presentation pathway such as TAP. Phylogenetic analyses of the entire SLA class II region revealed interesting facts, such as the existance of a higher degree of sequence homolgy with their HLA counterparts than with each other (Smith et al. 2005b).

Expressed DR and DQ

SLA class II antigens are mainly expressed on the surface of APC such as macrophages, B cells and DCs. The function of those antigens is to present exogenous antigens to $CD4^+$ T-helper cells. (see 1.2.2). In respect of the genomic structure, DQA and DRA consist of four exons of which exon 2 and exon 3 encode the corresponding extracellular α_1 and α_2 domain. On the other hand DQB1 and DRB1 are made of five and six exons, respectively but display the same encoding genetic structure for the β-chain.

Based on the IPD-MHC SLA database a total of 167 SLA class II alleles have been identified to date. The highest degree of polymorphism among the entire SLA coding region shows the SLA-DRB1 gene with 82 alleles. 44 alleles have been characterized within in the SLA-DQB1 loci, followed by SLA-DQA and SLA-DRA with 20 and 13, respectively identified alleles so far. In regard to the haplotypes, 21 SLA class II haplotypes have been identified so far by using the SBT-method. On the other hand, 43 SLA class II haplotypes have been identified with the PCR-SSP typing method so far (Lunney et al. 2009).

1.3.3 SLA class III Genes

The SLA class III region is centromeric, spans about 700kb of DNA and contains over 60 characterized loci which are important in innate immune defense mechanisms. Representative gene families and factors are TNF, Hsp70, the steroid cytochrome P450 21-hydrolase enzyme and components of the complement cascade (Chardon et al. 2000).

1.4 SLA typing

As described above, MHC molecules exhibit a high degree of polymorphism, making the SLA genes an interesting topic to investigate. In the recent years, several methods evolved which helped to identify the complex and manifold allelic variants of certain SLA genes. Serologic typing methods using allo-antisera and monoclonal antibodies (Lunney et al. 1994), as well as molecular-based techniques, such as restriction fragment length polymorphism (RFLP) (Fang et al. 2005) have been documented.

In the context of this diploma thesis, a novel method was used besides the high resolution typing method which is based on the molecular cloning of the gene of intrest. A PCR-based SLA typing method was established using PCR with sequence specific primers (PCR-SSP) (Ho et al. 2006). Based on 48 discriminatory primer pairs, 27 Pietrain pigs were investigated in regard to their allelic SLA repertoire. One of the major advantages of this new emerging typing method is the time consuming aspect. In contrast to the high resolution typing method, the PCR-SSP allows the analyzation of two individuals simultaneously by using a 96 well plate.

Due to previously published data, the results of the characterization of SLA class I and class II haplotypes give rise to an accumulated and limited haplotype repertoire within the tested outbred pig population, e.g. Large White, Yucatan, Meishan, Korean native pig etc. (Lee et al. 2008 and Lunney et al. 2009). Referring to the 27 Pietrain pigs tested in this study using the PCR-SSP typing method, the most prevalent SLA haplotype is Lr-43.14 (see 4.3.2).

1.5 SBT vs. PCR-SSP

1.5.1 SBT

The prevalent method to characterize and identify sequence specific alleles is the sequence-based typing method (SBT). A major part of this method involves the molecular cloning steps such as RNA isolation, Reverse Transcriptase - Polymerase chain reaction (RT-PCR), ligation, transformation and plasmid mini-prep.

In this thesis, two loci of the SLA class II complex (DQB1 and DRB1) of twelve Pietrain pigs were sequence based typed. Using previously published primers (Fang et al. 2005, Smith et al. 2005 and Ho et al. 2006; see 2.2.6), the obtained PCR-products were ligated in appropiate vectors, transformed in *Escherichia coli* (*E. coli)* and isolated plasmid-DNA was sequenced (see 2.2). Subsequently the sequences were aligned according to their corresponding reference alleles present in the IPD (see appendix). Based on this results and alignments, it is possible to identify the sites of increased polymorphism as well as the allele frequency within the twelve pigs tested.

1.5.2 PCR-SSP

In contrast to the SBT-method, the Polymerase chain reaction - Sequence specific primer method (PCR-SSP) is based on the analyses of DNA. In order to identify not allele specific sequences but certain allele groups, well-defined primer pairs build the basis of the characterization of SLA class I and class II loci. This typing method allows identifying precisely the SLA class I and II allele groups and the common class I and II haplotypes present in the Pietrain pigs.

In this study, a total of 27 Pietrain pigs were tested using the PCR-SSP typing method. Premixed primer pairs were kindly provided by Chak-Sum Ho from Gift of Life Michigan, Histocompatibility Lab, Ann Arbor, Michigan (USA). Allele groups of SLA class I (SLA1, SLA2, SLA3) as well as of class II (DQB1, DRB1, DQA) were characterized using 47 discriminatory primer pairs in a 96-well plate allowing to type two individuals simultaneously. Prior PCR, an

essential step of DNA extraction from whole blood samples (see 2.3.1) was carried out to detect accurately certain SLA allele groups. Due to the huge amount of SLA alleles it was necessary to combine the alleles that have similar sequence motifs into consolidated allele groups (Smith et al. 2005b, Ho et al. 2009b).

The identifaction of the allele groups was followed by the designation of the haplotypes. Haplotypes are combinations of alleles of multiple loci on the same chormosome.

Nomenclature

To date, a considerable number of alleles and allele groups have been determined by numerous investigators around the world. In 2002, the Nomenclature Committee of Factors of the SLA System was formed in order to assign the collected alleles and to establish a systematic nomenclature system for the already known alleles of the SLA class I and class II genes (Lunney et al. 2009).

Nomenclature	Indication
SLA	The SLA region and prefix for a SLA gene
SLA1	A particular SLA locus
SLA1*01	A group of SLA alleles
SLA*0101	A specific confirmed SLA allele

Table 1. Assignment of names and numbers for SLA allleles, based on Ho et al. 2009a.

The designation of alleles and allele groups is shown in **Table 1**. The first and two digits are used to designate groups of alleles that have similar DNA sequences. The third and fourth digits are used to designate alleles with different protein sequences (Ho et al 2009a).

In order to compare results of researcher on a more convenient level, it is more appropriate to match the findings in terms of haplotypes. High resolution haplotypes defined by DNA sequencing are named with a prefix “Hp-“, and a number for the class I haplotype followed by a number for the class II haplotype separated by a period (e.g. Hp-1.1). Closely related haplotypes within a class are indicated by a lower letter added to the haplotype number (e.g. Hp.0.15a vs Hp-0.15b; Lunney et al 2009). Based on the fact that in this study a additional typing method (PCR-SSP) was applied, found allele groups were designated as low

resolution haplotypes ("Lr-"). Once the allele groups are characterized the SLA class I haplotype of each individual is assigned in the following order: SLA1 (e.g. 01XX) - SLA3 (01XX) - SLA2 (01XX) resulting in Lr-1.0 haplotype. Corresponding SLA class II haplotype is assigned in the order of: DRB1 (e.g. 04XX) – DQB1 (02XX) – DQA (02XX) resulting in Lr-0.15a haplotype. Thus, one of the two haplotypes present in this exemplarily individual is finally defined as Lr-1.15a.

1.6 Features of Pietrain pigs

One of the major reasons to characterize the polymorphic sequence of the SLA complex in Pietrain pigs is because there is no typing data to date. The Pietrain race plays an important role in the three-rotational crossbreeding application in Austria, representing the most important male crossbreeding race. After a Large White is crossed with a Landrace pig, the generated sow in the F1 generation is paired with a Pietrain boar resulting in F2 descendants used for meat production. The use of Pietrain pigs within this crossbreeding guarantees high meat and low fat concentrations in the offsprings. In regard to Pietrain pigs used in the three-rotational crossbreeding, generated offsprings bear about 50% of the MHC outfit, making the Pietrain pig an interesting race to characterize.

1.7. Characteristics of porcine intestinal epithelial cells

Another aspect to this diploma thesis was to characterize porcine intestinal epithelial cells which which were kindly provided by Michael Burwinkel (Institute of Immunology and Molecular Biology, Faculty of Veterinary Medicine, Freie Universität Berlin, Germany).
Porcine *in vitro* studies with transmissible gastroenteritis virus (TGEV) suggest that the intestine is a crucial area where the infection resides. Therefore, two different types of cells have been isolated. According to the attributes of growth rate and morphology they were termed Type I and Type II cells (Ephraim et al. 2009).

The main attributes of Type I cells are rapid growth, homogenous granluarity and small in size. On the other hand Type II cells exhibit humble growth, inhomogenous granularity and a large size compared to Type I cells. Furthermore, Type I cells show a less frequent occurance of organelles than Type II cells. A similarity of both cell types is the typical epithelial morpholgy with microvilli on the surface (Ephraim et al. 2009).

1.7 Aim of the Study

The extreme diversity of the expressed vertebrate MHC loci is essential in identifying and processing a large number of pathogenic antigens. In fact, the MHC genes influence both resistance and susceptibility to a variety of diseases ranging from gastrointestinal helminths to viruses (Apanius et al. 1997). Hence, a precise characterization of the SLA complex in pigs is necessary in order to facilitate the design of more effective vaccines.

In this study the high degree of MHC polymorphism of three classical SLA class I (SLA1, SLA2, SLA3) and SLA class II (DQA, DQB1, DRB1) is characterized by using two typing methods. The assignment of alleles and allele groups and the definition of certain haplotypes are the basis of specifying the MHC repertoire in each individual.

Furthermore this study provides insights into Pietrain breeding stocks in regard to their MHC haplotype diversity. Pietrain boars play an important role in cross-breeding procedures in Austria. Large White pigs are used to generate F1 hybrids which are further paired with a Pietrain boar to obtain F2 descendants used for meat production. To date, no surveys examining the SLA complex of the Pietrain race have been carried out so far. Hence this study gives a first overview of the contribution of SLA haplotypes in Pietrain pigs in Austria.

The assignment of alleles and allele groups, respectively, by the sequence based and low resolution typing method both generate results which can be easily compared on the level of haplotypes. Ascertained and characterized haplotypes are the first step in establishing improved breeding strategies. Accumulated haplotypes within the Pietrain breeding stock are essential for the development of certain vaccination strategies in order to induce an appropriate immune response in each individual upon infection. Data emerging from this study will substantially contribute to future-planned projects addressing the design of novel vaccines based on a more detailed knowledge of MHC-haplotype-specific peptide binding

motifs and T-cell specific antigenic regions of porcine viral pathogens (Armengol et al. 2002, Gerner et al. 2006).

An additional aim of this thesis was to characterize the gene expression of four SLA class I genes (SLA1, SLA2, SLA3, SLA6) and four SLA class II genes (DQA, DRA, DQB1, DRB1) of porcine intestinal epithelial cells by using Southern Blot hybridization method. The main aspect of this study was to identify whether or not those cells constitutively express SLA class II genes which may point towards a certain immunological function.

II Material and Methods

2.1 Studied animals

Blood samples of 27 Pietrain pigs (**Table 2**), which were kindly provided by the "Österreichische Schweineprüfanstalt" (Streitdorf, Austria), have been analyzed by the Sequence based typing (SBT)-method and by the Polymerase chain reaction-Sequence specific primer (PCR-SSP)-method.

Internal number	Streitdorf number	Race
36	4752	Pietrain
37	4738	Pietrain
38	4602	Pietrain
39	4813	Pietrain
40	4832	Pietrain
41	4973	Pietrain
42	4918	Pietrain
43	4844	Pietrain
44	4833	Pietrain
45	4845	Pietrain
46	4840	Pietrain
47	5128	Pietrain
48	4801	Pietrain
49	4494	Pietrain
50	4750	Pietrain
51	4721	Pietrain
52	4431	Pietrain
53	4524	Pietrain
54	4600	Pietrain
55	4722	Pietrain
56	4520	Pietrain
57	4673	Pietrain
58	4736	Pietrain
59	4775	Pietrain
109	4739	Pietrain
110	4611	Pietrain
111	4583	Pietrain

	SBT & PCR-SSP
	PCR-SSP only

Table 2. Investigated animals. 27 animals have been typed according to their MHC class I and class II haplotype repertoire. Green marked animals were analyzed only by PCR-SSP; in contrast, light blue marked animals were typed by the SBT-method as well as by PCR-SSP. In the course of this thesis, the animals are further designated according to their internal numbers.

2.2 SBT-method

2.2.1 RNA-Extraction

peqGOLD Blood RNA Kit

Method

The very first step in the molecular cloning procedure was to purify and isolate RNA from whole blood samples. Therefore, cells were first lysed with corresponding reagents RNA was collected and subsequently washed with ethanol. The isolation of RNA was carried out by the peqGOLD Blood RNA Kit (PEQLAB Biotechnologie GmbH). Erythrocytes were first selectively lysed and the leukocytes were then collected by centrifugation. After lysis of the white blood cells, RNA was selectively bound on a sicila matrix of a HiBind® spin column, washed with Ethanol-completed wash buffer and RNA was finally eluted in DEPC-water.

Material - peqGOLD Blood RNA Kit (PEQLAB Biotechnologie GmbH)

- ERL-Buffer (10x)
- TRK-Lysis-Buffer
- RNA-washbuffer I
- RNA-washbuffer II

} supplied with the Kit

Procedure

5 volumes of 1x ERL-Buffer were mixed with 1 volume of blood (maximum of 1ml) by vortexing. Samples were incubated on ice for 15 min. White blood cells were collected by centrifugation (10 min, 2000rpm, 4°C, Heraeus Multifuge 1®) and washed with 2 volumes of 1x ERL-Buffer. After vortexing, the cell suspension was centrifugated again (10 min, 2000rpm, 4°C). Cells were then resuspended in 400µl TRK-Lysis-Buffer if less than 500µl blood have been used. Blood volumes of 0.5-1ml were thoroughly vortexed in 650µl of Lysis-Buffer. After transfer of the cell-lysate into a Homogenizer Column and centrifugation of 5 min at 13000rpm (Heraeus Biofuge®), a same volume of 70% EtOH was added. 650µl of the probe was loaded on a collection tube and centrifugated for 15 sec at 13000rpm. After discarding the flow-through, the step was repeated one more time and the column was washed with RNA-washbuffer I. After centrifugation with 500µl of RNA-washbuffer II for 15

sec at 13000rpm, the column was dried at 13000rpm and RNA was eluted with 100µl of nuclease-free DEPC-treated water.

PBMC-RNA extraction with TRI REAGENT

Method

A second approach to isolate RNA from a different source than blood was the extraction of RNA from enriched Peripheral Blood Mononuclear Cells (PBMCs). The use of TRI REAGENT leads to the lysis of the cells and the inactivation of RNases. RNA was separated from DNA and proteins and purified by the addition of chloroform and finally washed with EtOH.

Procedure

PBMCs were thawed at 4°C and centrifugated at 1300rpm for 10 min at 4°C (Heraeus Multifuge 1®). TRI REAGENT was added to the cells in the pellet and then cells were lysed by repeated pipeting. After incubation on ice for 5 min, 0.2ml chloroform was added and shaked vigorously for 15 sec. During the following incubation at room temperature for 15 min, RNA was collected in the aqueous phase. A centrifugation step at 15000rpm for 15 min at 4°C led to a two phase separation, of which the aqueous phase (upper phase) was transferred to a fresh tube and mixed with 0.5ml isopropanol to precipitate the RNA. After incubation at room temperature for 10 min and centrifugation at 15000rpm for 10 min at 4°C, the supernatant was transferred to a new tube and stored at -30°C. The remaining RNA pellet was washed with 70% EtOH, vortexed and centrifugated as above. The RNA was air dried and resuspended in 100µl DEPC-treated water.

Photometric determination of RNA quality

Method

The quantitative determination of extracted RNA was assessed by spectralphotometry (Spectronic GENESYS 10 Vis Scanning, Thermo Spectronic). Measurement of the concentration of RNA was performed by an absorbance at 260nm. An A_{260} value of 1 corresponds to 40µg RNA per ml water.

Procedure

The concentrations of the RNA samples were calculated as follows:

$$\textbf{Total RNA (µg/ml)} = A_{260} \textbf{ x 40µg/ml x dilution factor}$$

The dilution factor is the total volume per aliquot measured. 10µl of the samples were diluted in 190µl DEPC-treated water resulting in a dilution factor of 10.

2.2.2 DNase I - Digest & cDNA Synthesis

Method

For several downstream applications, a preparation of DNA-free RNA was neccesary. Therefore, a reaction was set up containing DNase I, an endonuclease that digests single- and double stranded DNA via hydrolization of phosphodiester bonds.

Procedure

The reaction was set up as follows:

Component	Volume (µl)
Total RNA (500ng)	1.0
10x reaction buffer with $MgCl_2$	1.0
DEPC water	to 9µl
DNase I, RNase-free (1u/µl, Fermentas Life Sciences)	1.0
Total Volume	**10µl**

After incubation at 37°C for 30 min, 1µl of 25mM EDTA was added and the reaction further incubated at 65°C for 10 min.

The DNase I-digested RNA was used as template for the cDNA synthesis reaction containing Reverse Transcriptase enzyme:

Component	Volume (µl)
RNA derived from "DNase I digestion reaction"	10
5x SuperScript Buffer	4.0
RNAsin (40U/µl)	0.5
50x dNTP-mixture (10mM each dNTP)	0.4
Random Primer (50ng/µl)	2.0
DTT	2.0
SuperScript™ II Reverse Transcriptase	1.0
DEPC-treated water (nuclease free)	0.1
Total Volume	**20µl**

The cDNA synthesis was performed at 50°C for 1 h in the thermal cycler (T-Gradient, Biometra). In order to inacitvate the Reverse Transkriptase, the reaction was finished at 70°C for 15 min. After cooling down the reaction to 4°C, the synthesized cDNA samples were stored at -20°C.

2.2.3 RT-PCR reactions

GAPDH Test RT-PCR

Method

In order to test the quality of the cDNA, a Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Test RT-PCR Reaction was performed. GAPDH is an essential enzyme which catalyses the the conversion of glyceraldehyde-3-phosohate to glycerate-1,3-bisphosphate (Barber et al. 2005). Amplification of the GAPH gene was carried out with previously published primers (Duvigneau et al. 2003).

Material

GAPDH Primers; T_A = 55°C, 318bp GAPDH RT-PCR product

20mer, T_M = 57.3°C sGAPDH/1$^+$ forward 5' - AAG TGG ACA TTG TCG CCA TC - 3'
19mer, T_M = 56.65°C sGAPDH/1$^-$ reverse 5' - TCA CAA ACA TGG GGG CAT C - 3'

Procedure

The reaction was set up as follows:

Component	Volume (µl)
DEPC-treated water (Nuclease free)	29.75
GoTaq® 5x Reactionbuffer (-$MgCl_2$)	10.0
$MgCl_2$ 25mM	5.0
50x dNTP-mixture (2mM each dNTP)	1.0
GAPDH Forward Primer (10pM/µl)	1.0
GAPDH Reverse Primer (10pM/µl)	1.0
cDNA	2.0
GoTaq® DNA Polymerase (5U/µl)	0.25
Total Volume	**50µl**

The PCR-tubes were placed in the thermal cycler (T-Gradient, Biometra®) and the PCR was performed with the following cycling parameters:

Step	**Temperature**	**Duration**	
Denaturation 1	95°C	2 min	
Denaturation 2	95°C	30 s	35 cycles
Annealing	55°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	5 min	
Hold	15°C	∞	

Proof-reading RT-PCR

Method

Genomic mutations in DNA can be detected by a Proof-reading PCR. For increased fidelity, a proofreading polymerase is suitable for detecting different alleles. This fidelity is about three times higher than that of standard enzymes. Therefore, a RT-PCR was carried out containing a KAPA HiFi^{TM} DNA-Polymerase (Peqlab) which generated less errors occuring during DNA amplification.

Material

Primers; DQB1 T_A = 58°C, 273bp RT-PCR product

T_M = 70.9°C DQB1/2$^+$ forward 5' - CGG AAT TCC CCG CAG/AGG ATT TCG TGT ACC - 3'
T_M = 58.8°C DQB1/2$^-$ reverse 5' - CGG TCG TGC CTT CCT CTA T – 3'

Primers; DRB1 T_A = 59°C, 475bp RT-PCR product

T_M = 56.67°C DRB1/1$^+$ forward 5' - TGT CCT CTC CTG TTC TCC A - 3'
T_M = 59.8°C DRB1/2$^-$ reverse 5' - AGA ACC CAG TCA CAG AGC AGA - 3'

Procedure

The reaction was set up as follows:

Component	Volume (µl)
DEPC-treated water (Nuclease free)	39.0
KAPA HiFi 5x Reactionbuffer	5.0
50x dNTP-mixture (2mM each dNTP)	1.0
Forward Primer (10pM/µl)	1.0
Reverse Primer (10pM/µl)	1.0
genomic DNA (1:10)	2.0
KAPA HiFi DNA Polymerase (1U/µl)	1.0
Total Volume	**50µl**

The PCR-tubes were placed in the thermal cycler (T-Gradient, Biometra®) and the PCR was performed with the following cycling parameters:

Step	Temperature	Duration	
Denaturation 1	95°C	2 min	
Denaturation 2	95°C	30 s	35 cycles
Annealing	58°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	
Hold	15°C	∞	

RT-PCR

Method

Previous published primers (Fang et al. 2005) were used to amplify the DQB1 gene. The primers were located at the beginning of exon 2 (SLA-DQB1/2^{+}; forward primer) and at the end of exon 2 (SLA-DQB1/2^{-}; reverse primer). According to the amplification of DRB1, the forward primer (SLA-DRB1/1^{+}), previously published (Smith et al. 2005 and Ho et al. 2006) was located at the beginning of exon 1 (3 bp upstream of the ATG-codon). The reverse primer (SLA-DRB1/2^{-}) (this study) was located in exon 3. This primer was designed using Primer3 software (http://frodo.wi.mit.edu/primer3).

Material

Primers; DQB1 T_A = 58°C, 273bp RT-PCR product

T_M = 70.9°C DQB1/2^{+} forward 5' - CGG AAT TCC CCG CAG/AGG ATT TCG TGT ACC - 3'
T_M = 58.8°C DQB1/2^{-} reverse 5' - CGG TCG TGC CTT CCT CTA T - 3'

Primers; DRB1 T_A = 59°C, 475bp RT-PCR product

T_M = 56.67°C DRB1/1^{+} forward 5' - TGT CCT CTC CTG TTC TCC A - 3'
T_M = 59.8°C DRB1/2^{-} reverse 5' - AGA ACC CAG TCA CAG AGC AGA - 3'

Procedure

The reactionwas set up as follows:

Component	Volume (µl)
DEPC-treated water (Nuclease free)	29.75
GoTaq® 5x Reactionbuffer	10.0
10x $MgCl_2$ (25mM)	5.0
50x dNTP-mixture (2mM each dNTP)	1.0
Forward Primer (10pM/µl)	1.0
Reverse Primer (10pM/µl)	1.0
GoTaq® DNA Polymerase (5U/µl)	1.0
Total Volume	**50µl**

The PCR-tubes were placed in the thermal cycler (T-Gradient, Biometra®) and the PCR was performed with the following cycling parameters:

Step	**Temperature**	**Duration**	
Denaturation 1	95°C	2 min	
Denaturation 2	95°C	30 s	35 cycles
Annealing	primer specific	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	
Hold	15°C	∞	

2.2.4 Agarose Gel Electrophoresis

Method

The seperation of DNA fragments according to their size was carried out by agarose gel electrophoresis. Negative-loaded DNA migrates towards the anode through a network of polymerized agarose. Smaller DNA fragments migrate faster than longer fragments, resulting in a sharp seperation. Due to the length of the fragments of intrest, different concentrations of agarose gels were used; analysis of RT-PCR products mostly required a concentration of 1.5% (w/v), whereas for the analysis of Plasmid-DNA 1% (w/v) agarose gels were used.

Material

- Electrophoresis Buffer
  50xTAE — 242g Tris-Base
  57.1ml Glacial Acetic Acid
  37.2g Na_2EDTA
  addition of distilled water to a total volume of 1l
  working solution: 1xTAE
- Fisherbrand® Standard Agarose — Fisher Scientific GmbH
- GelRed™ Nucleic Acid Gel Stain (Biotium Inc.): working solution: 1:20, 10µl of working solution is used for 100ml gel solution
- FastRuler™ DNA Ladder Markers (Fermentas Life Sciences) — Low Range (50-1500bp)
  Middle Range (100-5000bp)
  High Range (500-10000bp)
- 6x Loading Dye (Fermentas Life Sciences) — 10mM Tris-HCl (pH 6.7)
  0.03% bromophenolblue
  0.03% xylene cyanol blue
  60% glycerol
  60mM EDTA
- Fotodocumentation — BioDocAnalyze 1.0, Biometra®

Procedure

The desired amount of agorse was dissolved in an appropiate volume of 1xTAE and melted in in a microwave oven. After cooling down the solution, GelRed™ Nucleic Acid Gel Stain was added (10µl per 100ml gel solution). Before pouring the gel solution into the geltray, the casting platforms were sealed and gel comb(s) were inserted. Before the gel was hardened, it was ensured that no bubbles were on the surface of the gels.

Separation of the samples was carried out in 1xTAE running buffer, making sure that the buffer covered the gel to a depth of about 5mm. One volume of 6x Loading Dye was added to 5 volumes of each DNA sample. After loading the samples, an appropiate DNA weight marker was included. After separation, DNA could be visualized by the use of UV-light in order to assess the approximate length of each DNA fragment. The gel was photographed and documented using BioDocAnalyse 1.0, Biometra®.

2.2.5 Recovery of the DNA

Method

For downstream applications such as transformation or restriction digest, it was neccessary to purify DNA fragments of interest after electrophoresis. DNA molecules were absorbed to a column and contaminations such as agarose and dyes were washed away.

DNA bands were cut out of the gel with a sterile razorblade, dissolved in XP2 Binding Buffer and applied to a HiBind® DNA spin-column. After several washing steps and elution with DEPC-treated water, purified DNA was ready for further downstream applications.

Material – peqGOLD Gel Extraction Kit (PEQLAB Biotechnologie GmbH)

- XP2 Binding Buffer
- SPW Wash Buffer
- HiBind® DNA Columns
- 2ml Collection Tubes

→ Supplied with the Kit

Procedure

Due to the fact that prolonged exposure to UV light results in DNA damage, DNA fragments of interest were quickly excised of the gel and transferred into a 1.5ml Eppendorf tube. After determination of the volume of the gel slice, the appropiate volume of Binding Buffer XP2 was added and the gel slice was dissolved by shaking and incubation at 65°C for several min on a heating block. A maximum volume of 750µl of the DNA/agarose solution was poured onto a HiBind® DNA Column and centrifugated for 1min at 13000rpm (Heraeus Biofuge®). The flow-through was discarded and 300µl of Binding Buffer XP2 were added. The HiBind® DNA Column was washed with SPW Wash Buffer twice and dried by centrifugation for 1min at 13000rpm. DNA was eluted with DEPC-treated water in a volume of 30µl.

2.2.6 Ligation Reaction

Method

To generate recombinant DNA sequences, it is necessary to ligate the eluted DNA fragment into a plasmid, an extrachromosomal DNA element. Important features of plasmids are the presence of a resistance gene (e.g. β lactacmase gene for Ampicillin resistance; Amp^r), an origin of replication (ori) and a multiple cloning site (MCS) which harbours serveral recognition sites for restriction enzymes.

Depending on which DNA polymerase has been used, two different ligation cloning systems (pGEM®-T Easy and GeneJET™) were used in this thesis. RT-PCR reactions catalyzed by the GoTaq® DNA Polymerase generate sticky ends whereas the use of the proof-reading KAPA HiFi DNA Polymerase (chapter 2.2.3) generates blunt ends. The protocols for the sticky-end cloning of PCR products with 3'dA overhangs (pGEM®-T Easy) as well as the blunt-end cloning of blunt end PCR products are given below.

pGEM®-T Easy T/A cloning systems

Material

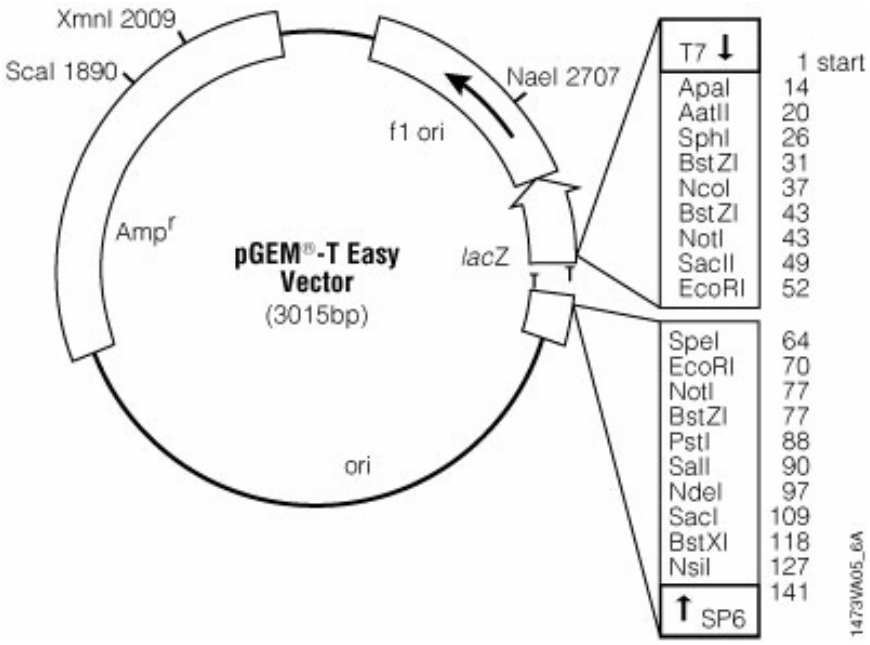

Figure 6. pGEM®-T Easy vector (Promega Corporation)

- 2x Rapid Ligation Buffer, T4 DNA Ligase
- pGEM®-T Easy Vector
- T4 DNA Ligase

The pGEM®-T Easy Vector contains an Ampicillin resistance gene to confirm the uptake of the PCR product on Ampicillin-containing LB plates. T7 and SP6 RNA polymerase promotors flank the MCS which contains several restriction sites for restriction enzymes e.g. EcoRI (**Figure 6**). Furthermore, the pGEM®-T Easy Vector expresses the *lacZ* gene, which allows a blue-white screening of positive transformed bacteria.

Procedure

Amplified and purified DNA was ligated into the pGEM®-T Easy vector and mixed together with the ligation components according to the following recipe:

Component	Volume (µl)
2x Rapid Ligation Buffer, T4 DNA Ligase	5.0
pGEM®-T Easy Vector (50ng/µl)	1.0
purified PCR product	3.0
T4 DNA Ligase (3 Weiss units/µl)	1.0
Total Volume	**10µl**

The reaction was incubated either at room temperature for a minimum of one hour or at 4°C over night.

GeneJET™ PCR cloning Kit

Material

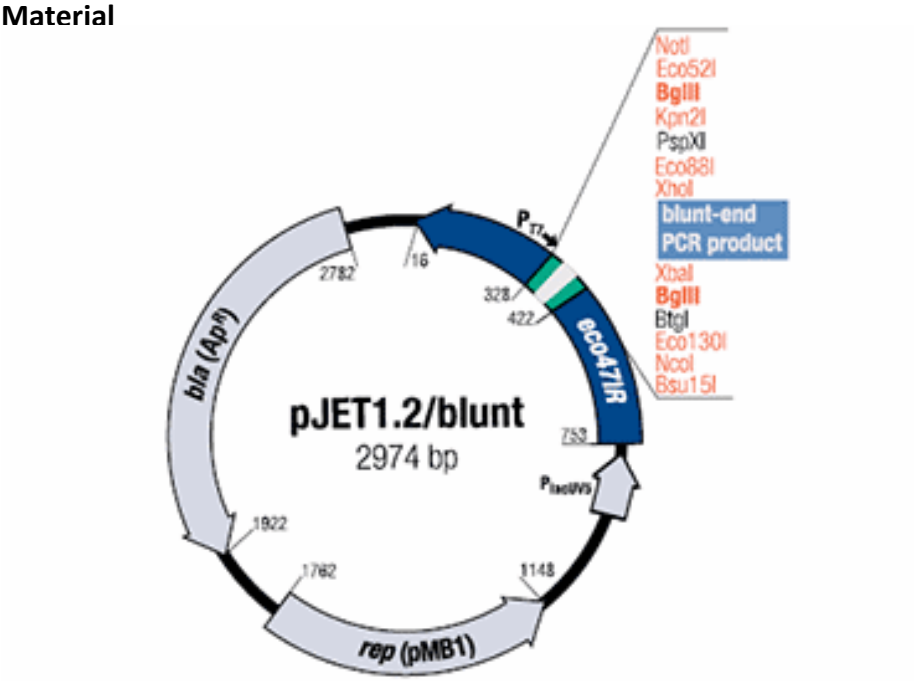

Figure 7. pJET1.2/blunt vector (Fermentas Life Sciences)

- 2x Rapid Ligation Buffer, T4 DNA Ligase
- pGEM®-T Easy Vector
- T4 DNA Ligase
- DNA blunting enzyme

The pJET1.2/blunt vector also contains an Ampicillin resistance gene *bla* (Ap^R) and a MCS for restriction enzymes within the MCS e.g. BglII. The MCS is incorporated into the eco47IR gene which produces a toxic protein, lethal for the host. Disruption of this gene by inserting the purified PCR product into the MCS leads to survival of the bacterial host (**Figure 7**).

Procedure

Due to the fact that *Taq* DNA polymerase generates PCR products with 3'-dA overhangs; a blunting reaction was required to make sure that the PCR product was ligated properly into the MCS:

Component	Volume (µl)
2x Rapid Ligation buffer, T4 DNA Ligase	10.0
purified PCR product	6.0
DEPC-treated water (Nuclease-free)	1.0
DNA blunting enzyme	1.0
Total Volume	**18µl**

The reaction was incubated at 70°C for 5 min and cooled down on ice for several min. Afterwards the ligation reaction was set up by adding the following ingredients for the blunting reaction mixture:

Component	Volume (µl)
pJET1.2/blunt Vector	1.0
T4 DNA Ligase (5 Weiss units/µl)	6.0

The ligation reaction was incubated at room temperature for 30 min or at 4°C overnight.

2.2.7 Transformation

To obtain transformed clones the introduction of ligated products into competent *Escherichia coli* cells was carried out as followed:

Transformation - pGEM®-T Easy vector

Method

The blue/white screening of transformants via the pGEM®-T Easy vector is dependent on the use of IPTG and X-Gal. IPTG works as an inducer of the lac operon, thereby activating the transcription of beta-galactosidase (encoded by *lacZ*). Beta-galactosidase is able to cleave X-Gal which leads to an oxidation step resulting in the production of 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product. Plasmids carrying the insert generate white clones, because the *lacZ* gene is interrupted and thereby unable to express beta-galactosidase. Clones which carry the plasmid without the insert appear as blue clones, expressing functional beta-galactosidase.

Material

- competent *E. coli* (JM109 > 10^7 cfu/µg, Promega)
- Luria broth (LB)-medium (per l)
  10g tryptone
  5g yeast extract
  5g NaCl
  adjusted to pH 7.0 with NaOH, after autoclaving, the medium was cooled down to 50°C before adding Ampicillin to a final concentration of 100µg/ml
- LB/Amp^{100} plates
  15g of agar was added to LB medium and approximately 30ml of medium was poured into 85mm petri dishes
- SOC medium (per liter)
  20g Trypton
  5g Yeast-Extract
  10mM NaCl
  2.5mM KCl
  10mM $MgCl_2$
  10mM $MgSO_4$
  20mM Glucose

Additional material for pGEM®-T Easy vector

- IPTG (40µl per LB/Amp^{100} plate) 100mM in DEPC-treated water
- X-Gal (40µl per LB/Amp^{100} plate) 50mg dissolved in 1ml Dimethylformamid

Procedure

2µl of pGEM®-T Easy ligation reaction was added to 50µl competent *E. coli* cells. After incubation of the transformation mixture for 20 min on ice, the mixture was quickly transferred in a water bath for 1 min at 42°C. After a retreat of the transformation mixture on ice (2 min), 500µl of SOC medium was added and the cells were shaked for 60 min at 750rpm and 37°C on the heating block. Meanwhile, the LB/Amp^{100} plates were prepared with IPTG/X-Gal (40µl each) by spreading 80µl over the surface. The solution was absorbed for about an h at room temperature and ready to use shortly after. 2x 250µl of the transformation mixture was spread onto LB/Amp^{100} plates and incubated at 37°C overnight.

Transformation - pJET1.2/blunt vector

Method

Transformation by the application of the pJET1.2/blunt vector does not require any IPTG/X-Gal pre-treated LB/Amp^{100} plates. Screening for positive transformants is obtained via the lethal gene eco47IR on the pJET1.2/blunt vector. The DNA fragment is ligated into the MCS which is located in the eco47IR gene. Disruption of this gene enables the positive selection of the recombinants by allowing the transformed *E. coli* to grow.

Procedure

4µl of the pJET1.2/blunt ligation reaction was added to 50µl competent *E. coli*. After incubation of the transformation mixture for 20 min on ice, the mixture was quickly transferred in a water bath for 1 min at 42°C. After a retreat of the transformation mixture on ice (2 min), 500µl of SOC medium was added and the cells were shaked for 60 min at 750rpm and 37°C on the heating block. 2x 250µl of the transformation mixture was spread onto LB/Amp^{100} plates and incubated at 37°C overnight.

2.2.8 Screening for positive transformants (Colony PCR)

Method

At least 16 clones per animal were picked from LB/Amp100 plates to check for positive transformants. The verification was done by PCR, using gene-specific primers and the bacteria suspension as DNA template.

Material

Primers; DQB1 T_A = 58°C, 273bp RT-PCR product

T_M = 70.9°C DQB1/2$^+$ forward 5' - CGG AAT TCC CCG CAG/AGG ATT TCG TGT ACC - 3'
T_M = 58.8°C DQB1/2$^-$ reverse 5' - CGG TCG TGC CTT CCT CTA T - 3'

Primers; DRB1 T_A = 59°C, 475bp RT-PCR product

T_M = 56.67°C DRB1/1$^+$ forward 5' - TGT CCT CTC CTG TTC TCC A - 3'
T_M = 59.8°C DRB1/2$^-$ reverse 5' - AGA ACC CAG TCA CAG AGC AGA - 3'

Procedure

Under semi-sterile conditions bacterial colonies were picked with a toothpick from the LB-plates and were first tipped on a backup-plate and then transferred into 100µl H_2O containing Ampicillin. In order to detach the remaining colony, the toothpick was vortexed shortly after. After a denaturation step of 5 min has led to lysis of the bacteria, the suspension served as DNA template. The colony-PCR reaction was set up as follows:

Component	Volume (µl)
DEPC-treated water (Nuclease free)	10.875
GoTaq® 5x Reactionbuffer (-$MgCl_2$)	5.0
$MgCl_2$ 25mM	2.5
50x dNTP-mixture (2mM each dNTP)	0.5
Forward Primer (10pM/µl)	0.5
Reverse Primer (10pM/µl)	0.5
GoTaq® DNA Polymerase (5U/µl)	0.125
Cell suspension	5.0
Total Volume	**25µl**

The PCR-tubes were placed in the thermal cycler (T-Gradient, Biometra®) and the PCR was performed with the following cycling parameters:

Step	**Temperature**	**Duration**	
Denaturation 1	95°C	5 min	
Denaturation 2	95°C	30 s	30 cycles
Annealing	60°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	5 min	
Hold	15°C	∞	

2.2.9 Plasmid Minipreparation (Plasmid Miniprep)

DirectPrep® 96 Miniprep

Method

For sequencing DRB1 and DQB1 alleles, 16 clones of each animal have been picked. In respect of 12 animals to be sequenced, this resulted in 196 different clones. In respect of material and time consuming aspects, the DirectPrep® 96 Miniprep-Kit (QIAGEN GmbH) was used to isolate Plasmid DNA. This kit enables the Plasmid-DNA purification of 16 clones of 6 different animals on a 96-well plate simultaneously.

Material

- DirectPrep 96 Plates
- Buffer P1
- Buffer P2
- Buffer DP3
- Buffer PE
- RNase A
- Flat-Bottom Blocks
- Lids for Flat-Bottom blocks
- AirPore™ Tape Sheets
- Tape Pads
- Elution Microtubes RS

} supplied with the Kit

- Isopropanol
- LB-medium

10g tryptone
5g yeast extract
5g NaCl
adjusted to pH 7.0 with NaOH, after autoclaving, the medium was cooled down to 50°C before adding Ampicillin to a final concentration of 100µg/ml

Procedure

Approximately 1.25ml of LB-Medium was poured into each well of the 96-well plate. 95µl of each cell suspension was filled into a single well and the cultures were incubated for 24 h at 37°C at 250rpm. Thereafter the bacteria were harvested by centrifugation for 5 min at 3600rpm (Heraeus Multifuge 1®). The medium was then removed by inverting the block. The bacteria pellets were resuspended in 150µl Buffer P1, completed with RNase A. The same volume of Buffer P2 was added and the block was sealed with an AirPore™ Tape Sheet and

gently inverted six times to mix. After removing the tape, 150µl of Buffer DP3 was added sealed again and gently inverted a second time. Again, the tape was removed and 300µl isopropanol were added into each well. Tightly sealed, the block was inverted the last time. The lysates were then pipeted into the DirectPrep 96 Plate, which was combined with a waste tray underneath, centrifugated at 5000rpm for five min and after addition of 0.75ml of Buffer PE the DirectPrep 96 Plate was centrifugated again under the same conditions. The waste tray was replaced by the elution microtube adapter and the elution was carried out by adding 75µl Buffer EB into each well of the DirectPrep 96 Plate.

peqGOLD Plasmid Miniprep Kit I

Method

For purifaction of Plasmid-DNA from a suspension number less than 96, the peqGOLD Plasmid Miniprep Kit I was used. The first steps included lysis of bacteria and neutralisation of the lysate. Shortly after binding of the plasmid-DNA on a HiBind® miniprep column and several washing steps, the plasmid-DNA was eluted with Elution buffer.

Material - peqGOLD Plasmid Miniprep Kit I (PEQLAB Biotechnologie GmbH)

- HiBind® Columns
- 2ml Collection Tubes
- Solution I
- Solution II
- Solution III
- Buffer HB
- DNA wash buffer
- Rnase A
- Elution Buffer (10mM Tris-HCl, pH 9.0)

} supplied with the Kit

- LB-medium

Procedure

About 95µl of each positively identified cell suspension were added into 4ml LB-Medium containing Ampicillin (1µl per 1ml of LB-Medium). Bacteria grew for at least 16 h at 37°C and 250rpm. After centrifugation (13000rpm, 5 min, Heraeus Biofuge®) of the cultures, bacterial pellets were ready for the Plasmid Miniprep.

The bacterial pellets were lysed by adding 250µl of SolutionI/RNase A via resuspension. Addition of 250µl of Solution II led to cleared lysates by inverting the tube 4-6 times and incubation for 2 min at room temperature. By addition of 350µl of Solution III a white precipitate was formed and subsquently the lysate was centrifugated at 13000rpm for 10 min. Binding on the HiBind® miniprep column was executed by transferring the supernatant into the column, ensuring that no cellular debris were carried over and centrifugation (13000rpm, 1 min). The column was washed once with 500µl of HB Buffer and twice with 750µl of Wash buffer and completed with ethanol. After drying the HiBind® column at 13000rpm for 1 minute, the Plasmid-DNA was eluted in 100µl of Elution Buffer.

Restriction Digest

Method

In order to check whether the vector has incorporated the purified PCR product properly, a restriction digest was performed. Several recognition sites for restriction enzymes help to detect the insert within the MCS. Depending on the vector, two different restriction enzymes were used. Application of the pGEM®-T Easy vector required a digest by EcoRI, pJET/blunt vector is cut by BglII (see MCS in **Figure 6** and **Figure 7**).

Material

The palindromic nucleic acid sequences which are cut by these enzymes, are listed below:

EcoRI 5' - G|AATTC - 3'
3' - CTTAA|G - 5'

BglII 5' - A|GATCT - 3'
3' - TCTAG|A - 5'

Both Eco RI and BglII cut after the first base of the 5’ strand and after fifth base of the 3’ strand, thereby generating “sticky ends” within the recognition site.

Procedure

The restriction digest-reaction was set up as follows:

Component	Volume (µl)
10x enzyme-specific digestion buffer	2.0
Restriction enzyme (EcoRI/BglII) (10U/µl)	1.0
DEPC-treated water (Nuclease free)	15.0
Plasmid DNA	2.0
Total Volume	**20µl**

The tubes were placed on a heating block at 37°C for at least 1 h. 3µl of 6x Loading Dye was added to the digestion mixture. The plasmid and its insert was visualized unter UV-light after running the samples on an agarose gel (1% (w/v) agarose, 1xTAE).

2.2.10 DNA-Sequencing of positive clones

Positive clones were sent to Eurofins MWG GmbH and sequenced with standard primers. Depending on the number of clones to be sequenced, 15µl of Plasmid DNA was either transferred into a 1.5µl Eppendorf tube or into a 96-well plate.

Primers for cloning with pGEM®-T Easy T/A cloning system

M13 sequencing primers: M13 uni (-21): 5’ - TGT AAA ACG ACG GCC AGT - 3’
M13 rev (-29): 5’ - CAG GAA ACA GCT ATG ACC - 3’

Primers for cloning with GeneJET™ PCR cloning Kit

pJET/blunt: pJET_fwd: 5’ - GCC TGA ACA CCA TAT CCA TCC - 3’
pJET_rev: 5’ - GCA GCT GAG AAT ATT GTA GGA GAT C - 3’

2.2.11 Sequence analysis

Sequences obtained by Eurofins MWG GmbH were analysed and interpreted using the sequencing alignment editors CodonCode Aligner (version 3.0.1; CodonCode Corporation, USA), BioEdit Sequence Alignment Editor (version 7.0.9.0; Hall 2001) and ClustalX (version 1.8; Thompson et al.). First, the primer sequences were clipped off in BioEdit, thereby generating only the coding sequence. Then, the sequnces were aligned with all known sequences from the Immuno Polymorphism Database (IPD) which served as reference alleles. Together, these sequences were further analysed in ClustalX which generated a phylogenetic tree of aligned sequences.

2.3 Polymerase chain reaction - Sequence specific primer (PCR-SSP)

2.3.1 DNA Extraction

Method

In contrast to the SBT-method, the PCR-SSP typing method is based on analyses of DNA. The lysis of cellular components is an essential step during extraction in order to obtain porcine DNA of interest. The isolation of genomic DNA was carried out by the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich®).

Material

- Lysis Solution C
- Column Preparation Solution
- Wash Solution Concentrate
- Elution Solution (10mM Tris-HCl; 0.5mM EDTA, pH 9.0)
- Proteinase K
- RNase A Solution
- GenElute Miniprep Binding Columns
- Collection tubes, 2.0ml capacity

} supplied with the Kit

Procedure

20µl of Proteinase K and up to 200µl of blood samples were placed into 1.5ml Eppendorf tubes. After the additon of 200µl RNase A Solution/per tube, the solutions were incubated for two min at room temperature. After the addition of Lysis Solution C the tubes were vortexed for 15 sec and incubated at 55°C for 5 min. 500µl of the Column Preparation Solution were added to GenElute Miniprep binding Column and centrifugated at 13000rpm for one min (Heraues Biofuge®). The flow-through was discarded and 200µl of EtOH (96%) was added to the lysates and mixed thoroughly by vortexing for 10 s. The entire content was transferred into the treated binding column and centrifugated at 8200rpm for one min. The binding column was placed onto a new Eppendorf tube and washed twice with 500µl Wash Solution at 8200rpm for one min. To dry the the binding colunm, it was centrifugated at 13000rpm for 3 min. In order to elute DNA, 200µl of Elution Soultion were placed in the center of the column and centrifugated at 8200rpm for one min. Subsquentely, purified DNA was quantified via spectralphotometry.

Photometric determination of DNA quality

Method

The quantitative determination of extracted DNA was assessed by spectralphotometry (Spectronic GENESYS 10 Vis Scanning, Thermo Spectronic). Measurement of the concentration of DNA was performed by an absorbance at 260nm. An A_{260} value of 1 corresponds to 50µg DNA per ml water.

Procedure

The concentrations of the DNA samples were calculated as follows:

$$\textbf{Total DNA (µg/ml)} = A_{260} \textbf{ x 50µg/ml x dilution factor}$$

The dilution factor is the total volume per aliquot measured. 20µl of the samples were diluted in 180µl DEPC water resulting in a dilution factor of 10.

2.3.2 PCR-SSP Typing

Method

94 discriminatory PCR primer pairs, which were kindly provided by Chak-Sum Ho, Department of Pathology, University of Michigan, were the basis for typing groups of alleles of the three classical SLA class I loci (SLA1, SLA3, SLA2) and the three classical SLA class II loci (DQB1, DRB1, DQA). These primer pairs helped to differentiate allele groups which share similar sequence motifs. Several primer pairs were designed to detect tentative alleles without group designation (e.g. *SLA1*rh03*). Most of the PCR primer pairs detected allele specificities characterized by the low-resolution group-specific SLA typing (e.g. *SLA1*01XX*). The first two digits (*01*) indicate the group of alleles, the third and fourth digit (*XX*) characterize low-resolution group specificity (Ho et al. 2009a).

Each primer pair comprised an internal control, the α-actin gene which displayed its band at 516 bp. Positive allele groups were displayed by size-characteristic bands which were used to be smaller than the positive control (α-actin). Most of the primer pairs were able to

identify one group of allele only. In case of SLA class I, eight primer pairs (P2079D2, N2002, P1172D3, P3116, P2046D3, P2054U1, P2174, P2140D1) were able to detect detect two different loci.

After the identification of the primer pairs and the subsequent assignment of the allele groups, it was neccesary to define the Low Resolution (Lr) Haplotype for each pig typed. For SLA class I (SLA1, SLA3, SLA2) as well as for SLA class II (DQB1, DRB1, DQA) genes, each detected allele group was assigned to its corresponding locus. The principle of the definition of Lr-haplotypes, were the combination of three allele groups within each SLA class.

In respect of nomenclature, inferred SLA class I haplotypes were termed Lr-XX.0, whereas SLA class II hapltoypes were termed Lr-0.XX.

Material

- SLA class I and class II primer pairs (Ho et al. 2009b and 2010)
- 96 well-plates
- Fotodocumentation, BioDocAnalyze 1.0, Biometra®

Procedure

For each pig to be typed, the DNA was diluted 1:10 and prepared to a total volume of 200µl, resulting in a final concentration of about 30ng. PCR reactions included an internal positive control (*ACTA1*) which helped to interpret a potential presence of smaller PCR products. The Master Mix (MM) was prepared for each DNA sample on ice according to the following recipe:

Component	Volume (µl)	Sample number	Master Mix (MM)
DEPC water	2.45	52	127.40
5x Green Go Taq flexi buffer	2.00		140.00
10x $MgCl_2$ (25mM)	0.80		41.60
BSA (1µg/µl)	0.50		26.00
50x dNTPs (10mM each dNTP)	0.20		10.40
GoTaq Flexi DNA Polymerase (5U/µl)	0.05		2.60
DNA (~30ng)	2.00		
Forward+Reverse Primer (5pmol/each)	2.00		
Total Volume	**10µl**		**312µl**

Before DNA samples were added, 8.0µl of the MM was transferred into the appropiate negative control well. Afterwards, 104.0µl of DNA was added to the MM and vortexed thoroughly. Ensuring that the negative control well was skipped, 8.0µl of the MM was transferred into each of the remaining 47 reaction wells. The PCR-plate was tightly sealed and kept on ice until the thermal cylcer (T-Gradient, Biometra) was ready.

The PCR-tubes were placed in the thermal cycler (T-Gradient, Biometra®) and the PCR was performed with the following cycling parameters:

Step	**Temperature**	**Duration**	
Denaturation 1	95°C	2 min	
Denaturation 2	95°C	15 s	30 cycles
Annealing	65°C	20 s	
Extension	72°C	20 s	
Final extension	72°C	3 min	
Hold	15°C	∞	

Positive allele groups were identified by electrophoresis (3% (w/v) agarose, 1xTAE).

2.4 SLA Expression Profiling

2.4.1 Studied cell lines

Porcine *in vitro* studies with transmissible gastroenteritis virus (TGEV) suggest that the intestine is a crucial area where the infection resides. Therefore, two different types of cells have been isolated and characterized. According to the attributes of growth rate and morphology they were termed Type I and Type II cells. (Ephraim et al. 2009) **Table 3** summerizes the main attributes of the two cell lines.

	Type I cells	**Type II cells**
Growth rate	rapid growth	humble growth
Morphology	small; homogenous monolayer	large; inhomogenous cells
Size and granularity	homogenous	inhomogenous

Table 3. Characteristics of Type I and Type II cells.

Both cell types display a typical epithelial morpholgy with microvilli on the surface. Type I cells show a less frequent occurance of organelles than Type II cells (Ephraim et al. 2009).
An additional aim of this thesis was to characterize the gene expression of four SLA class I genes (SLA1, SLA2, SLA3, SLA6) and four SLA class II genes (DQA, DRA, DQB1, DRB1). cDNA of Type I and Type II cells, which was kindly provided by Michael Burwinkel (Institute of Immunology and Molecular Biology, Faculty of Veterinary Medicine, Freie Universität Berlin, Germany) were characterized in respect of their SLA expression. The main aspect of this study was to identify whether those cells were active in any immunological manner. For this analysis, RT-PCRs with SLA-specific primers were carried out and SLA expression was detected by Southern Blot hybridization with gene specific probes.

2.4.2 Primers

(Non)-labeled Primer

To investigate the SLA expression in Type I and Type II cells, two different forward primers for each SLA gene were used (**Table 4**). Each of the six forward primers has been labeled with Digoxigenin and was used in a RT-PCR together with unlabeled reverse primers. Additionally, a second RT-PCR approach was set up as a control, including unlabeled forward primers as well as unlabeled reverse primers.

DIG-labeled Primers

In order to avoid radioactive labeling and detection of nucleic acids, the DIG-System provides a safe detection technology. Many advantages such as high sensitivity, low background and stability of labeled probes made the DIG system to a convient method for detecting nucleic acids. Primers are modified via addition of a single DIG-11-ddUTP to the 3'- end of the oligonucleotide by a terminal transferase.

After hybridization, the detection of DIG probe-target hybrids on membranes was visualized by α-DIG-Alkalnie Phosphatase.

Locus	Forward primer (5' → 3')	Reverse primer (5' → 3')	T_A (°C)	Reference	Amplicon size (bp)	Extension time (seconds)
DQA	GCC TGT GGA GGT GAA GAC AT	CCA CAT GAC AGA TGA GGG TG	59	this study	349	30
DRA	CCT TCA GAA ATC ATG GGC TA	ACA GGC TTG TCT GAG AGC AC	59	this study	309	30
DQB1	CGG AAT TCC CCG CAG/AGG ATT TCG TGT ACC	CGG TCG TGC CTT CCT CTA T	58	Fang et al. 2005	273	30
DRB1	TGT CCT CTC CTG TTC TCC A	AGA ACC CAG TCA CAG AGC AGA	59	this study & Smith et al. 2005a	475	30
SLA1	CCT CTT CCT GCT GCT GTC G	ACT CCA CAC ACA GTC CCT GC	60	Ando et al. 2003	548	45
SLA2	GCC ATC CTC ATT CTG CTG TC	AGC GTG TCC TTC CCC ATC T	55	Ando et al. 2003	587	45
SLA3	GAG CCC TCT TCT TGC TGC TGT C	CCT CCA GGT AGC TTC TCA TTT GCT	58	this study	537	45
SLA6	CCT CCC TGT TAT AGG ATC CCA CTC G	CAG CAC GTC CTT CCC CTT CT	60	this study	550	45

Table 4. Primer pairs used in this experiment.

2.4.3 RT-PCR

Procedure

Following recipe was set up for each SLA gene (SLA1, SLA2, SLA3, SLA6, DQA, DRA, DQB1, DRB1) and proceeded under different conditions. The different parameters, which depend on the primers used, are listed above. The reaction, which was mixed on ice, was set up as follows:

Component	Volume (µl)
DEPC-treated water (Nuclease free)	29.75
GoTaq® 5x Reactionbuffer (-$MgCl_2$)	10.0
$MgCl_2$ 25mM	5.0
50x dNTP-mixture (2mM each dNTP)	1.0
(DIG labeled) Forward Primer (10pM/µl)	1.0
Reverse Primer (10pM/µl)	1.0
cDNA (200ng/µl)	2.0
GoTaq® DNA Polymerase (5U/µl)	0.25
Total Volume	**50µl**

The PCR-tubes were placed in the thermal cycler (T-Gradient, Biometra®) and the PCR was performed with the following cycling parameters:

Step	**Temperature**	**Duration**	
Denaturation 1	95°C	2 min	
Denaturation 2	95°C	30 s	30 cycles
Annealing	primer specific	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	
Hold	15°C	∞	

RT-PCR products were separated via agarose gel electrophoresis (2% (w/v), 1xTAE) and isolated under UV-light.

2.4.4 Southern Blot

Method

Southern Blotting, which was developed by Edward M. Southern, is a common used strategy for detection of DNA sequences. Based on the seperation of DNA samples through agarose gel electrophoresis (see 2.2.4) and the subsequently transfer onto a nylon membrane, this technique is an essential element for the analysis of specific DNA samples.

DNA of intrest was first amplified by PCR and followed by agarose gel electrophoresis resulting in separated DNA accoring to size. After several washing steps and a denaturation step, DNA was transferred onto a nylon membran overnight. The membrane was then exposed to a specific hybridization probe (DIG-labeled primers) with specificity of DNA samples of intrest. α-DIG antibodies were applied to locate positions of bands complementary to the probe.

Material

- cDNA (Type I + Type II)
- DIG-labeled Primers
- Nylon membrane, Hybond^{TM}-N (Amersham Biosciences)
- Blotting Paper, Grade 703 (VMR)
- α-DIG Antibody, 150U (200µl) (Roche)
- DNA Molecular Weight Marker (Roche) — DIG-labeled (VI), Fragment sizes: 0.15-2.17kbp
- Denaturation solution — 0.5M NaOH
1.5M NaCl
- Neutralization solution — 0.5M Tris-Base
3M NaCl, pH 7.3
- Pre-hybridization solution — 5xSSC
Blocking reagent 1% (w/v)
N-Lauroylsarcosin 0.1% (w/v)
SDS 0.02% (w/v)
- Buffer 1 — 0.1M Tris/HCl
0.15M NaCl

- Buffer 2 — Blocking reagent 1% (w/v) in Buffer 1
- Buffer 3 — 0.1M Tris-Base
0.1M NaCl
0.005M $MgCl_2$, pH 9.5
- Buffer 4 (=TE) — 0.01M Tris-Base
0.001mM Na_2-EDTA
- Staining solution — 10ml Buffer 3
45µl NBT (50mg NBT/ml DMF 70%) (Promega)
35µl X-Phosphate = BCIP (50mg/1ml DMF 100%) (Promega)
- Other solutions — 0.5M HCl
20xSSC
2xSSC
1xSSC + 1% SDS
1xSSC

Procedure

The gel was soaked in 0.5M HCl and shaked (50rpm) until a colour change to yellow was observed. The gel was then washed with Aqua dest. and shaked in denaturation solution for 40 min. After again washing with Aqua dest., the gel was shaked twice in neutralization solution and swilled with Aqua dest. in between and afterwards. Gel, blotting paper and nylon membrane were soaked in 2xSSC and the membrane was blotted overnight.

The next day, the membrane was washed with 2xSSC and dried on the heating block. To ensure that DNA is tightly bound, the membrane was irradiated with UV-light for 4 min. The membrane was then incubated in pre-hybridization solution in the hybridization oven at 50-65°C (T_m -5°C) for 2 hours. Afterwards, the DIG-Oligo was added to the pre-hybridization solution and the membrane was incubated under the same conditions as before.

The membrane was then washed four times with 1xSSC; 1% SDS (5 min each) and once with 1xSSC only. After shaking the membrane in Buffer 1 for 1 min, it was blocked in buffer 2 for 1 h. α-DIG antibody was diluted 1:5000 in buffer 2 and incubated on the membrane for 30 min. Then, the membrane was washed twice in Buffer 1 for 15 min and incubated in buffer 3 for 2 min. To visualize potential DNA bands, the nylon membrane was shrinkwraped together with the staining solution. The staining reaction was stopped by buffer 4.

2.5 Isolation of PBMCs

Method

For several downstream applications - such as flow cytometry (FCM) - it is necessary to isolate mononuclear cells form whole blood samples.

The isolation of PBMCs from heparinized blood was carried out according to the SOP (Standard Operatitng Procedure) of “Isolation of procine Peripheral Blood Mononuclear Cells (PBMC)” of the Institute of Immunology, Vetmeduni Vienna.

Material

- Heparinized blood
- PBS
- Lymphocyte separation medium (LSM, density 1,077g/liter)
- Cell culture medium
- Wash medium

Procedure

15ml of LSM were placed into a sterile 50ml conical centrifuge tubes. Blood, together with the same volume of PBS was gently mixed in an Erlenmayer flask. In order to avoid separation prior to centrifugation, the blood/PBS mixture was carefully layed over the LSM and filled up to 50ml. The mixture was then centrifugated for 30 min at 2100rpm (Heraeus Multifuge 1®), with an acceleration of 4 and decelaration of 1. Meanwhile new sterile 50ml tubes were placed on ice. After centrifugation the upper layer, which contains the plasma, was removed and collected in a sterile bottle. PBMCs, which are collected in the interphase, were carefully removed using a 5ml short pipet and transferred into the ice cooled 50ml tubes. With 2.5 volumes of the PBMC layer, the cells were washed with PBS and centrifugated for 30 min at 1300rpm at 4°C. After removing the supernatant and resuspending the cells in PBS, the cells from the same animal were pooled into one tube filled with PBS and centrifugated as above. The supernatant was removed again and after resuspending, the cells were washed with wash medium and centrifugated. Depending on the amount of blood used at the beginning, 5 to 20ml of cell culture medium was added to the cells. An amount of <100µl of the cell suspension was transferred into an Eppendorf cup and freezed down for future use.

In order to count isolated cells, 90µl of each Trypan Blue and Türk's solution were placed into two wells of a microtiter plate. After addition of 10µl of cell suspension, dead cells were checked in the Trypan Blue chamber and cells in the chamber with Türk's solution were counted.

III Results

3.1 DNA sequence-based SLA typing

3.1.1 RNA Isolation and Reverse Transcription

RNA of 12 Pietrain pigs was isolated from frozen blood samples (-80°C) via the peqGOLD Blood RNA Kit. To quantify the RNA concentration, RNA was diluted in 100ml DEPC water and the A_{260} and A_{280} were determined with the Spectrophotometer (Spectronic GENESYS 10 Vis Scanning, Thermo Spectronic). A 5µl aliquot of eluted RNA was analyzed by agarose gel electrophoresis (1.5% (w/v) agarose gel) (data not shown). Due to the A_{260} and A_{280} determination, the volume of RNA used in cDNA synthesis was calculated, the remaining RNA was stored at -80°C.

To check the quality of obtained RNA in regard to DNA contaminations, a GAPDH Test RT-PCR reaction was performed. Usually, a band at 318bp is expected after amplification of mRNA of the GAPDH gene. Possible DNA contamination would exhibit a PCR product of 640bp in length due to one additional intron being amplified. In order to obtain pure RNA without any contamination, a DNase I digest is necessary.

Figure 8 shows a GAPDH RT-PCR reaction after DNase I digest. RNA was isolated from the homozygous pig dd1156 which was used as positive control throughout the molecular cloning steps. All of the 4 aliquots display the GAPDH band at 318bp, as well as no contamination of DNA. Brighter bands (#1 and #2) indicate higher RNA concentrations in contrast to the samples #3 and #4. In order to introduce an additional control, the 4 RNA samples were either transcribed with (RT^+) or without (RT^-) reverse transcriptase during cDNA synthesis. As expected, all 4 RT^- samples display no GAPDH specific bands.

The 12 Pietrain pigs were tested at two different SLA loci (SLA-DQB1 and SLA-DRB1) and further haplotyped according to their identified alleles. Due to reverse transcription of SLA-DQB1 published primers were used (Fang et al. 2005), as well as for reverse transcription of SLA-DRB1 (Ho et al. 2006). The obtained PCR products showed a length of 475bp (DRB1) and 273bp (DQB1).

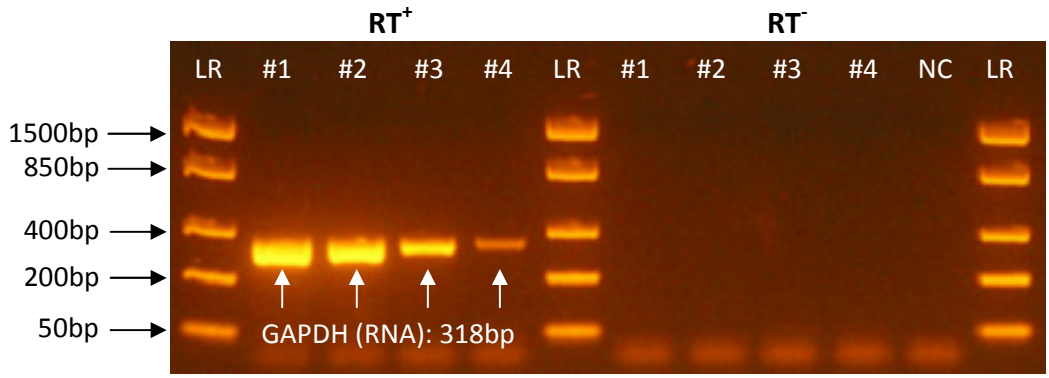

Figure 8. GAPDH RT-PCR. 4 aliquots of RNA (dd1156) were isolated. The previous step of cDNA synthesis was carried out either with (RT^+) or without (RT^-) reverse transcriptase. LR: Low Range; RT: reverse transcriptase; NC: negative control; 1.5% (w/v) agarose.

3.1.2 Molecular Cloning of PCR products

The cloning of DNA sequences obtained by RT-PCR required an extrachromosomal vector to ligate PCR products into plasmids. After the ligation of DQB1 and DRB1-PCR products into the pJET1.2/blunt vector, vector plus insert were transformed into *E. coli* JM109. To test, whether the ligation was successful, a Colony-PCR with the DQB and DRB primers described above, was carried out. **Figure 9** shows a representative picture of a colony PCR screening for positive DQB1 transformants in animal #43 and #47. Every clone which has been picked, displays a distinct band, indicating a successful ligation of the PCR product into the pJET1.2/blunt vector.

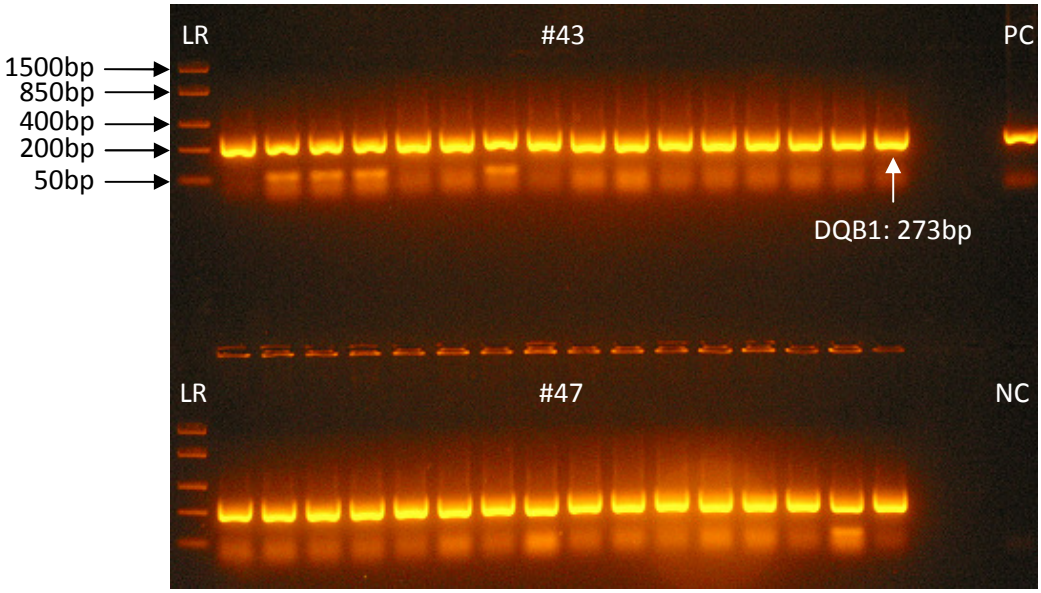

Figure 9. Colony-PCR. Representative picture of a colony PCR (animal #43 and #47; DQB1). In this particular case, each of the 16 clones picked was positively transformed. LR: Low Range; PC: positive control, NC: negative control, 1.5% (w/v) agarose.

3.1.3 Plasmid-Miniprep and Restriction digest

16 positively identified transformants per SLA-loci were selected for Plasmid Minipreparation to obtain plasmid-DNA. To check the integrity of the insert, the plasmids were either cut with EcoRI or BglII, depending on which vector has been used for ligation. **Figure 10** shows cutted pGEM-T Easy vector (with EcoRI), previously ligated with the DRB1 insert.

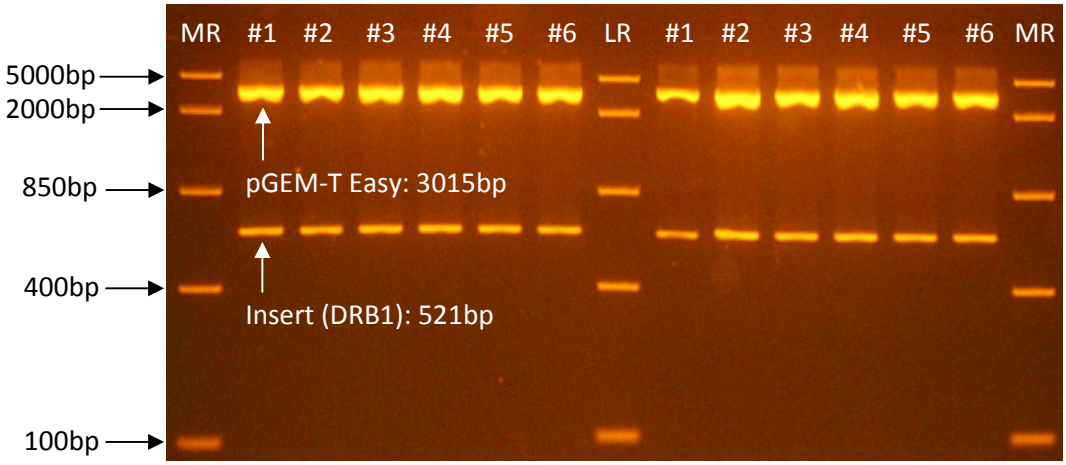

Figure 10. Restriction digest. Representative picture of Plasmid-DNA of 12 clones which were purified via Plasmid Minipreparation and subsequently cut with EcoRI performing a restriction digest. MR: Middle Range; 2% (w/v) agarose.

3.1.4 Sequencing Analysis

16 clones per animal were sequenced with M13 sequencing primers. Obtained sequences were subsequently clipped, ensuring that both vector sequence and primer sequence were deleted, thereby generating only the sequence of interest. All sequences were analyzed using Codon Code Aligner, BioEdit and ClustalX in order to do a complete alignment with the reference alleles available in the IPD (http://www.ebi.ac.uk/ipd/mhc). Finally, a phylogenetic tree was generated including found alleles and reference alleles.

3.1.5 DQB1 Sequencing Outcome

In the 12 sequenced Pietrain pigs, 2 alleles are the major key player within the DQB1-Locus, SLA-DQB1*0801/*08ch01 and SLA-DQB1*0701. In 8 animals, at least one of those alleles could be identified and 5 animals even expressed both alleles. Other found alleles were SLA-DQB1*0101, present in 3 animals and the alleles SLA-DQB1*0601 and SLA-DQB1*0302/0303, each found only in one animal, respectively. Two animals, #43 and #57 were found to be homozygous for the DQB1-locus. Obtained results are displayed in **Table 5** and **Figure 11**.

	0801	0701	0101	0601	0302	0201
	08ch01				0303	
#38						
#39						
#40						
#41						
#42						
#43						
#47						
#51						
#53						
#54						
#57						
#59						

Table 5. DQB1 Annotation table. Found DQB1-alleles in 12 Pietrain pigs, using the SBT-method.

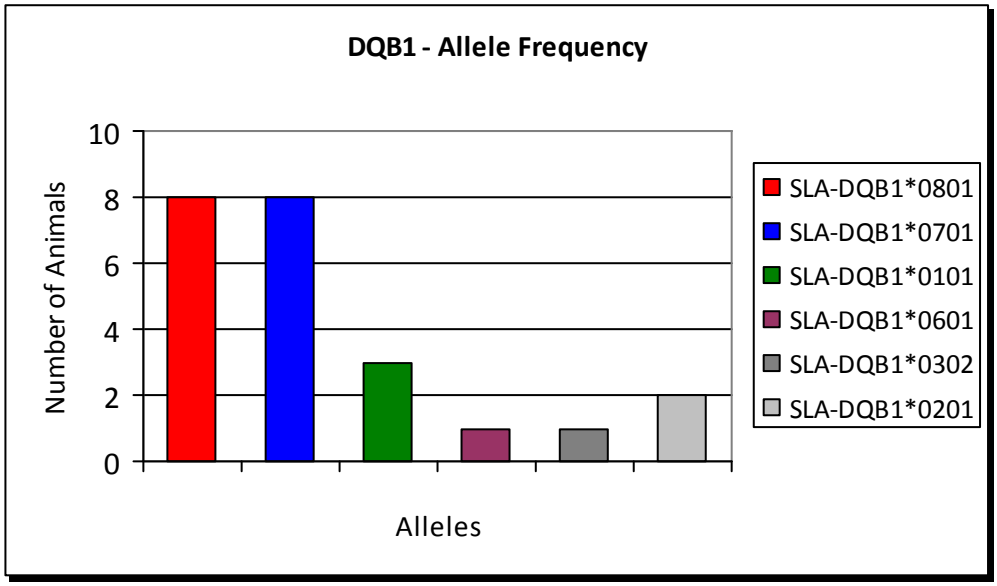

Figure 11. DQB1 - Allele Frequency in 12 Pietrain pigs studied.

Nucleotide Alignment

Figure 12 gives an overview of the binding-sites of the DQB1-primers in relation to the amplified exon 2. The reference allele SLA-DQB1_0101 was used to illustrate the position of the primer binding sites and the exon borders. The position of the DQB1-forward primer (yellow/turquois) is located upstream of exon 2 and overlaps with exon 2. The borders of exon 2 are marked with vertical bars at bp 110 and bp 379. The exptected DQB1-amplicon size (273bp) is confirmed by the amplified sequence, ranging from bp 95-368.

DQB1 sequenced alleles of 12 Pietrain pigs were identified by comparison with the reference alleles listed in the IPD. (**Figure 22;** see appendix). A nucleotide alignment was generated using BioEdit (version 7.0.9.0) and ClustalX (version 1.8). In this alignment, the forward primer as well as the reverse primers were clipped off, thereby generating the core sequence of exon 2 of the DQB1-locus (224bp). Two allelic sequences of each pig were picked and aligned with their reference alleles according to the highest consistency. SLA-DQB1_0101 serves as “major allele” in order to identify nucleotide exchanges.

Observation of the sequenced alleles leads to the identification of several single nucleotide exchanges displayed at bp 54, 110, 143, 151 in most of these alleles. Double or higher nucleotide exchanges are found in the area of bp 81-85 and 142-143. Highly polymorphic sites are located in the area of bp 49-54 and 110-191. One sequenced allele (DQB1_39_1) is even identical with the SLA-DQB1_0101 allele.

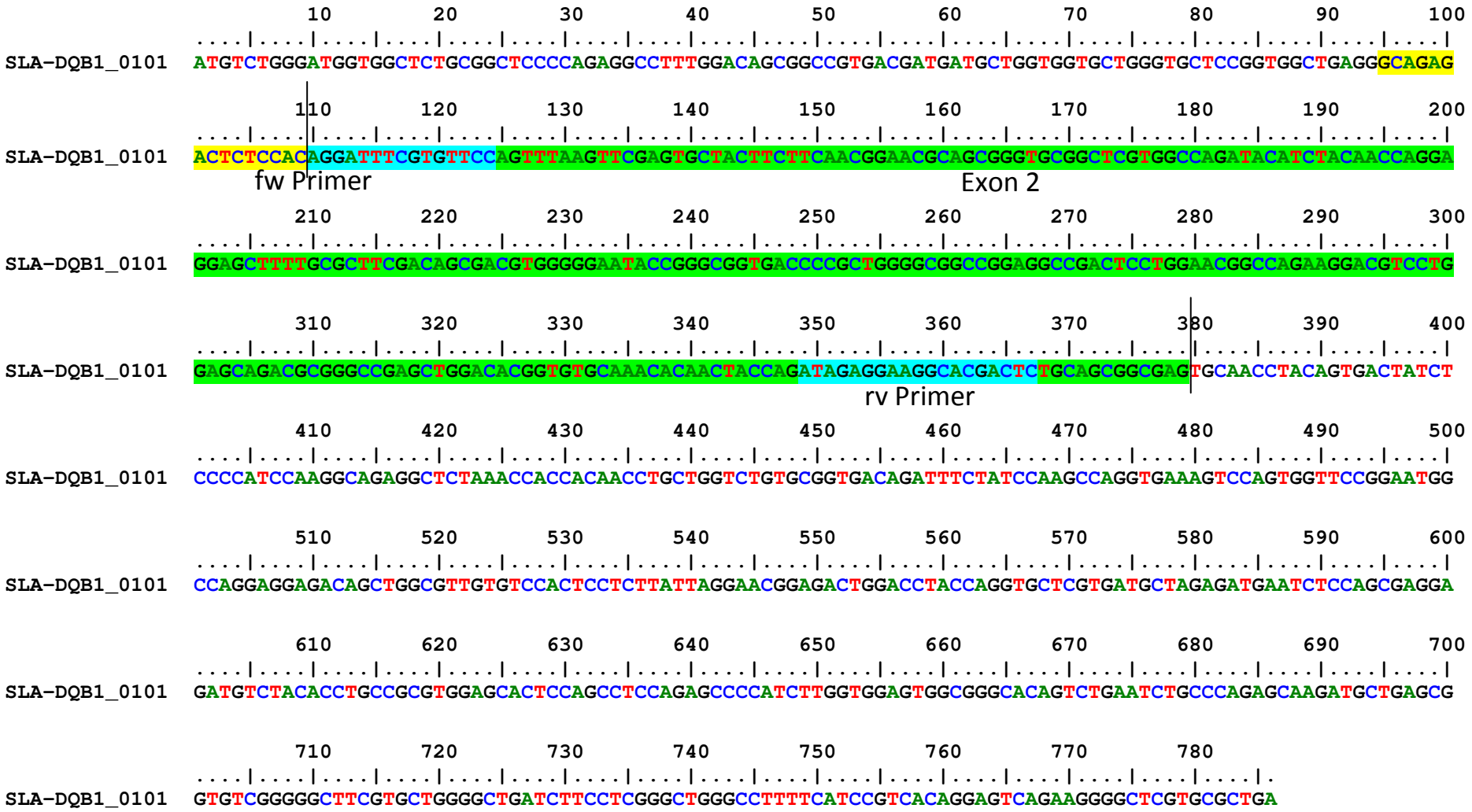

Figure 12. DQB1-Nucleotide Alignment. The reference allele SLA-DQB1_0101 is cited as an example to illustrate the primer binding sites and the exon borders. The forward primer is marked yellow and the exons are displayed in green. Overlapping sequence of DRB1-reverse primer and exon 2 is highlighted in turquoise.

3.1.6 DRB1 Sequencing Outcome

For the DRB1-Locus, 7 different alleles were found. The most prevalent allele in the 12 Pietrain pigs studied, is the SLA-DRB1*0901 allele, which is present in 7 animals. The SLA-DRB1*0602 was discovered in 5 pigs and the SLA-DRB1*0404 allele was found in 3 animals out of 12. Further alleles such as SLA-DRB1*1001, SLA-DRB1*0403, SLA-DRB1*1301 and SLA-DRB1*0206 seem to be less frequent in Pietrain pigs, which can be observed in **Table 6** and **Figure 13.**

	0901	0602	0404	1001	0403	1301	0206
#38							
#39							
#40							
#41							
#42							
#43							
#47							
#51							
#53							
#54							
#57							
#59							

Table 6. DRB1 Annotation table. DRB1-allele distribution in the 12 Pietrain pigs studied.

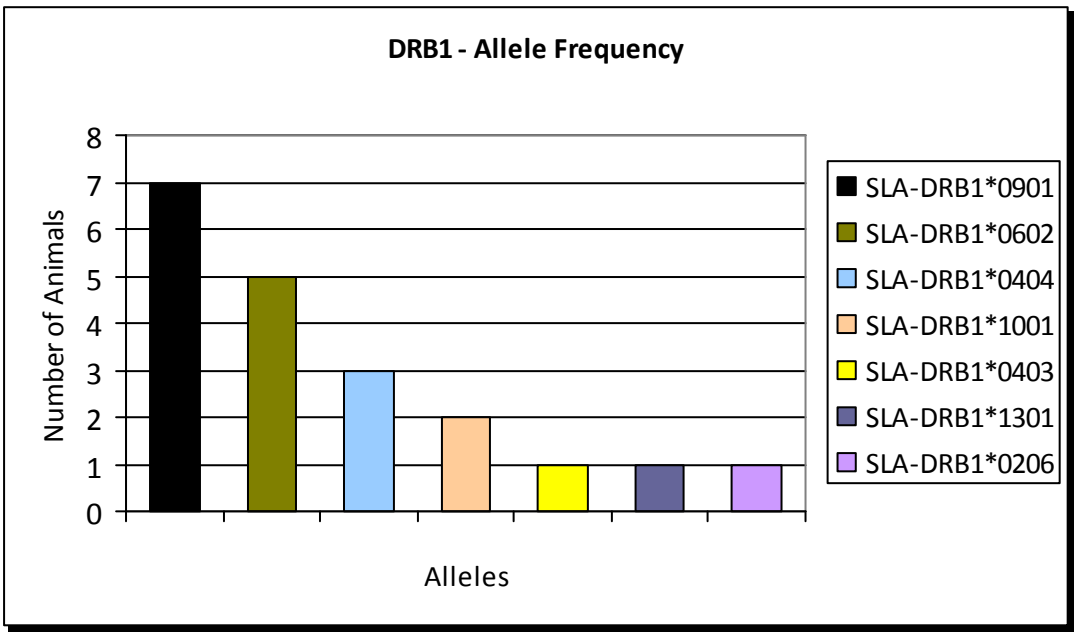

Figure 13. DRB1 - Allele frequency in 12 Pietrain pigs studied.

Figure 14 gives an overview of the binding-sites of the DRB1-primers in relation to the amplified exon 1 and exon 2. The reference allele SLA-DQB1_0101 was used to illustrate the position of the primer bindig sites and the exon borders. The position of the DRB1-forward primer (yellow/turquois) is located 3 bp upstream of exon 1. The borders of exon 1, 2 and 3 are marked with vertical bars. The exptected DRB1-amplicon size (475bp) is confirmed by the amplified sequence, ranging from bp 1-475.

DRB1 sequenced alleles of 12 Pietrain pigs were identified by comparison with the reference alleles listed in the IPD. (**Figure 23,** see appendix). A nucleotide alignment was generated using BioEdit (version 7.0.9.0) and ClustalX (version 1.8). In this alignment, the forward primer as well as the reverse primers were clipped off, thereby generating the core sequence of exon 1, 2 and the beginning of exon 3 of the DRB1-locus (433bp). Two allelic sequences of each pig were picked and aligned with their reference alleles according to the highest consistency. SLA-DQB1_0101 serves as “major allele” in order to identify nucleotide exchanges.

Observation of the sequenced alleles leads to the identification of several single nucleotide exchanges displayed at bp 169, 173, 308 in most of these alleles. Double or higher nucleotide exchanges are found in the area of bp 124-125, 196-197, 142-143, 165-167 and 111-113. Highly polymorphic sites are located in the area of bp 110-180 and 225-300. One sequenced allele (DRB1_51_10) is even identical with the SLA-DQB1_0101 allele.

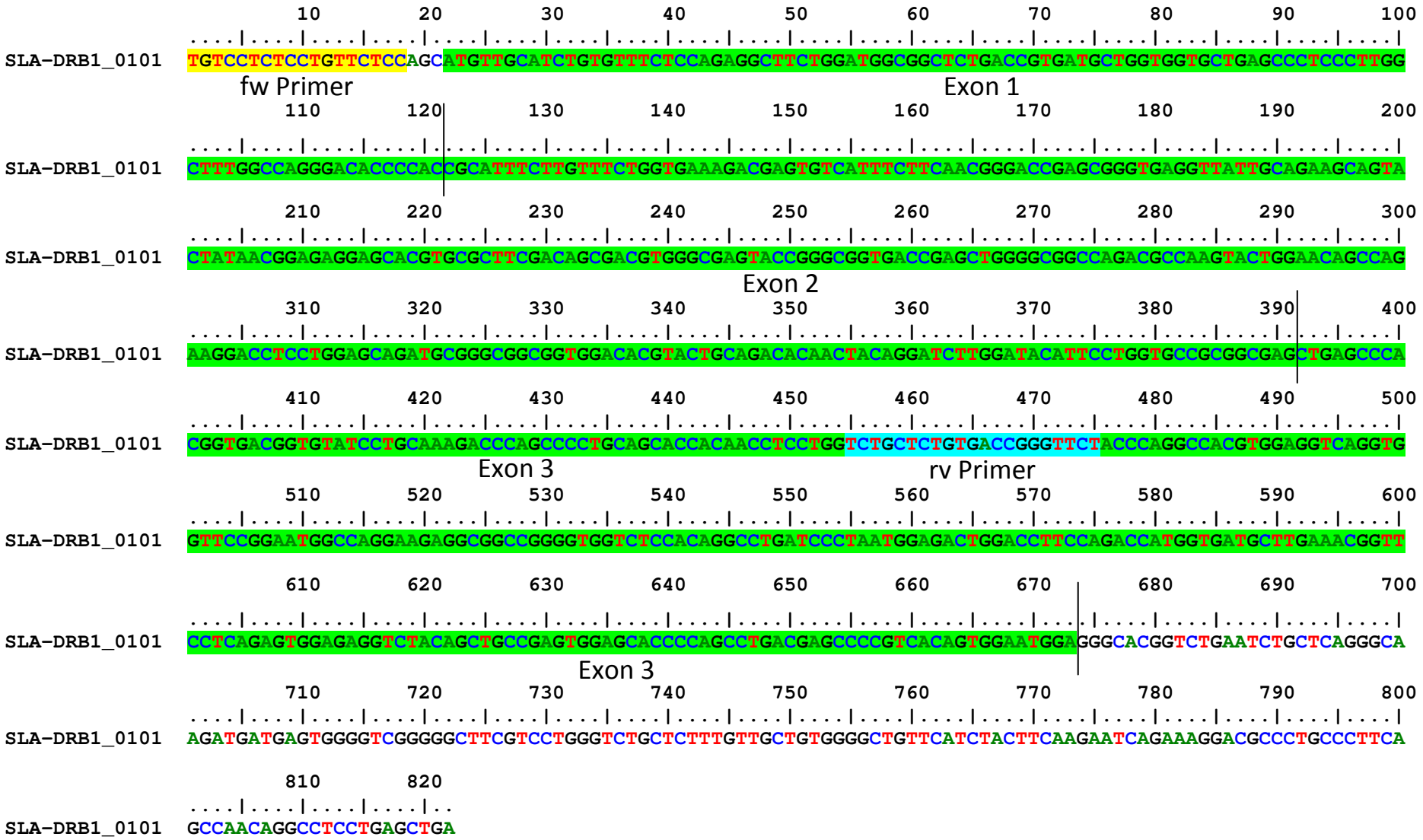

Figure 14. DRB1-Nucleotide Alignment. The reference allele SLA-DRB1_0101 is cited as an example to illustrate the primer binding sites and the exon borders. The forward primer is marked yellow and the exons are displayed in green. Overlapping sequence of DRB1-reverse primer and exon 3 is highlighted in turquoise.

3.2 SLA typing by PCR-SSP

The typing assay of PCR-SSP required sequence specific primers, which helped to identify allele and allele groups present in 27 Pietrain pigs. Based on preliminary designed primer pairs (Ho et al. 2009b), allele groups were determined through a single PCR step, thereby generating allele specific bands. Positively identified bands were the origin for the designation of haplotypes, inferred by allele groups found.

In order to SLA genotype as many animals as possible, a 96-well plate was used which allowed the typing of 2 animals simultaneously. This time consuming aspect of the PCR-SSP method is one of the huge advantages in respect of the typing of alleles.

Each of the primer pairs comprised the α-actin gene (*ACTA1*) as an internal control. Allele specific primers were designed to amplify products smaller than the positive control (516bp).

According to the low resolution SLA class I typing assay, 17 primer pairs were SLA1-specific, 14 primer pairs were SLA2-specific, 8 primer pairs were SLA3-specific and 8 primer pairs responded to more than one locus. SLA class II typing assay involved 23 DRB1-specific primer pairs, 17 DQB1-specific primer pairs and 7 DQA-specific primer pairs.

During the observation of the typing assay results, clear bands as well as weak bands could be observed.

Figure 15 and **Figure 16** show representative pictures of a SLA class I and SLA class II PCR-SSP typing the animal #54. The α-actin gene is visible in all primer pairs, indicated by the band at 516bp. Allele groups detected by the sequence specific primers were designated as positive since the allele specific bands exhibit the expected product size. In this particular case of animal #54, SLA class I PCR-SSP displayed 9 allele specific bands identified as positive. SLA class II PCR-SSP results suggest, that 5 allele groups exist in animal #54.

After running the gel electrophoresis, the gels were photographed under the UV light and the primer pairs were first identified. Due to the known amplicon size of the PCR products, each band observed was checked whether it was positive or not. Primer pairs were designed either to give rise to a single group of alleles, or to several different groups of alleles. **Table 7** shows an overwiev of the assigned allele groups and the inferred SLA class I and class II haplotypes.

The identification of alleles of the three 3 SLA class I genes (SLA1, SLA3 and SLA2) is summerized in the left part of the column, inferred SLA class II alleles and haplotypes (DRB1, DQB1 and DQA1) are summerized in the right part.

Each SLA class I and class II haplotype is made up of of three alleles, each representing a single SLA-locus. Inferred PCR-SSP haplotypes are either designated as Lr-XX.0 (SLA class I) or as Lr-0.XX (SLA class II). In a diploid organism, a single gene is usually presented by two alleles. All typed animals exhibit 2 different alleles per locus, resulting in a heterozygous haplotype.

Although optimized primers were used, there are plenty of tested pigs, which could not be identified and characterized precisely. Several question marks display this problem of allele group designations. Due to the fact, that several SLA class I primer pairs detect allele groups of different SLA class I loci, the characterization of certain allele groups are more complicated compared to the SLA class II designation.

The derivation of SLA class I and class II haplotypes revealed particular haplotypes, which occur in a more common frequency than other haplotypes. The most prevalent SLA class I haplotype in the 27 Pietrain pigs tested, is Lr-43.0 (11XX, 04XX/hb06, 04XX). Observation of inferred SLA class II haplotypes displayed the Lr-0.14 haplotype (09XX/La02, 08XX, 03XX) as the most common one in the analyzed pigs (**Table 7.**).

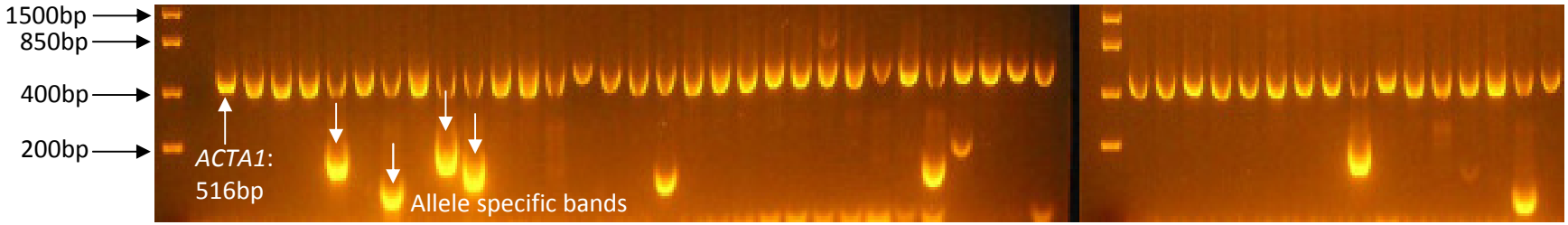

Figure 15. SLA class I PCR-SSP; animal #54. In this representative figure, 47 discriminatory primer pairs were used to differntiate SLA class I alleles by groups that have similar sequence motifs in animal #54. A negative control without DNA was introduced to check for contaminations of used reagents (lane 2). α-actin which served as positive control - exptected product size: 516bp - is visible in all primer pairs. The presence of bands smaller than the positive control indicates that these reactions were positive. Marker: Low Range, 3% (w/v) agarose.

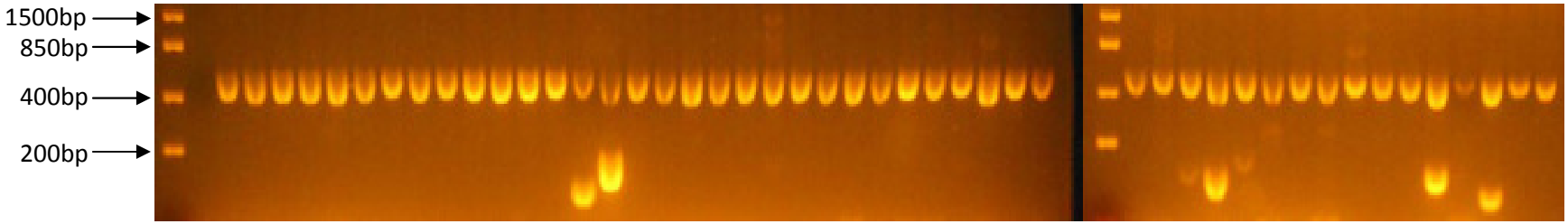

Figure 16. SLA class II PCR-SSP; animal #54. Same approach as in Figure 15, with primer pairs that differntiate SLA class II alleles by groups. Marker: Low Range, 3% (w/v) agarose.

SLA class I					SLA class II			
animal	**SLA1**	**SLA3**	**SLA2**	**inferred haplotype**	**DRB1**	**DQB1**	**DQA**	**inferred haplotype**
#36	blank	05XX	10XX	Lr-39.0	04XX	02XX	02XX	Lr-0.15a
	11XX	04XX/hb06	04XX	Lr-43.0	09XX/La02	08XX	03XX	Lr0.14
#37	11XX	03XX	07XX	Lr-25.0	04XX	02XX	02XX	Lr-0.15a
	16XX	04XX/hb06	03XX/es22 or 06XX?	?	13XX	09XX	04XX+w05XX	Lr-0.25
#38	12XX, 13XX	05XX	10XX	Lr-35.0	06XX	07XX	01XX	Lr-0.12
	11XX	04XX/hb06	04XX	Lr-43.0	09XX/La02	08XX	03XX	Lr-0.14
#39	01XX	01XX	01XX	Lr-1.0	01XX/be01/ha04	01XX	01XX	Lr-0.1
	11XX	04XX/hb06	04XX	Lr-43.0	09XX/La02	08XX	03XX	Lr-0.14
#40	08XX	05XX	10XX	Lr-26.0	06XX	03XX	01XX	Lr-0.20
	11XX	04XX/hb06	04XX	Lr-43.0	09XX/La02	08XX	03XX	Lr-0.14
#41	08XX	05XX	10XX	Lr-26.0	06XX	03XX	01XX	Lr-0.20
	11XX	04XX/hb06	04XX	Lr-43.0	09XX/La02	08XX	03XX	Lr-0.14
#42	08XX	05XX	10XX	Lr-26.0	04XX	blank	03XX	?
	blank	04XX/hb06	06XX	Lr-24.0	10XX/er01	06XX/zs12	01XX	Lr-0.23
#43	11XX	04XX/hb06	04XX	Lr-43.0	04XX	07XX	03XX	Lr-0.19a
	15XX or (09XX)	07XX	05XX	Lr.28.0	10XX/er01	06XX/zs12	01XX	Lr-0.23
#44	01XX	01XX	01XX	Lr-1.0	01XX/be01/ha04	blank	01XX	?
	15XX	05XX	10XX	Lr-67.0	04XX	02XX	02XX/ka01	Lr-0.15a
#45	15XX	04XX/hb06	es22	Lr-55.0	09XX/La02	08XX	03XX	Lr-0.14
	11XX or es11	blank	01XX or 04XX	?	04XX or 05XX?	02XX or 09XX	04XX+w05XX	?
#46	11XX	04XX/hb06	04XX	Lr-43.0	04XX	03XX	02XX/ka01	Lr-0.13
	14XX	05XX	10XX	Lr-64.0	09XX/La02	08XX	03XX	Lr-0.14

SLA class I					SLA class II			
animal	**SLA1**	**SLA3**	**SLA2**	**inferred haplotype**	**DRB1**	**DQB1**	**DQA**	**inferred haplotype**
#47	11XX	04XX/hb06	04XX	Lr-43.0	04XX	07XX	03XX	Lr-0.19a
	16XX or an02	blank	06XX or 03XX or es22?	?	09XX/La02	08XX	03XX	Lr-0.14
#48	01XX	01XX	01XX	Lr-1.0	01XX/be01/ha04	01XX	01XX	Lr-0.1
	15XX	04XX/hb06	es22	Lr-55.0	04XX	07XX	03XX	Lr-0.19a
#49	08XX	05XX	10XX	Lr-26.0	06XX	03XX	03XX	?
	09XX	07XX	05XX or w14XX or 16xx or jh02?	Lr-11.0 od Lr-28.0?	10XX	06XX/zs12	01XX	Lr-0.23
#50	16XX	05XX	10XX	Lr-40.0	04XX	03XX	02XX/ka01	Lr-0.13
	an02	04XX/hb06	03XX or es22 or 06XX?	?	04XX	07XX	03XX	Lr-0.19a
#51	14XX	05XX	10XX	Lr-64.0	04XX	03XX	02XX	Lr-0.13
	15XX	04XX/hb06	es22	Lr-55.0	13XX	09XX	04xx+w05XX	Lr-0.25
#52	07XX or 16XX/an02?	04XX/hb06	03XX/es22 or 16XX?	?	10XX/er01	06XX/zs12	01XX	Lr-0.23
	blank	blank	blank	?	04XX	07XX	03XX	Lr-0.19a
#53	blank	04XX/hb06	06XX	Lr-24.0	04XX	07XX	01XX	Lr-0.19b
	16XX or an02	blank	03XX or es22 or w09XX	?	09XX/La02	08XX	03XX	Lr-0.14
#54	11XX	03XX	07XX	Lr-25.0	06XX	07XX	01XX	Lr-0.12
	12XX, 13XX	05XX	10XX	Lr-35.0	13XX	09XX	04xx+w05XX	Lr-0.25
#55	14XX	05XX	10XX	Lr-64.0	04XX	03XX	02XX/ka01	Lr-0.13
	blank	blank	blank	?	blank	blank	03XX	?
#56	11XX	04XX/hb06	04XX	Lr-43.0	06XX	blank	01XX	?
	12XX or 13XX or cs02?	blank	10XX	?	09XX/La02	blank	03XX	Lr-0.27
#57	11XX	03XX	07XX	Lr-25.0	09XX/La02	08XX	03XX	Lr-0.14
	blank	04XX/hb06	06XX	Lr-24.0	13XX	09XX	04xx+w05XX	Lr-0.25

SLA class I					SLA class II			
animal	**SLA1**	**SLA3**	**SLA2**	**inferred haplotype**	**DRB1**	**DQB1**	**DQA**	**inferred haplotype**
#58	11XX	03XX	07XX	Lr-25.0	10XX/er01	06XX	01XX	Lr-0.23
	09XX	07XX	01XX	Lr-28.0	13XX	09XX/La02	04XX+w05XX	Lr-0.25
#59	08XX	05XX	10XX	Lr-26.0	06XX	07XX	01XX	Lr-0.12
	12XX, 13XX	blank	blank	?	09XX/La02	08XX	03XX	Lr-0.14
#109	15XX	04XX/hb06	es22	Lr-55.0	04XX	07XX	03XX	Lr-0.19a
	es11 or 16XX/an02?	blank	06XX or w09XX? or 16XX? 01XX or	?	05XX	08XX	01XX	Lr-0.6
#110	11XX	04XX/hb06	04XX	Lr-43.0	07XX	02XX	02XX	Lr-0.24
	blank	05XX	w09XX	Lr-29.0	13XX	09XX/La02	03XX	?
#111	16XX/an02	04XX/hb06	03XX/es22 or 06XX?	?	04XX	02XX/zs13	03XX	?
	blank	blank	blank	?	13XX	blank	blank	?

Table 7. Overview of inferred SLA class I and class II haplotypes by PCR-SSP. All 27 Pietrain pigs were typed and characterized by using the PCR-SSP method. After performing gel electrophoresis (**Figure 15, 16**), positive allele specific bands were assigned according their allele groups which were the basis of the definition of low-resolution (Lr) haplotypes. Animals with inferred haplotypes signed with a question mark are doubtful. The most common SLA class I haplotype observed in the 27 Pietrain pigs seems to be Lr-43.0, appeared in ten pigs and the most prevalent SLA class II haplotype Lr-0.14 appeared in eleven pigs. The assignment of allele groups for the DQB1-locus is fragmentary because several DQB1 specific primer pairs seemed to miss certain allelegroups, resulting in an incomplete DQB1 haplotype characterization.

3.3 SLA expression profiling

The cDNA of two different types of porcine intestinal epithelial cells (Type I and Type II), which were kindly provided by Michael Burwinkel, were analyzed due to SLA class I and class II expression. **Figure 17** shows a SLA class I Southern Blot, displaying DIG-labeled SLA specific bands after incubation with α-DIG antibody. Four different SLA class I genes (SLA1, SLA2, SLA3, SLA6) were analysed using cDNA of Type I and Type II cells. According to the Type I and Type II cells, the RT-PCR reactions were performed in duplicates (termed Ia, Ib and IIa, IIb). Additionally, positive controls (pC, cDNA of dd1156) as wells as negative controls (nC, DEPC-water) were conducted.

In all four SLA class I genes specific bands were observed almost exclusively in Type II cells. Type II cells and the positive controls display the SLA specific band consistently to the expected amplicon size. Amplification of the SLA1 locus lead to the identification of artefact bands (indicated as red arrows) which has further been proven by subsequent cloning and sequencing

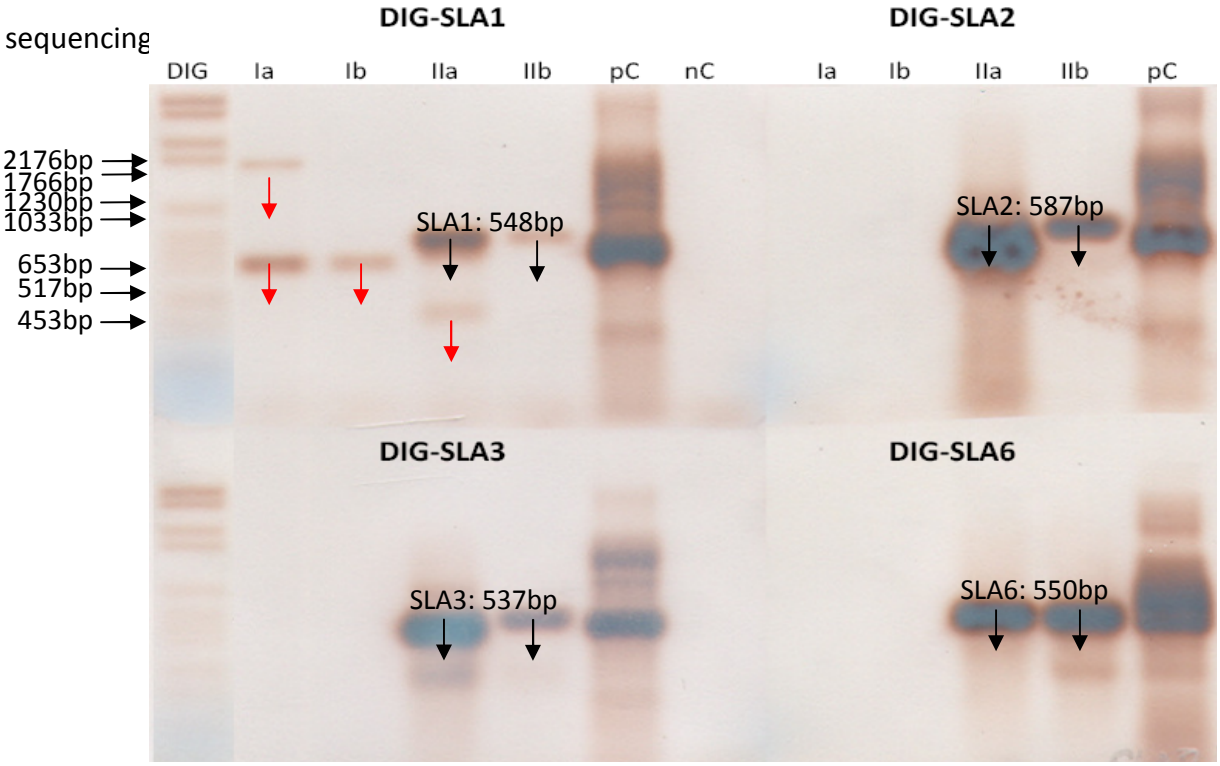

Figure 17. SLA class I Southern Blot. Four different SLA class I loci were analyzed in Type I and Type II cells. After running a RT-PCR with DIG-labeled forward primers, the bands were transferred to a Hybond™-N nylon membran, incubated with α-DIG antibody and visualized using a staining solution. The most apparently result in respect of detecting SLA specific bands is the fact, that Type Ia and Ib cells do not express any SLA class I genes. SLA specific bands are marked with black arrows including their expected amplicon size. Bands marked with red arrows are amplified artifacts, which are related to unspecific binding of the SLA1 primer pairs.

Figure 18 shows the SLA class II Southern Blot (SLA-DQA, SLA-DRA, SLA-DQB1, SLA-DRB1). The results of SLA class II expression are similar to those of SLA class I. As observed, mainly Type II cells show SLA class II gene expression. Missing SLA-DQA specific bands in Type I and Type II cells are the result of unspecific amplification of the SLA-DQA locus. During amplification of the SLA-DQA locus, two RT-PCRs with different annealing temperatures (52.0°C, 53.0°C) were performed. None of them revealed distinct bands which may be related to SLA-DQA. SLA-DRA, SLA-DQB1 and SLA-DRB1 display distinct bands in Type II cells, Type I cells seem to express low amounts of SLA-DQB1 and SLA-DRB1.

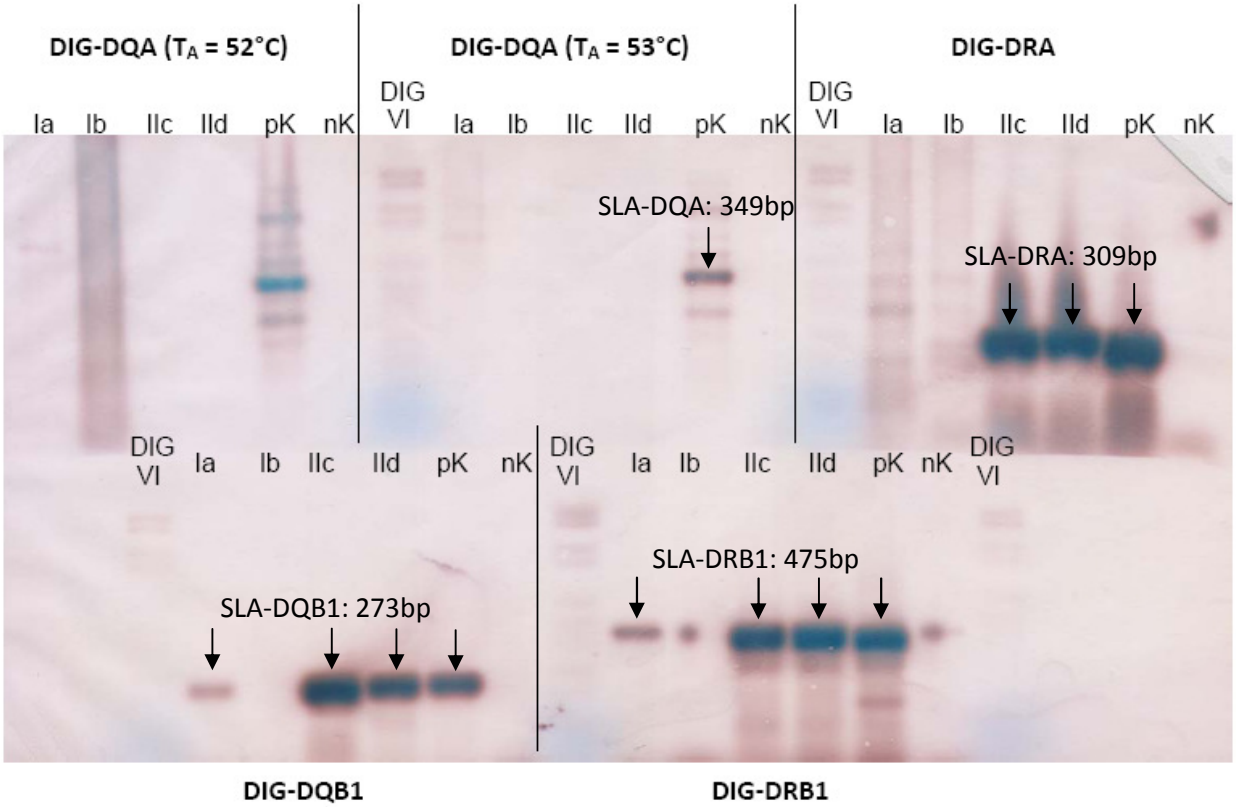

Figure 18. SLA class II Southern Blot. Four different SLA class II genes were analyzed. In case of SLA-DQA, two different RT-PCRs with varied annealing temperatures (52.0°C, 53.0°C) were carried out. Basically, Type I cells tend to express SLA class II genes (SLA-DQB1, SLA-DRB1).

According to Type II cells, differences in RT-PCRs between labeled and non-labeled primer pairs could not be detected (data not shown). Slight differences were observed during the comparison of Type I cells. In some cases, such as SLA6 and SLA-DRB1, those genes display bands in RT-PCRs using non labeled forward primers whereas RT-PCRs performed with DIG-labeled forward primers did not display any SLA specific bands at all (data not shown).

However, another important aspect of this expression profile was the comparison of Type I and Type II cells in respect of their varying SLA class II and class II expression. In **Table 8**, the two cell types are compared, distinct and SLA specific bands occurred in the cell type are

marked with a "+". In contrast, RT-PCRs which displayed no positive signals in any of the SLA loci were marked with a "-". **Table 8** summerizes - with few exceptions - that the SLA class I and class II gene expression is sparsed in Type I cells and abundant in Type II cells.

	SLA Class I				SLA Class II			
	SLA1	**SLA2**	**SLA3**	**SLA6**	**DQA**	**DRA**	**DQB1**	**DRB1**
Ia	+	–	–	–	–	–	+	+
Ib	–	–	–	+	–	–	–	+
IIc	++	++	+	+	-	++	+	+
IId	+	+	+	+	-	++	+	+

Table 8. SLA class I and class II expression in Type I and Type II cells.

Due to the inconsistent results between Type I and Type II cells, a GAPDH RT-PCR was carried out in order to check the cDNA concentrations in both cell types (**Figure 19**). The first four aliquots (#1 - #4) represent the GAPDH band at 318bp in the positive control (dd1156). As expected, bands of Type I cells display a less visible GAPDH band than in Type II cells. This leads to the assumption that the cDNA concentration in Type II cells is obviously higher than in Type I cells.

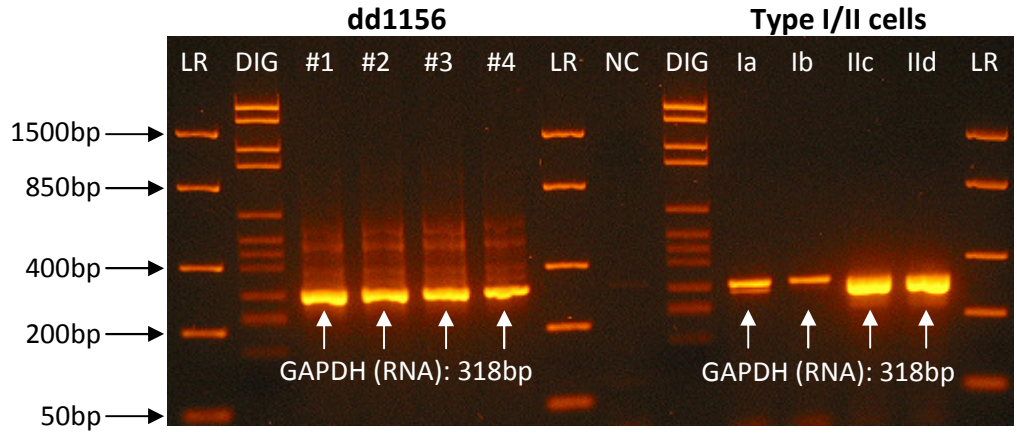

Figure 19. GAPDH RT-PCR. 4 aliquots of each dd1156 and Type I and Type II cells were tested for cDNA concentration. LR: Low Range; DIG: Digoxigenin; NC: negative control; 1.5% (w/v) agarose.

IV Discussion

4.1 SLA diversity in Pietrain pigs

In order to characterize the highly polymorphic SLA complex of 27 Pietrain pigs, two different methods were used in this thesis. Three SLA class II related genes (DRB1, DQB1, DQA) were investigated using both the high- and low-resolution typing method. Additionally three SLA class I genes (SLA1, SLA3, SLA2) were investigated by the PCR-SSP method only.
Twelve out of the 27 pigs were characterized using the sequence based typing method as well as the PCR-SSP typing method (**Table 2**). The high-resolution haplotyping was carried out in order to obtain the sequences of DQB1 and DRB1 and further to characterize the extent of the polymorphism within those genes. Due to the amplification of DQB1 and DRB1, primers were chosen which were located at the beginning and at the end of exon 2 (**Figure 12**, **Figure 13**). The exon 2 plays a critical role in building the peptide binding cleft, so primers were chosen which amplified only the part of the locus with the highest extent of polymorphism.

DQB1

Sequence based typing of the DQB1 locus of twelve Pietrain pigs revealed the existance of six different alleles: SLA-DQB1*0801/08ch01, SLA-DQB1*0701, SLA-DQB1*0101, SLA-DQB1*0601, SLA-DQB1*0302/0303, SLA-DQB1*0201 (**Table 5**). Eight out of twelve pigs exhibit the most common SLA-DQB1*0801/08ch01 and SLA-DQB1*0701 alleles (**Figure 11**). Considering the SLA-DQB1*0801, SLA-DQB1*0601 and SLA-DQB1*0302/0303 alleles, the high-resolution haplotyping results were confirmed by the PCR-SSP method in all of the corresponding pigs (**Table 7**). Further investigation and comparison of the low-resolution haplotyping results showed that in six out of eight pigs the SLA-DQB1*0701 allele and in two out of three pigs the SLA-DQB1*0101 allele were found. The detection of the SLA-DQB1*0101 and SLA-DQB1*0201 has to be considered carefully since the high-resolution results show the emergence of three alleles in one single animal (**Table 5**).

Based on these results, **Figure 20** displays the percental DQB1-correspondency between alleles which were detected by the high-resolution and further confirmed by the low-resolution method. For the DQB1 locus 78% of the identified alleles were verified by the PCR-SSP method. The minority of 22% of the six detected alleles remain unconfirmed.

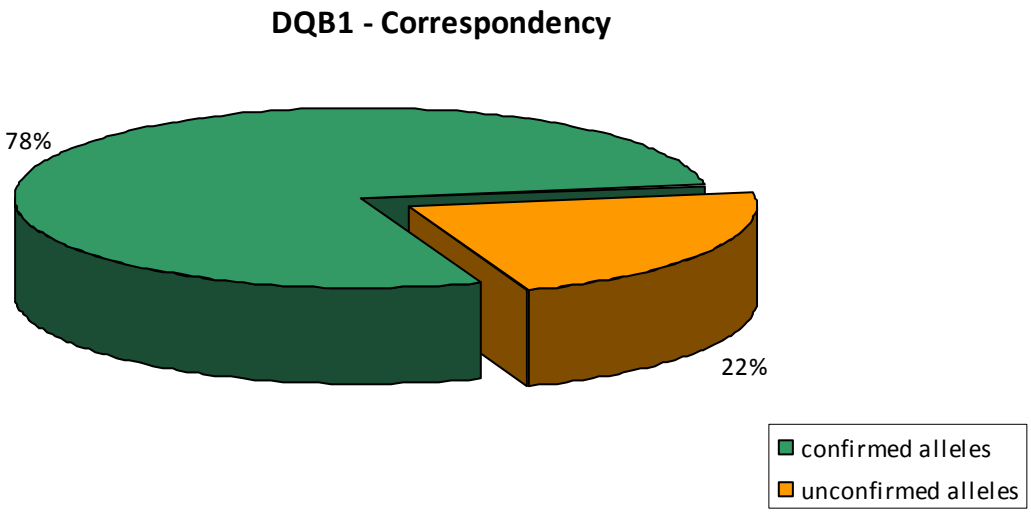

Figure 20. DQB1 – Correspondency.

DRB1

Sequence based typing of the DRB1 locus of twelve Pietrain pigs revealed the existance of eight different alleles: SLA-DRB1*0901, SLA-DRB1*0602, SLA-DRB1*0404, SLA-DRB1*1001, SLA-DRB1*0403, SLA-DRB1*1301, SLA-DRB1*0206 (**Table 6**). Seven out of twelve pigs exhibit the most common SLA-DRB1*0901 allele (**Figure 13**). When compared to the DQB1 locus, there is a slight increase of the allele frequency within the DRB1 locus (**Figure 13**). Considering the SLA-DRB1*0901, SLA-DRB1*0602, SLA-DRB1*0404, SLA-DRB1*0403 and SLA-DRB1*1301 alleles, the high-resolution haplotyping results were confirmed by the PCR-SSP method in all of the corresponding pigs (**Table 7**). SLA-DRB1*1001 and SLA-DRB1*0206 alleles were not detected by the low-resolution method.

Correspondency of confirmed DRB1 alleles by the high- and low-resolution typing method is displayed in **Figure 21**. In contrast to DQB1, the low-resolution haplotyping of DRB1 revealed a much higer consistency between the two typing methods.

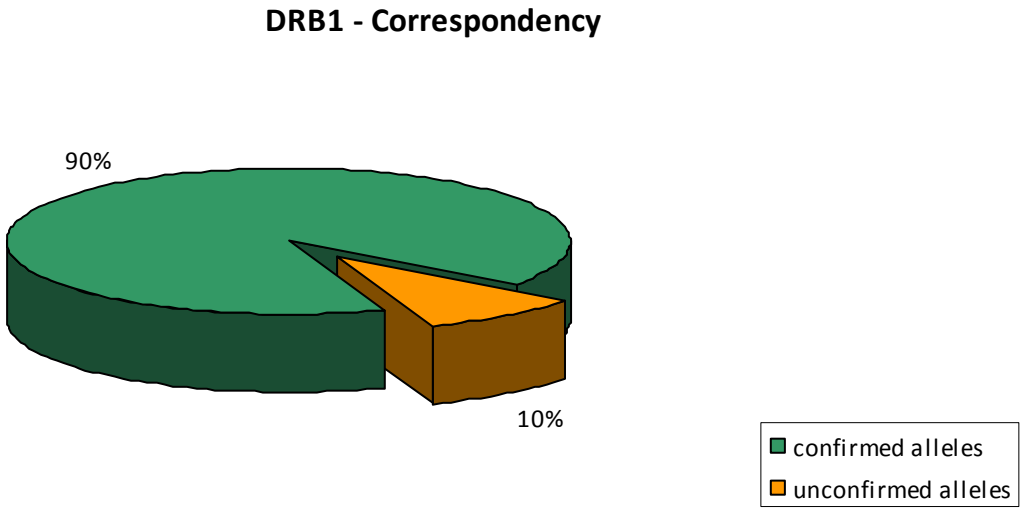

Figure 21. DRB1 – Correspondency.

Although these correspondency results suggest a high extent of verification of found alleles between the two methods, there are factors which may be limited due to the consistency of alleles. The application of the PCR-SSP method revealed some alleles, which could not be detected by the sequence based method. One of the reasons might be the use of RNA which functioned as starting product in the sequence based method. This is linked to the expression of the alleles to be characterized, hence low expressed alleles are much more difficult to detect.

A major difference between the two methods is the fact that the high-resolution typing method is able to detect single alleles in contrast to the low-resolution typing method which only identfies certain allele groups (see 2.3.2). This consequently increases the sensitivity of the SBT-method.

Three SLA class I (SLA1, SLA3, SLA2) and three SLA class II (SLA-DRB1, SLA-DQB1, SLA-DQA) loci were characterized in this thesis. The highest degree of polymorphism of the SLA class I genes is concentrated in the exons 2 and 3 representing the coding region which form the class I peptide binding cleft. Increased polymorphism of SLA class II genes also occurs in exon 2 (Lunney et al. 2009).

4.2 SLA diversity in outbred pig populations

Several outbred pig populations have been characterized due to their SLA diversity. Some studies used the SBT- and the PCR-SSP typing method to identify differences in SLA polymorphism in Meishan pigs (Ho et al. 2006), others only applied the sequence typing method to characterize class I and class II alleles in Korean native pigs (Lee et al. 2008). Due to the association of the SLA halpotype *B* in homozygous pigs with increase penetrance of the tumor trais in Sinclair swine cutaneous melanoma (SSCM), a total of 469 animals (Sinclair x Hanford crosses), were characterized (Ho et al. 2010a). In respect of wild boars recently published data focuses on the SLA class II DRB1 locus (Barbisan et al. 2009).

Investigation of three class I (SLA1, SLA2, SLA3) and two SLA class II (SLA-DQB1, SLA-DRB1) loci, revealed only four different haplotypes found 20 Meishan pigs. The most common haplotypes appeared to be Hp-18.14 and Hp-19.15, in all of the investigated animals at least one of these two haplotypes could be detected. (Ho et al. 2006). Relative to the investigated 27 Pietrain pigs in this thesis, none of the Meishan SLA class I haplotypes (Hp-18.0 and Hp-19.0) could be characterized neither by the SBT-method nor by the PCR-SSP. In sharp contrast, the SLA class II Hp-0.14 appeared in eleven, SLA class II Hp-0.15 in three Pietrain pigs.

Four class I (SLA1, SLA2, SLA2, SLA6) and four class II (SLA-DQA, SLA-DRA, SLA-DQB1, SLA-DRB1) loci were studied in three Korean native pigs. Intrestingly, two out of three animals were homozygous for two novel SLA haplotypes (Hp-56.30, Hp-59.1), the third was characterized as heterozygous for SLA haplotypes Hp-7.23 and Hp-56.30 (Lee et al. 2008). Again, none of the SLA class I haplotpyes (Hp-7.0, Hp-56.0, Hp-59.0) found in Korean native pigs could be detected in Pietrain pigs. SLA class II haplotypes Hp-0.1 and Hp-0.23 occur in two and four Pietrain pigs, respectively.

Examination of haplotypes present in the Sinclair and Hanford pigs by a combination of SBT and PCR-SSP typing revealed the existance of seven SLA haplotypes (Hp-9.10, Hp-2.2, Hp-10.11, Hp-11.12, Hp-12.13, Hp-12.10, Hp-12.3). Also these breeds of pigs only show similarities in Pietrain SLA class II haplotypes (Hp-0.12, Hp-0.13).

Concerning the SLA diversity in wild boars, there are marginal published data to date. The direct sequencing cloning of four wild boar populations (57 individuals in total) led to the detection of 18 alleles. A huge difference of allele distribution between the four populations

could be observed. Due to the simultaneous presence of up to three alleles in the same individual, it could be assumed that a duplication event at the DRB1 locus occurred at least in one of the four populations examined (Barbisan et al. 2009).

4.3 High-resolution vs. low-resolution haplotyping

One of the major aspects of this thesis was the comparison of results of two SLA typing methods. The high-resolution (SBT) and low-resolution haplotyping (PCR-SSP) method are two totally different approaches in order to characaterize the SLA complex in swine. Considering the SBT-method, the sequencing of certain alleles within an individual is time comsuming, expensive and inefficient. The PCR-SSP-method on the other hand is fast, cheap and allows the typing of two animals simultaneously. In order to take the advantage of comparison both methods directly, 12 out of 27 Pietrain pigs were characterized by the SBT- as well as the PCR-SSP-method.

4.3.1 SBT

The basis of the high-resolution typing method was based on the molecular cloning of DQB1 and DRB1 related alleles. These cloning steps included, amongst other things, the purifaction of DNA, amplification of exons which form the peptide binding cleft and the transformation in *E. coli* followed by a screening for positive transformants (see 2.2). Sequenced alleles are aligned with reference alleles listed in the IPD (see appendix).

As already mentioned, the amplifications of the DQB1 and DRB1 locus were carried out by previously published primers (see 2.2.3; Fang et al. 2005, Smith et al. 2005, Ho et al. 2006). The primers were designed to amplify exon 2 of the DQB1 gene (**Figure 12**) and exon 2 and parts of exon 3 of the DRB1 gene (**Figure 14**). Both exons are the major key players in building the highly polymorphic residues within the peptide binding cleft. In order to detect the high degree of polymorphism in the SLA system, a nucleotide alignment with the reference alleles from the IPD was carried out. As expected, most of the sequenced alleles

show single or multiple nucleotide exchanges (**Figure 22 and Figure 23;** see appendix). Two sites of high polymorphism could bei identified in DQB1 alignment located at bp 49-54 and 110-191. Results of the DRB1 alignment revealed high diversity at bp 110-180 and 225-300.
Based on the fact that these informations are the first results in the course of characterization the SLA complex in Pietrain pigs, further studies may be performed with different PCR primers in order to confirm this data. In regard to the amplification of DQB1 and DRB1, following primer pairs could be used (Luetkemeier et al.):

Primers; DQB1 T_A = 64°C, 400bp RT-PCR product
forward 5' - CGG GCG GAG GCC TGA CTG - 3'
reverse 3' - CGG CGG GCA AGC ACT CAC - 5'

Primers; DRB1 T_A = 68°C, 700bp RT-PCR product
forward 5' - GGG CGA ATC CTT GGG GAG C - 3'
reverse 3' - ACA CAC ACT CTG CCC CCC G-5'

4.3.2 PCR-SSP

Fourty-seven primer pairs (plus negative control) form the basis of the low-resolution typing method which helps to characterize certain allele groups within the SLA complex. The straightforward and cost-effective aspects are the major advantages of this typing method. On the other hand it has to be considered that this typing assay only allows identifying groups of alleles with preknown sequences. In consideration of the fact that a huge amount of alleles have to be identified, it was obvious to combine similar alleles into groups which can be detected by certain primer pairs.
All primer pairs were designed to have their melting temperatures slightly lower than the annealing temperatures to increase their specificties (Ho et al. 2006).
The most prevalent SLA class I haplotype in Pietrain pigs tested, is Lr-43.0 (SLA1*11XX-SLA3*04XX/hb06-SLA2*04XX), ten out of 27 individuals exhibt this haplotype. SLA class I haplotypes of eleven pigs could not be assigned clearly. The reason for this may be the low specificities of the used primer pairs resulting in too many ambiguities. As can be observed, a

lot of allele groups in SLA1, SLA3 and SLA2 did not respond to any of the attributed primer pairs. As a result, unidentified allele groups within the corresponding locus are termed “blank”. Consequently, no inferred haplotype could be assigned in these individuals (question mark) (**Table 7**). Another problem which makes the identification of inferred haplotypes diffucult is the fact, that some SLA class I primer pairs are able to respond to two loci simultaneously.

This matter of fact makes the characterization of SLA class II haplotypes easier. PCR-SSP revealed the most common SLA class II haplotype in eleven Pietrain pigs: Lr-0.14 (DRB1*09XX/La02-DQB1*08XX-DQA*03XX). Unidentified allele groups are restricted on the DQB1 locus. Plenty of the designed DQB1 primers are very sensitive and tolerate only humble variability in the annealing and melting temperatures during the PCR cycle. This is an explanation why some DQB1 primers seem to miss several DQB1 allelegroups. Nearly all of the 27 Pietrain pigs could be assigned to a certain SLA class II haplotype expect for pig #49. Besides the inferred SLA class II haplotype Lr-0.23 (DRB1*10XX-DQB1*06XX/zs12-DQA*01XX) a second one could be identified clearly: DRB1*06XX-DQB1*03XX-DQA*03XX. Because there is no designated haplotype with these allele groups, it can be assumed that pig #49 exhibts a new and unknown SLA class II haplotype.

The most prevalent SLA haplotype in the 27 pigs tested is Lr-43.14 (SLA1*11XX-SLA3*04XX-SLA2*04XX-DRB1*0901-DQB1*0801-DQA*03XX) which was found in seven pigs.

Concerning the characterization of the SLA complex in swine, there is an additional approach using the SBT method in eight commercially available porcine cell lines. Novel alleles were confirmed by PCR-SSP. (Ho et al. 2009a,b).

4.4 SLA expression profiling

Another aim of this thesis was to detect SLA acitvity in two intestinal epithelial cell lines. Previously isolated cell lines were analyzed using the Southern Blot to examine the expression of SLA class I (SLA1, SLA2, SLA3, SLA6) and SLA class II (SLA-DQA, SLA-DRA, SLA-DQB1, SLA-DRB1) genes. Results show that the expression of SLA genes in Type II cells is mainly higher than in Type I cells (**Figure 17, 18**). The reason for this might be different cDNA concentrations in the delivered aliqouts. This is partly confirmed by the performance of a GAPDH RT-PCR (**Figure 19**) which revealed much stronger bands in Type II than in Type I cells. In contrast, the positive control using cDNA of inbred pig dd1156 showed uniform bands in Type I as well as in Type II cells.

Further study shows, IL-2 and IL-4 in Type I cells show a high constituive expression, whereas TNF-α expression occurs in Type II cells. Due to the fact that only Type II cells promote TGEV infection, probiotic treatment of these cells resulted in an increased cell survival and a decreased virus titre. Additionally, IL-6, IL-8 and IFN-γ cytokine production decreased after TGEV infection. Based on this observations it seems, that Type I cells share similar features with crypt cells whereas Type II cells are comparable with M cells and further have potential to be used as a cell model for in vitro studies on TGEV (Ephraim et al. 2009).

4.5 Outlook

The application of the low resolution typing method (PCR-SSP) comprises time consuming and straight forward characteristics. These essential features make this new type of typing approach interesting and help to improve the identification of novel allelegroups and haplotypes in future studies. Due to the huge amount of different breeds to investigate, it may be advantageous to develop breed specific typing panels in order to detect allelegroups within a population more precisely. Furthermore, the application of allele-specific primers would turn the PCR-SSP into a high-resolution method.

One of the huge disadvantages of using the SBT method are its limitations, including RNA isolation and extensive DNA cloning requirements. Recently a new SBT approach was

developed by Park et al. A combined application of genomic PCR and direct sequencing (GSBT) may aid in the study of SLA diversity. Based on DNA, this newly evolved method should help to avoid allelic dropouts. For example, in one experiment using Korean native pigs (Cho et al. 2010) it could be confirmed that genotyping results of GSBT were identical to those using PCR-SSP typing (Park et al. 2010).

In regard to the outbred pig populations available in Austria, it is also necessary to focus and enhance the SLA typing of Large White and Landrace pigs in order to obtain an entire repertoire of all breeds existing in Austria.

Due to the fact that the SLA expression profiling is a semi-quantitative method, the development of real-time PCR experiments might be an interesting aspect of propective studies in terms of expression of SLA genes. Sepcific SLA q/RT-PCR probes or qPCR primer panels might give a better look inside the expression of SLA related genes.

V Abbreviations

Amp	Ampicillin
APC	Antigen presenting cell
bp	Base pairs
CD	Cluster of differentation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CTL	$CD8^+$ cytolytic T-lymphocytes
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
E.coli	Escherichia coli
EDTA	Ethylenediamintetraacidic acid
ER	Endoplasmatic reticulum
EtOH	Ethanol
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSBT	Genomic sequence based typing
H_2O	Water
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
IFN	Interferon
I_i	Invariant chain
IL	Interleukin
IPD	Immnuo polymorphism database
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCl	Potassium chloride
LB	Luria broth

Lr	Low-resolution
LSM	Lymphocyte separation medium
Mb	Megabase
MCS	Multiple cloning site
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MHC	Major histocompatibility complex
MIC	MHC class I chain-related genes
ml	Milliliter
MM	Master mix
mRNA	Messenger ribonucleicacid
Na_2EDTA	Disodium ethylenediaminetetraacidic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
NK	Natural killer cells
o/n	Over night
ori	Origin of replication
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-SSP	Polymerase chain reaction-sequence specific primer
PCV2	Porcine circovirus type 2
pH	Potentia Hydrogenii
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase - Polymerase chain reaction
rpm	Rotations per minute
SOC	Super optimal broth with catabolite repression
SBT	Sequence-based typing
SDS	Sodiumdodecyl sulfate

SLA	Swine leukocyte antigen
SSC	Saline-sodium citrate
T_A	Annealing temperature
TAP	Transporter associated with antigen processing
TAE	Tris-acetate ethylendiaminetetraacdic acid
TCR	T-cell receptor
TGEV	Transmissible gastroenteritis virus
Taq	Thermus aquaticus
T_M	Melting temperature
TNF	Tumor necrosis factor
UV	Ultraviolet
X-Gal	Bromo-chloro-indolyl-galactopyranoside
µl	Microliter

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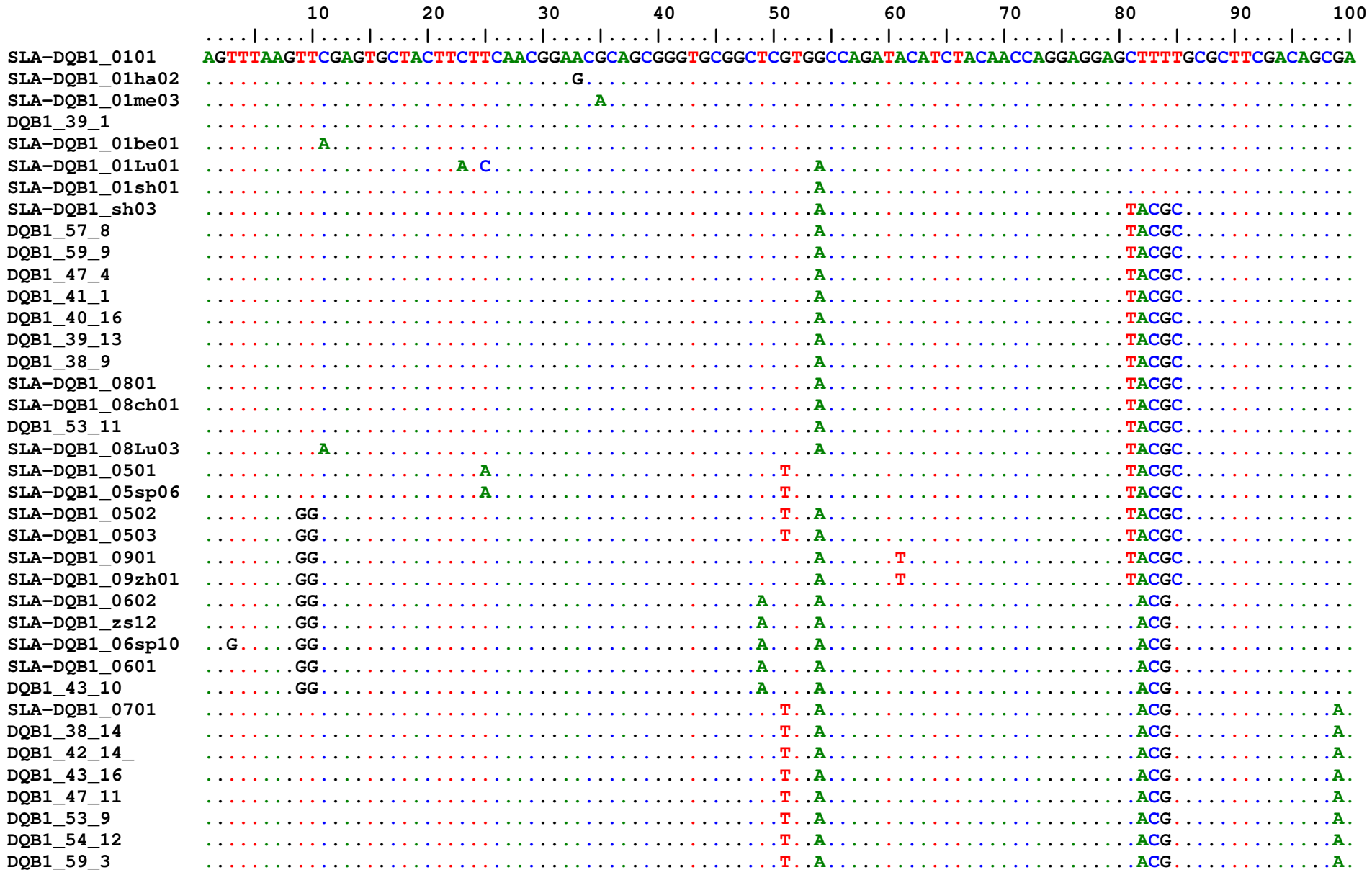
VII Appendix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DQB1_39_1
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DQB1_59_9
DQB1_47_4
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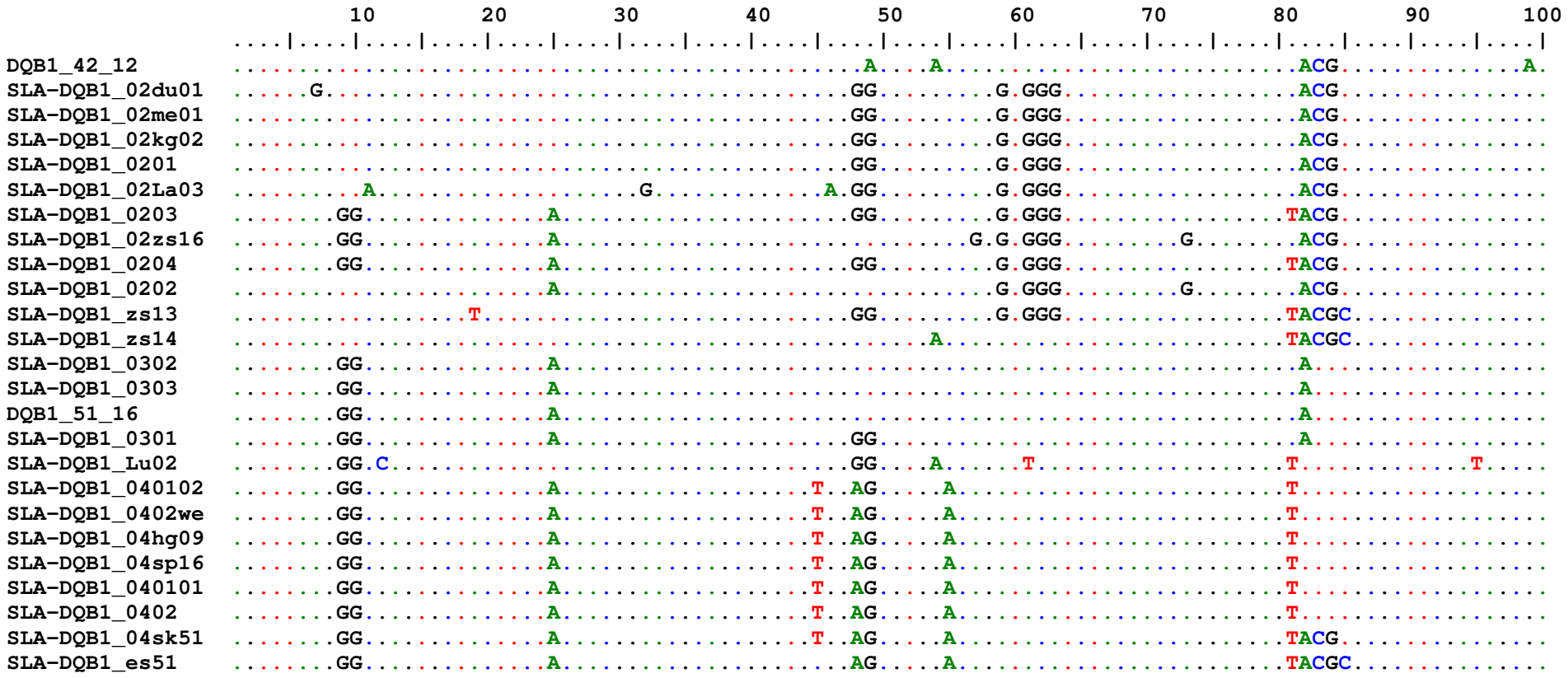
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SLA-DQB1_02me01
SLA-DQB1_02kg02
SLA-DQB1_0201
SLA-DQB1_02La03
SLA-DQB1_0203
SLA-DQB1_02zs16
SLA-DQB1_0204
SLA-DQB1_0202
SLA-DQB1_zs13
SLA-DQB1_zs14
SLA-DQB1_0302
SLA-DQB1_0303
DQB1_51_16
SLA-DQB1_0301
SLA-DQB1_Lu02
SLA-DQB1_040102
SLA-DQB1_0402we
SLA-DQB1_04hg09
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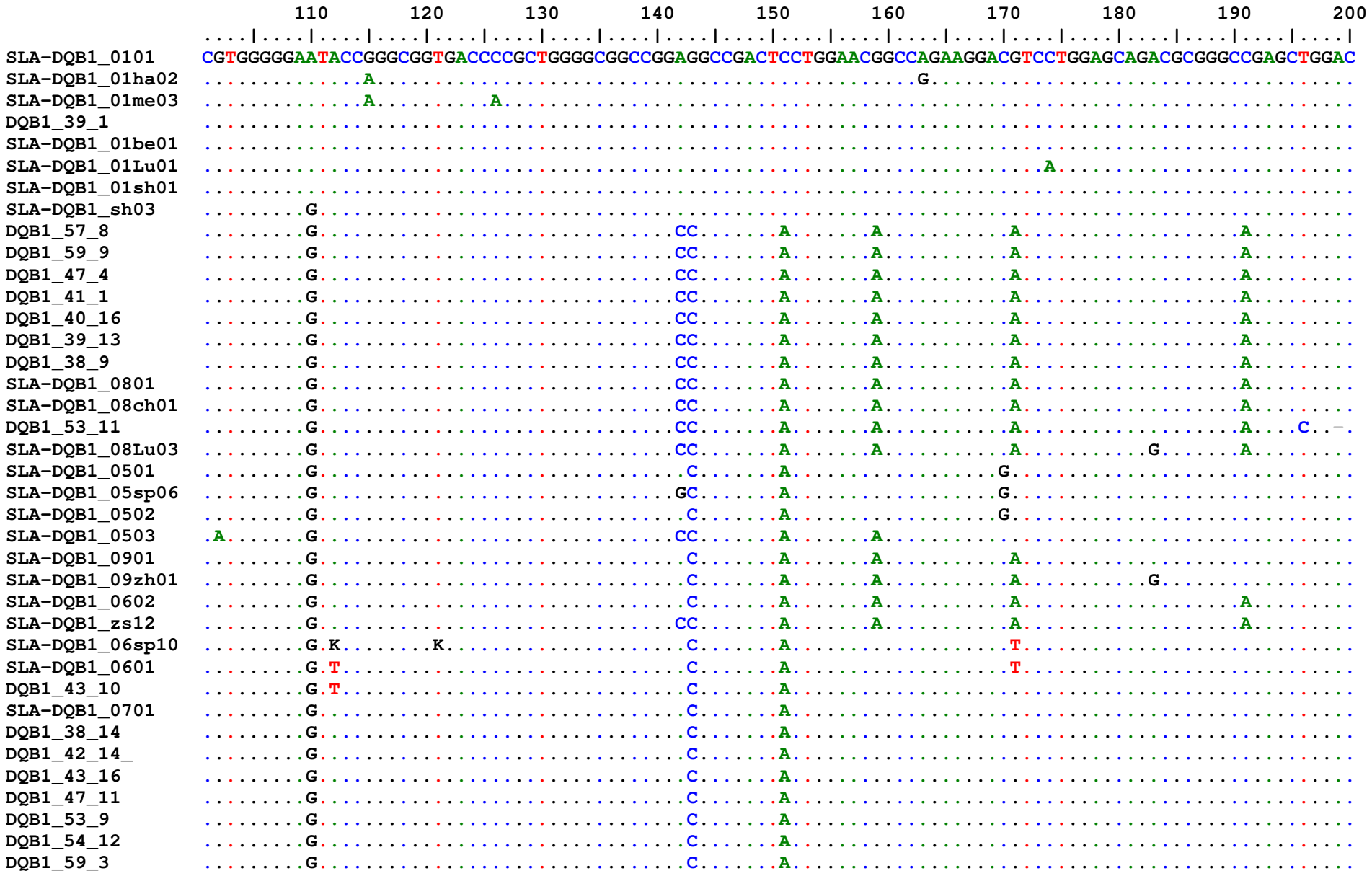
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SLA-DQB1_01ha02
SLA-DQB1_01me03
DQB1_39_1
SLA-DQB1_01be01
SLA-DQB1_01Lu01
SLA-DQB1_01sh01
SLA-DQB1_sh03
DQB1_57_8
DQB1_59_9
DQB1_47_4
DQB1_41_1
DQB1_40_16
DQB1_39_13
DQB1_38_9
SLA-DQB1_0801
SLA-DQB1_08ch01
DQB1_53_11
SLA-DQB1_08Lu03
SLA-DQB1_0501
SLA-DQB1_05sp06
SLA-DQB1_0502
SLA-DQB1_0503
SLA-DQB1_0901
SLA-DQB1_09zh01
SLA-DQB1_0602
SLA-DQB1_zs12
SLA-DQB1_06sp10
SLA-DQB1_0601
DQB1_43_10
SLA-DQB1_0701
DQB1_38_14
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DQB1_53_9
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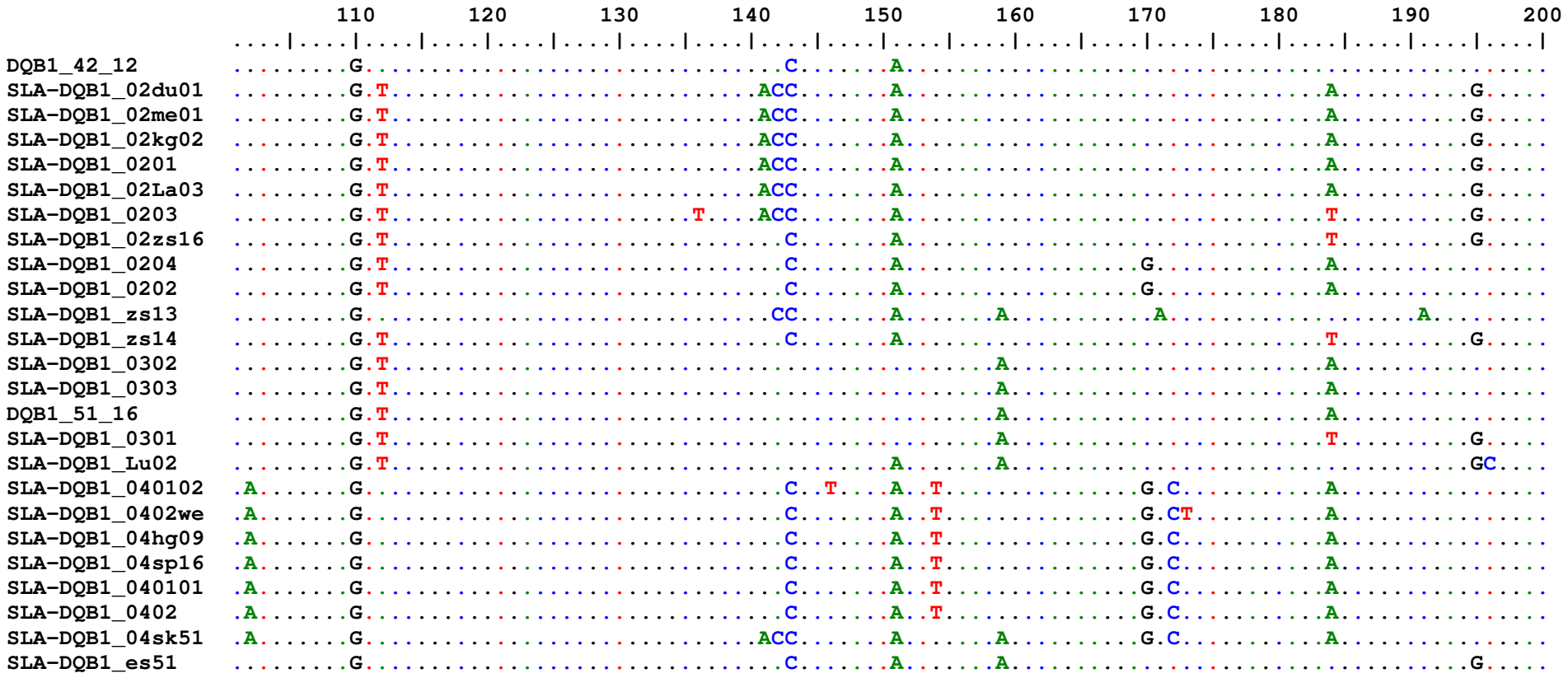
DQB1_59_3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SLA-DQB1_02La03
SLA-DQB1_0203
SLA-DQB1_02zs16
SLA-DQB1_0204
SLA-DQB1_0202
SLA-DQB1_zs13
SLA-DQB1_zs14
SLA-DQB1_0302
SLA-DQB1_0303
DQB1_51_16
SLA-DQB1_0301
SLA-DQB1_Lu02
SLA-DQB1_040102
SLA-DQB1_0402we
SLA-DQB1_04hg09
SLA-DQB1_04sp16
SLA-DQB1_040101
SLA-DQB1_0402
SLA-DQB1_04sk51
SLA-DQB1_es51

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                          210       220
                    ....|....|....|....|....|
SLA-DQB1_0101       ACGGTGTGCAAACACAACTACCAG.
SLA-DQB1_01ha02     ........................
SLA-DQB1_01me03     ........................
DQB1_39_1           ........................
SLA-DQB1_01be01     ........................
SLA-DQB1_01Lu01     ........................
SLA-DQB1_01sh01     ........................
SLA-DQB1_sh03       ........................
DQB1_57_8           ........................
DQB1_59_9           ........................
DQB1_47_4           ........................
DQB1_41_1           ........................
DQB1_40_16          ........................
DQB1_39_13          ........................
DQB1_38_9           ........................
SLA-DQB1_0801       ........................
SLA-DQB1_08ch01     ........................
DQB1_53_11          C.C.....................
SLA-DQB1_08Lu03     ........................
SLA-DQB1_0501       ........................
SLA-DQB1_05sp06     ........................
SLA-DQB1_0502       ........................
SLA-DQB1_0503       ........................
SLA-DQB1_0901       ........................
SLA-DQB1_09zh01     ........................
SLA-DQB1_0602       ..T.....................
SLA-DQB1_zs12       ........................
SLA-DQB1_06sp10     ..T.....................
SLA-DQB1_0601       ..T.....................
DQB1_43_10          ..T.....................
SLA-DQB1_0701       ..T.....................
DQB1_38_14          ..T.....................
DQB1_42_14_         ..T.....................
DQB1_43_16          ..T.....................
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DQB1_53_9           ..T.....................
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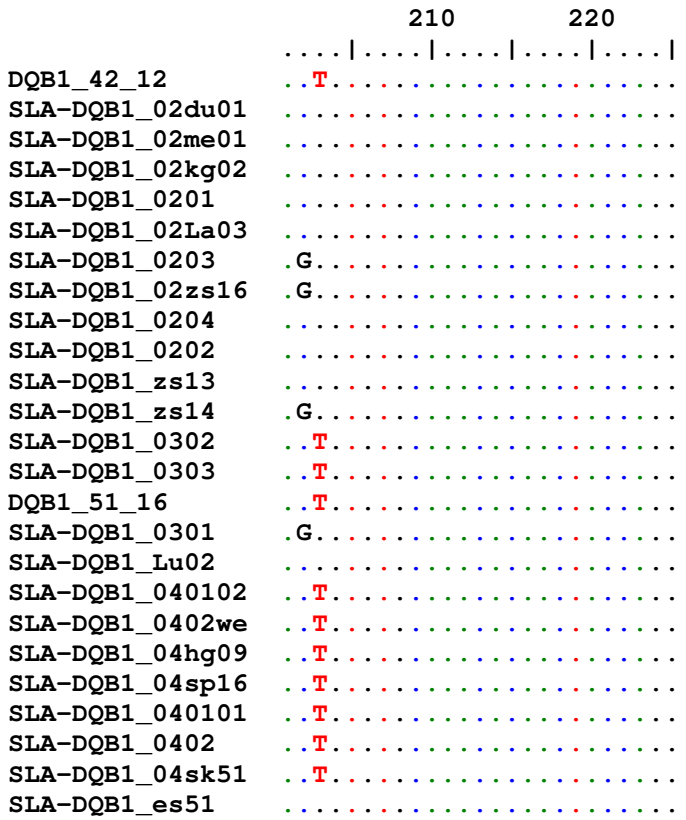
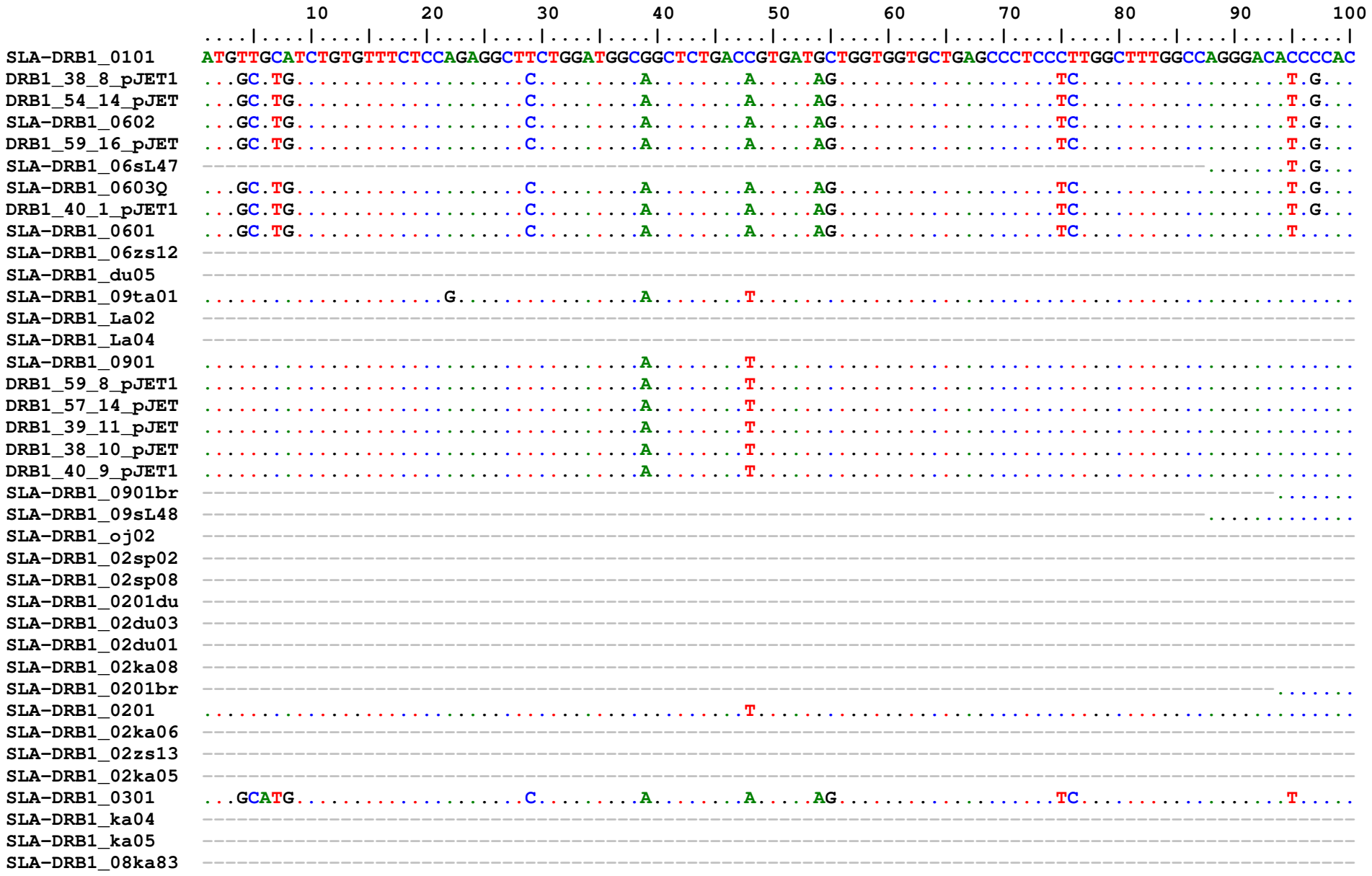


Figure 22. DQB1-Nucleotide Alignment of the 12 SBT-typed Pietrain pigs. An alignment including the identified DQB1-alleles and the reference alleles listed in the IPD was generated by the Neighbour-Joining method.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DRB1_54_14_pJET ...GC.TG.....C.....A.....A....AG.....TC.....T.G...
SLA-DRB1_0602 ...GC.TG.....C.....A.....A....AG.....TC.....T.G...
DRB1_59_16_pJET ...GC.TG.....C.....A.....A....AG.....TC.....T.G...
SLA-DRB1_06sL47 --.....T.G...
SLA-DRB1_0603Q ...GC.TG.....C.....A.....A....AG.....TC.....T.G...
DRB1_40_1_pJET1 ...GC.TG.....C.....A.....A....AG.....TC.....T.G...
SLA-DRB1_0601 ...GC.TG.....C.....A.....A....AG.....TC.....T.....
SLA-DRB1_06zs12 --
SLA-DRB1_du05 --
SLA-DRB1_09ta01 .....G.....A.....T.....
SLA-DRB1_La02 --
SLA-DRB1_La04 --
SLA-DRB1_0901 .....A.....T.....
DRB1_59_8_pJET1 .....A.....T.....
DRB1_57_14_pJET .....A.....T.....
DRB1_39_11_pJET .....A.....T.....
DRB1_38_10_pJET .....A.....T.....
DRB1_40_9_pJET1 .....A.....T.....
SLA-DRB1_0901br --.....
SLA-DRB1_09sL48 --
SLA-DRB1_oj02 --
SLA-DRB1_02sp02 --
SLA-DRB1_02sp08 --
SLA-DRB1_0201du --
SLA-DRB1_02du03 --
SLA-DRB1_02du01 --
SLA-DRB1_02ka08 --
SLA-DRB1_0201br --.....
SLA-DRB1_0201 .....T.....
SLA-DRB1_02ka06 --
SLA-DRB1_02zs13 --
SLA-DRB1_02ka05 --
SLA-DRB1_0301 ...GCATG.....C.....A.....A....AG.....TC.....T.....
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SLA-DRB1_ka05 --
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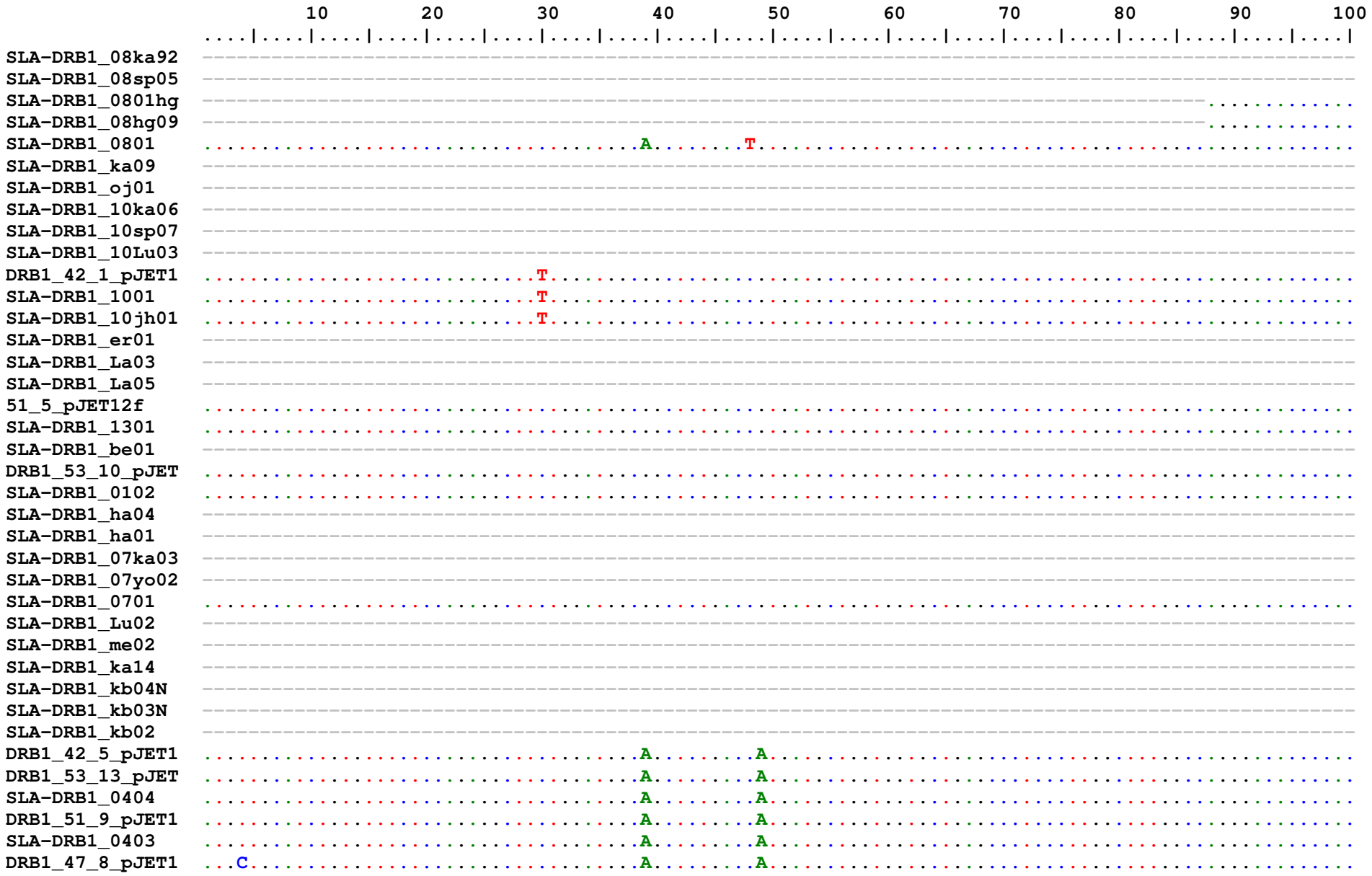
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80
90
100
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SLA-DRB1_08hg09
SLA-DRB1_0801
SLA-DRB1_ka09
SLA-DRB1_oj01
SLA-DRB1_10ka06
SLA-DRB1_10sp07
SLA-DRB1_10Lu03
DRB1_42_1_pJET1
SLA-DRB1_1001
SLA-DRB1_10jh01
SLA-DRB1_er01
SLA-DRB1_La03
SLA-DRB1_La05
51_5_pJET12f
SLA-DRB1_1301
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DRB1_53_10_pJET
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SLA-DRB1_07ka03
SLA-DRB1_07yo02
SLA-DRB1_0701
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SLA-DRB1_kb02
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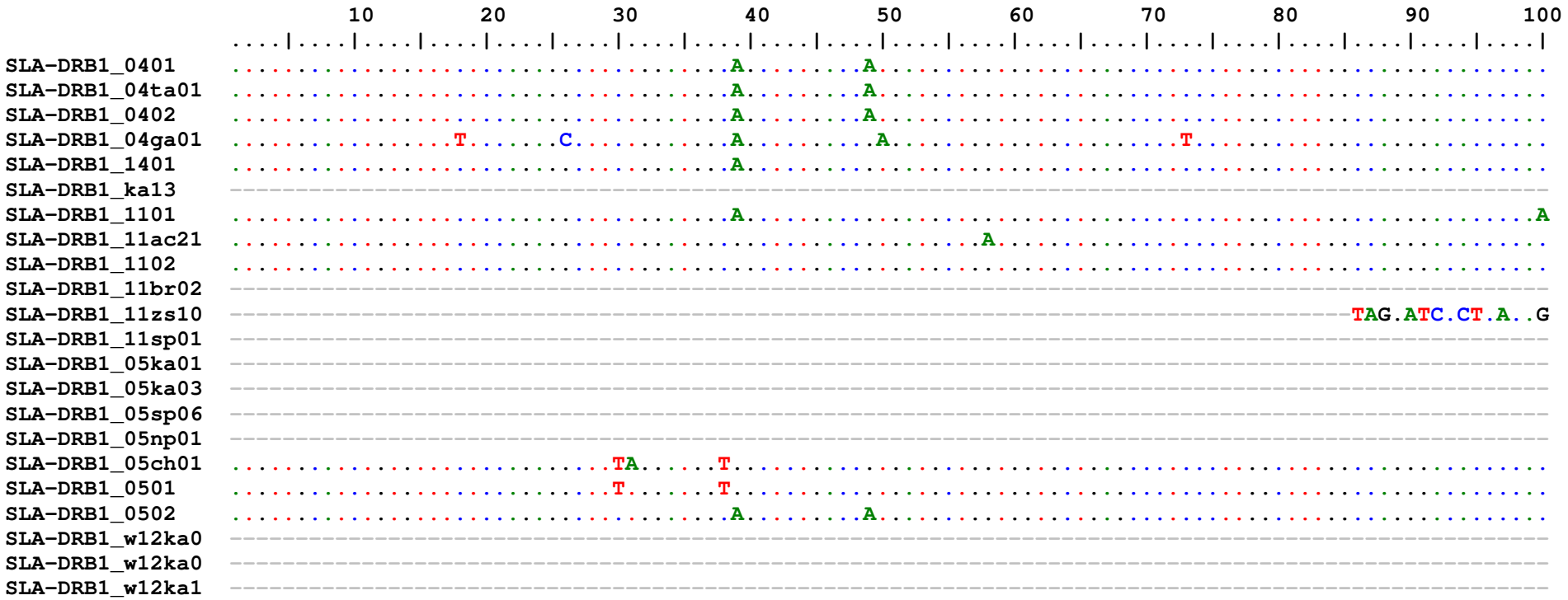
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SLA-DRB1_0402
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SLA-DRB1_1401
SLA-DRB1_ka13
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SLA-DRB1_11ac21
SLA-DRB1_1102
SLA-DRB1_11br02
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SLA-DRB1_11sp01
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SLA-DRB1_05ka03
SLA-DRB1_05sp06
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SLA-DRB1_05ch01
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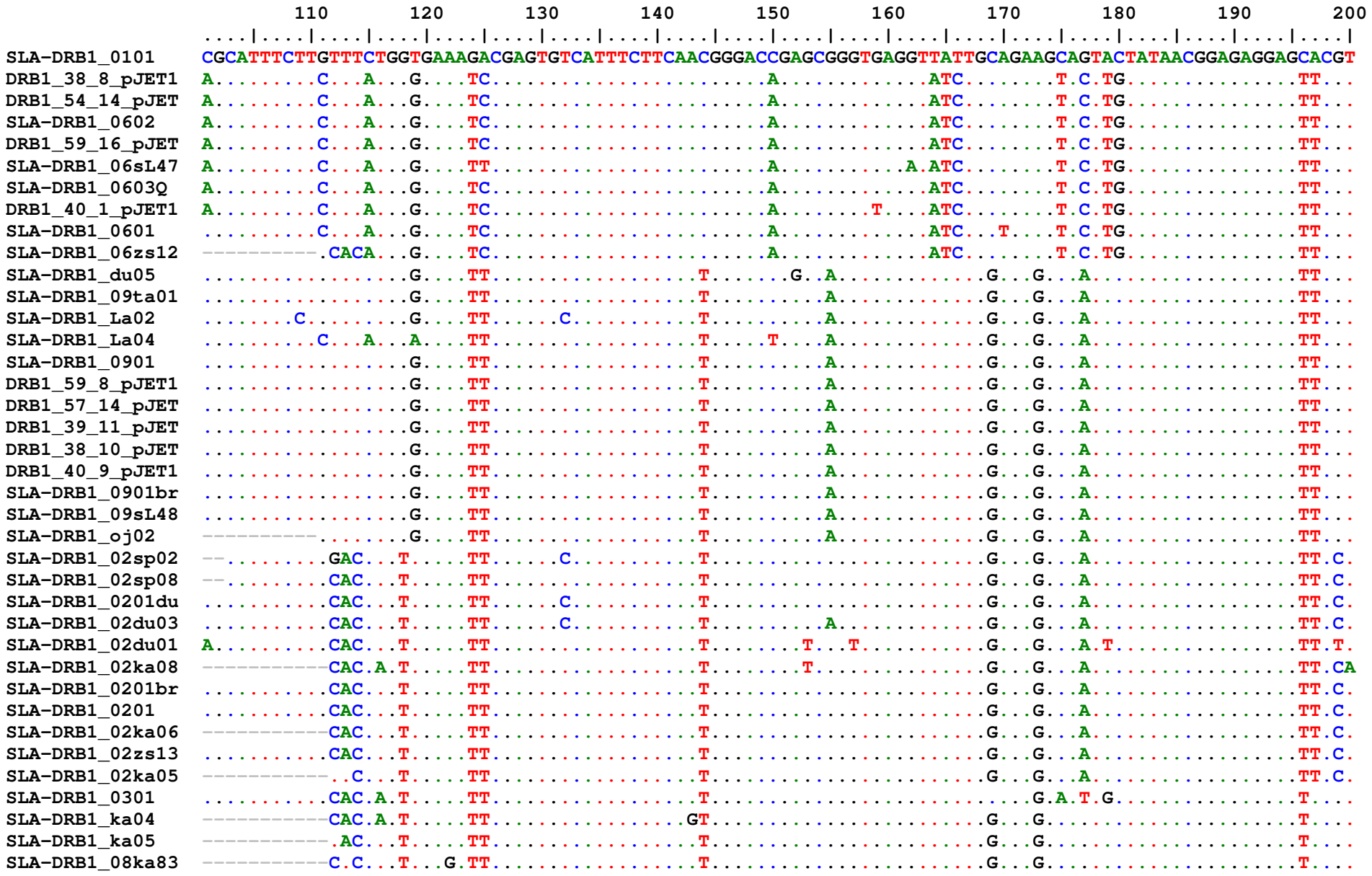
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DRB1_54_14_pJET
SLA-DRB1_0602
DRB1_59_16_pJET
SLA-DRB1_06sL47
SLA-DRB1_0603Q
DRB1_40_1_pJET1
SLA-DRB1_0601
SLA-DRB1_06zs12
SLA-DRB1_du05
SLA-DRB1_09ta01
SLA-DRB1_La02
SLA-DRB1_La04
SLA-DRB1_0901
DRB1_59_8_pJET1
DRB1_57_14_pJET
DRB1_39_11_pJET
DRB1_38_10_pJET
DRB1_40_9_pJET1
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SLA-DRB1_02sp08
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SLA-DRB1_02du03
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SLA-DRB1_ka04
SLA-DRB1_ka05
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SLA-DRB1_10sp07
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SLA-DRB1_10jh01
SLA-DRB1_er01
SLA-DRB1_La03
SLA-DRB1_La05
51_5_pJET12f
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SLA-DRB1_be01
DRB1_53_10_pJET
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SLA-DRB1_ha04
SLA-DRB1_ha01
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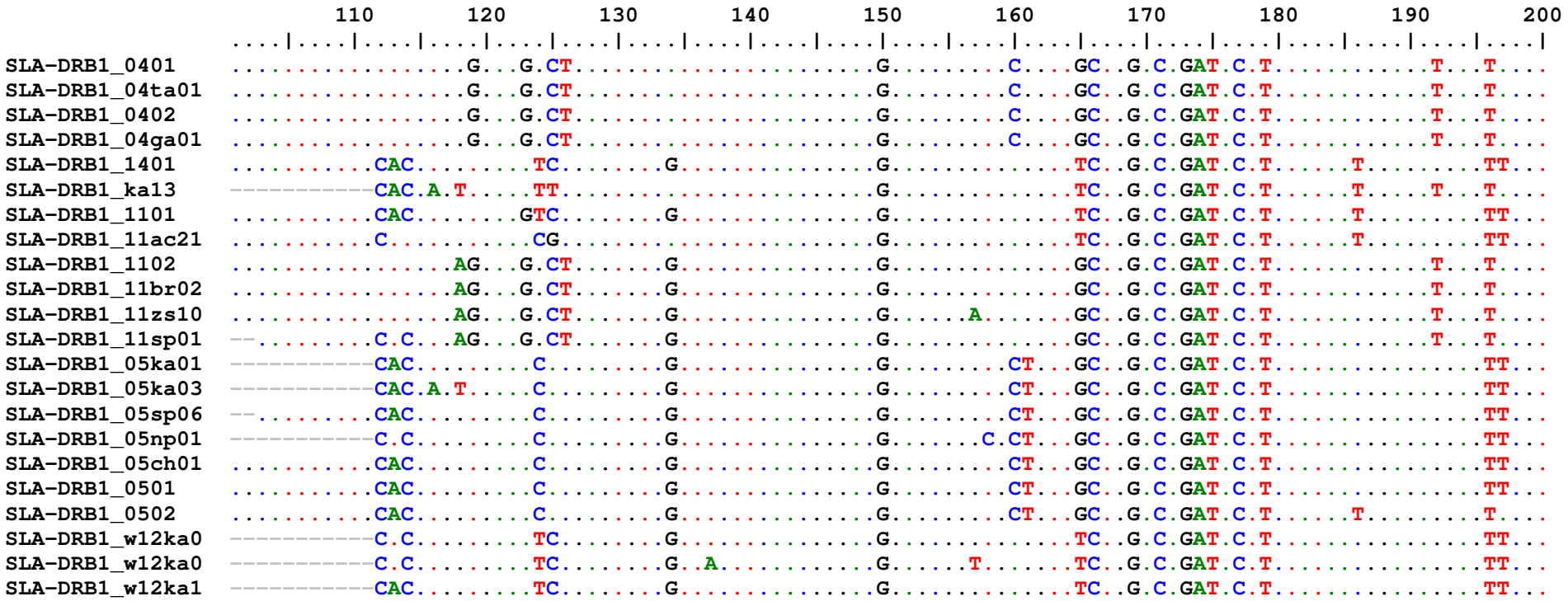
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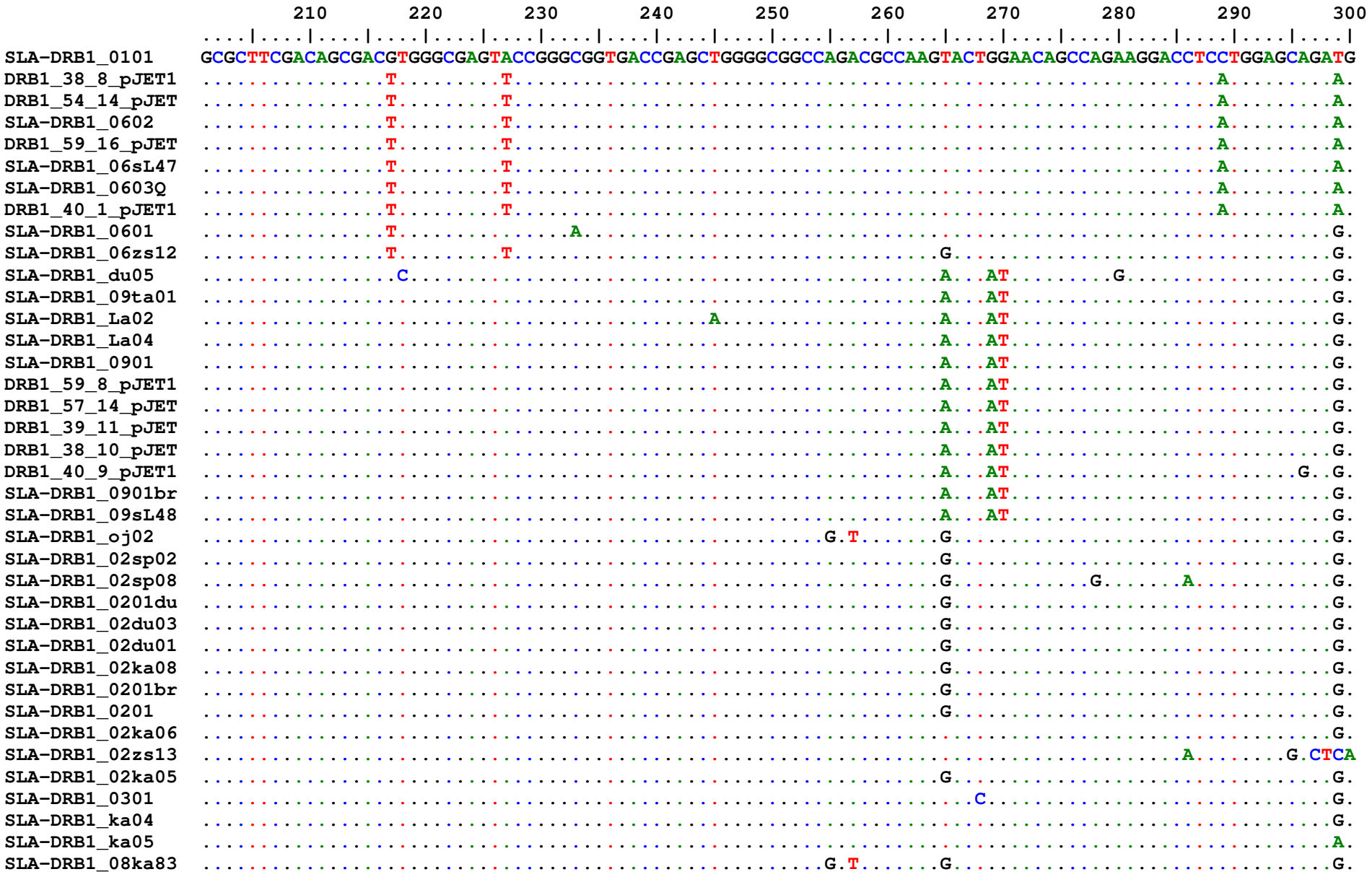
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DRB1_59_16_pJET
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DRB1_39_11_pJET
DRB1_38_10_pJET
DRB1_40_9_pJET1
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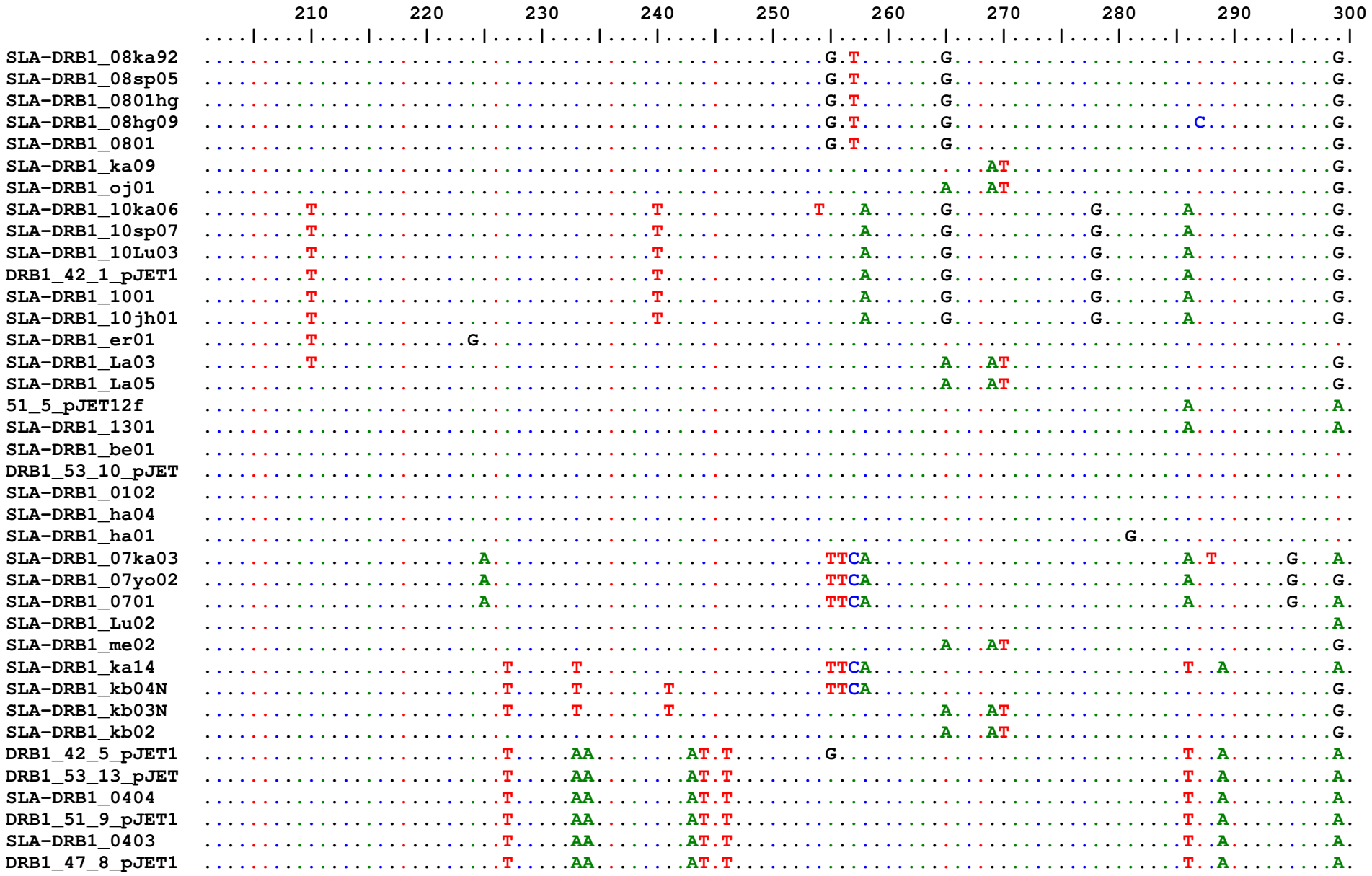
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SLA-DRB1_oj01
SLA-DRB1_10ka06
SLA-DRB1_10sp07
SLA-DRB1_10Lu03
DRB1_42_1_pJET1
SLA-DRB1_1001
SLA-DRB1_10jh01
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SLA-DRB1_La05
51_5_pJET12f
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DRB1_47_8_pJET1


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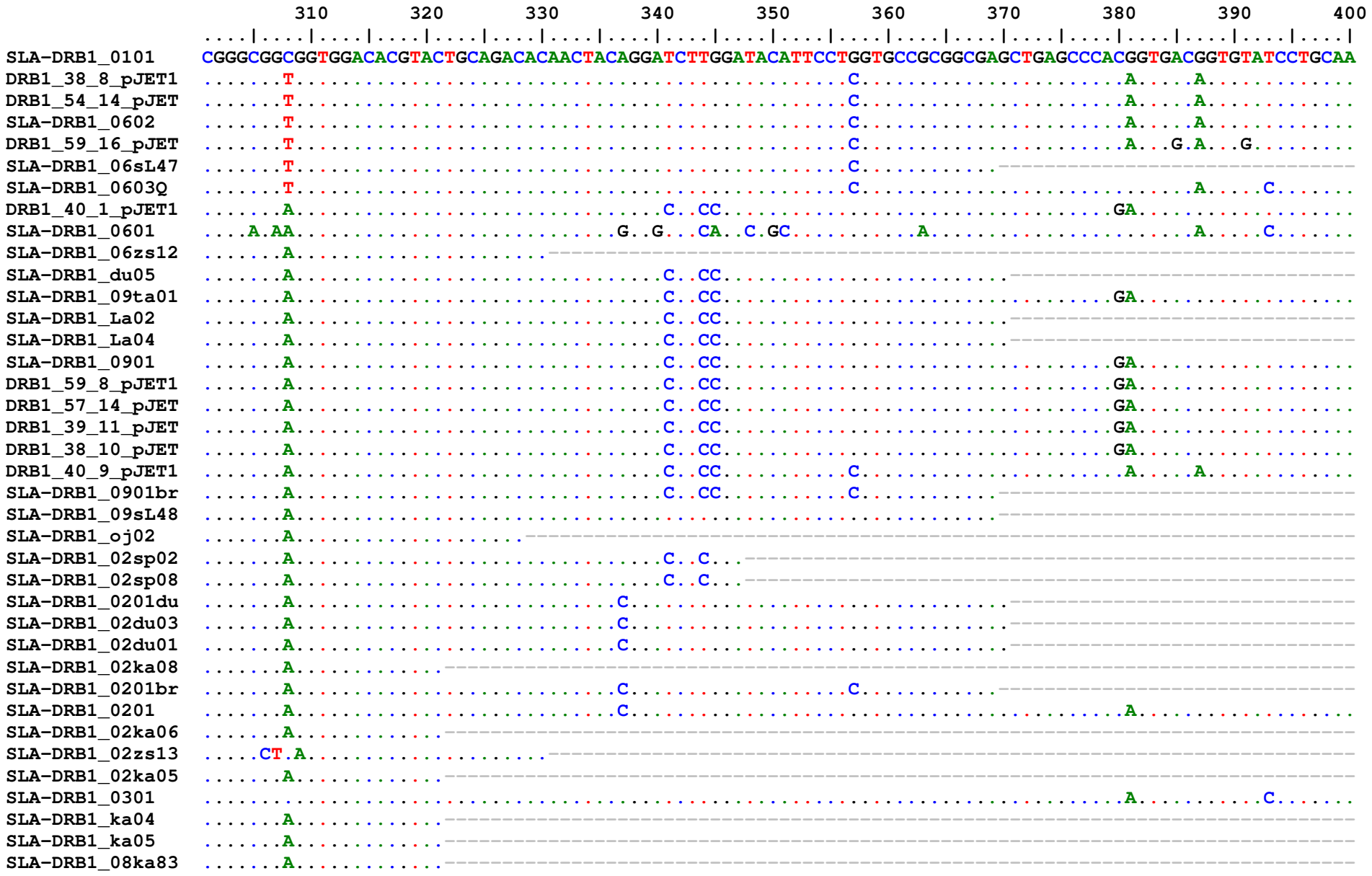
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SLA-DRB1_0602
DRB1_59_16_pJET
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SLA-DRB1_0603Q
DRB1_40_1_pJET1
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SLA-DRB1_06zs12
SLA-DRB1_du05
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SLA-DRB1_La02
SLA-DRB1_La04
SLA-DRB1_0901
DRB1_59_8_pJET1
DRB1_57_14_pJET
DRB1_39_11_pJET
DRB1_38_10_pJET
DRB1_40_9_pJET1
SLA-DRB1_0901br
SLA-DRB1_09sL48
SLA-DRB1_oj02
SLA-DRB1_02sp02
SLA-DRB1_02sp08
SLA-DRB1_0201du
SLA-DRB1_02du03
SLA-DRB1_02du01
SLA-DRB1_02ka08
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SLA-DRB1_02zs13
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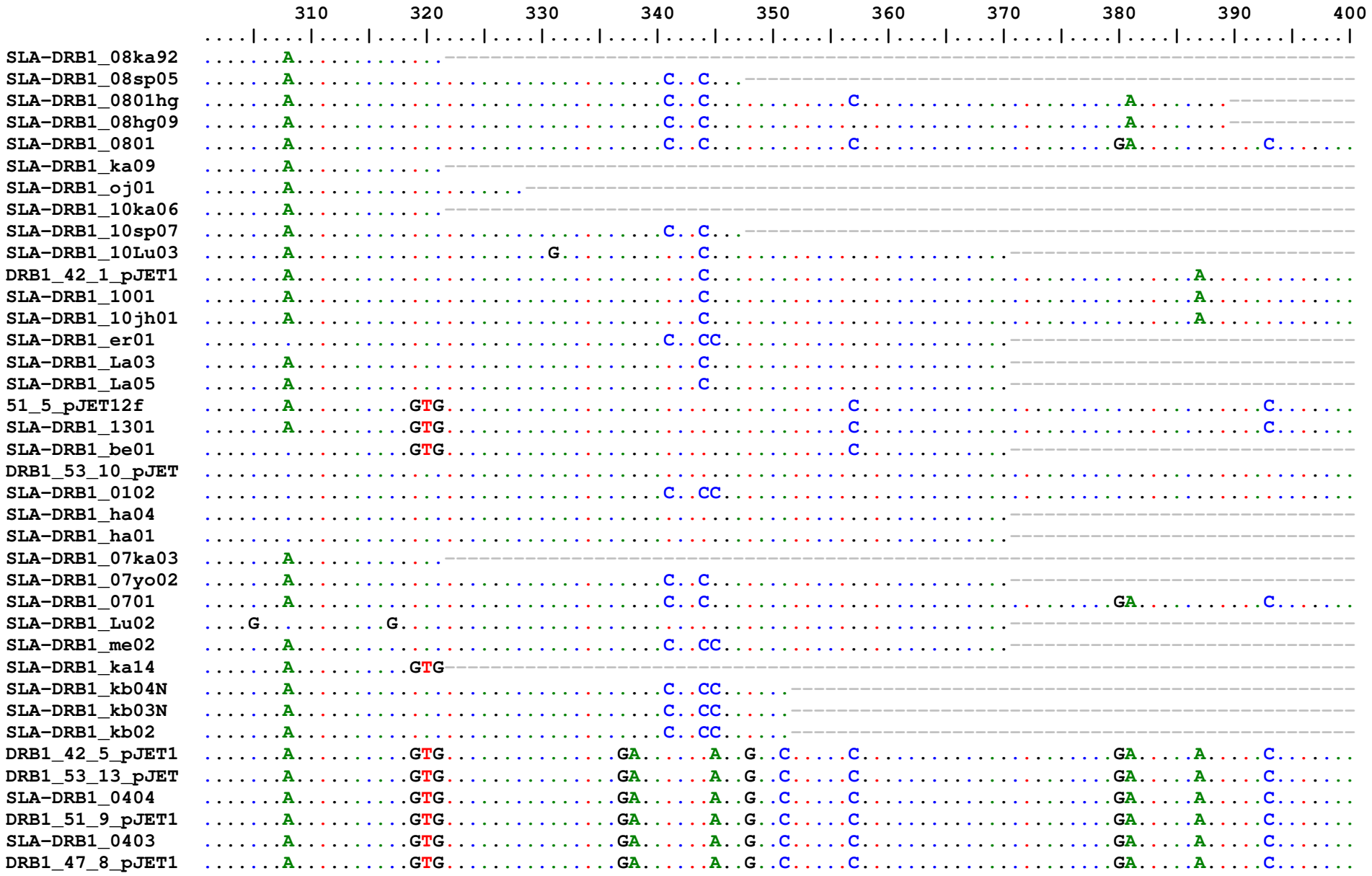
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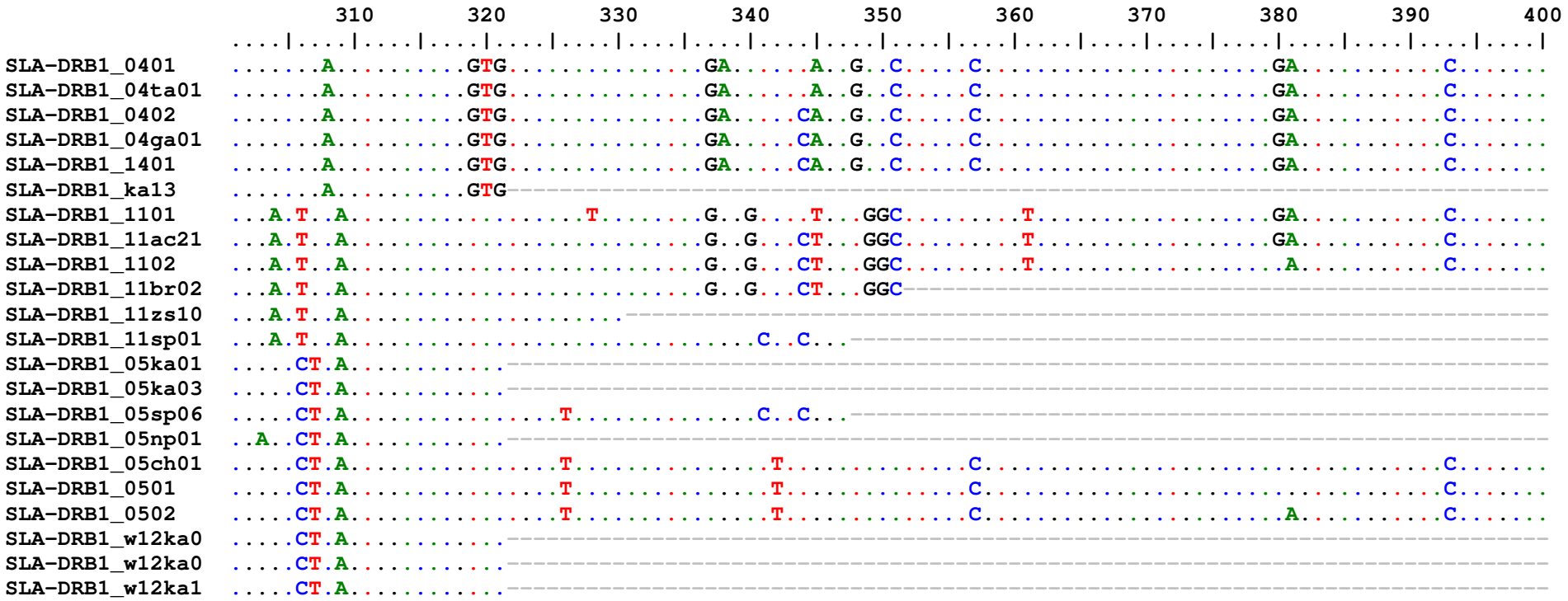
SLA-DRB1_0403
DRB1_47_8_pJET1


310
320
330
340
350
360
370
380
390
400
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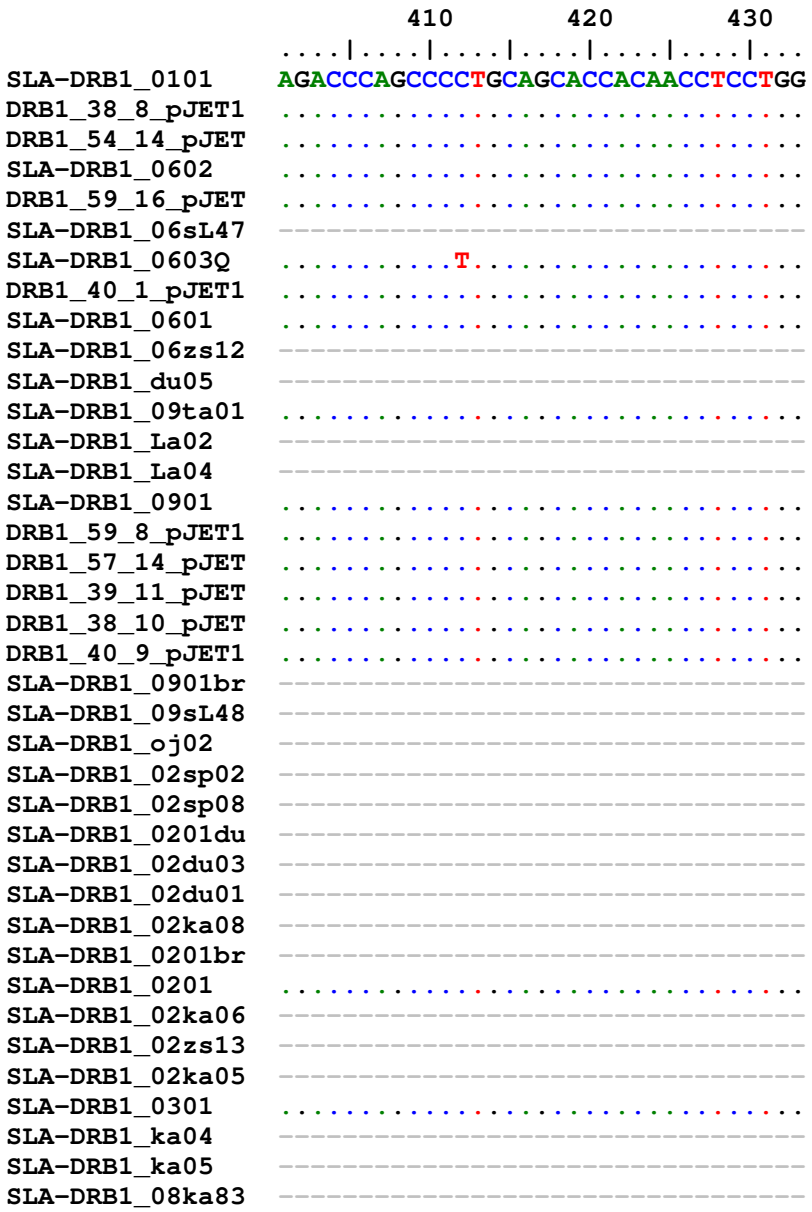
SLA-DRB1_04ga01
SLA-DRB1_1401
SLA-DRB1_ka13
SLA-DRB1_1101
SLA-DRB1_11ac21
SLA-DRB1_1102
SLA-DRB1_11br02
SLA-DRB1_11zs10
SLA-DRB1_11sp01
SLA-DRB1_05ka01
SLA-DRB1_05ka03
SLA-DRB1_05sp06
SLA-DRB1_05np01
SLA-DRB1_05ch01
SLA-DRB1_0501
SLA-DRB1_0502
SLA-DRB1_w12ka0
SLA-DRB1_w12ka0
SLA-DRB1_w12ka1


410
420
430
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DRB1_38_8_pJET1
DRB1_54_14_pJET
SLA-DRB1_0602
DRB1_59_16_pJET
SLA-DRB1_06sL47
SLA-DRB1_0603Q
DRB1_40_1_pJET1
SLA-DRB1_0601
SLA-DRB1_06zs12
SLA-DRB1_du05
SLA-DRB1_09ta01
SLA-DRB1_La02
SLA-DRB1_La04
SLA-DRB1_0901
DRB1_59_8_pJET1
DRB1_57_14_pJET
DRB1_39_11_pJET
DRB1_38_10_pJET
DRB1_40_9_pJET1
SLA-DRB1_0901br
SLA-DRB1_09sL48
SLA-DRB1_oj02
SLA-DRB1_02sp02
SLA-DRB1_02sp08
SLA-DRB1_0201du
SLA-DRB1_02du03
SLA-DRB1_02du01
SLA-DRB1_02ka08
SLA-DRB1_0201br
SLA-DRB1_0201
SLA-DRB1_02ka06
SLA-DRB1_02zs13
SLA-DRB1_02ka05
SLA-DRB1_0301
SLA-DRB1_ka04
SLA-DRB1_ka05
SLA-DRB1_08ka83

```
                          410        420        430
                 ....|....|....|....|....|....|...
SLA-DRB1_08ka92  ----------------------------------
SLA-DRB1_08sp05  ----------------------------------
SLA-DRB1_0801hg  ----------------------------------
SLA-DRB1_08hg09  ----------------------------------
SLA-DRB1_0801    ..................................
SLA-DRB1_ka09    ----------------------------------
SLA-DRB1_oj01    ----------------------------------
SLA-DRB1_10ka06  ----------------------------------
SLA-DRB1_10sp07  ----------------------------------
SLA-DRB1_10Lu03  ----------------------------------
DRB1_42_1_pJET1  ..................................
SLA-DRB1_1001    ..................................
SLA-DRB1_10jh01  ..................................
SLA-DRB1_er01    ----------------------------------
SLA-DRB1_La03    ----------------------------------
SLA-DRB1_La05    ----------------------------------
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SLA-DRB1_1301    ..................................
SLA-DRB1_be01    ----------------------------------
DRB1_53_10_pJET  ..................................
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SLA-DRB1_kb03N   ----------------------------------
SLA-DRB1_kb02    ----------------------------------
DRB1_42_5_pJET1  ..................................
DRB1_53_13_pJET  ..................................
SLA-DRB1_0404    ..................................
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SLA-DRB1_0403    ..................................
DRB1_47_8_pJET1  ..................................
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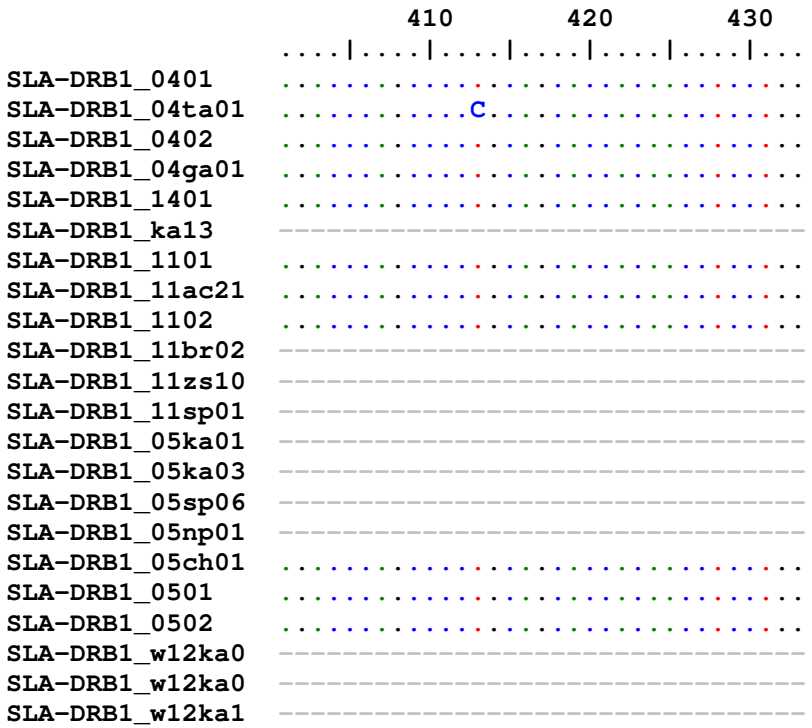

Figure 23. DRB1-Nucleotide Alignment of the 12 SBT-typed Pietrain pigs. An alignment including the identified DRB1-alleles and the reference alleles listed in the IPD was generated by the Neighbour-Joining method

VIII Curriculum Vitae

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Education

Sep 1989 – Jun 1993	Elementary school, VS Zennerstraße 1, 1140 Vienna
Sep 1993 – Jun 2001	High school, BRG7 Kandlgasse 39, 1070 Vienna Matura, 8th June 2001
Oct 2002 –	Molecular Biology, specialized in immunology, biochemistry and organic chemestry, University of Vienna
Mar 2009 – Oct 2009	Diploma thesis at University of Veterinary Medicine Vienna, Institute of clinic immunology, Topic: „Characterization of swine leukocyte antigen polymorphism by sequence-based and low-resolution typing methods"

Oral presentations

Hammer SE[1], **Ertl W**, Rütgen BC, Groiß S, Deutsch J, Gerner W, Ho C-S, Saalmüller A. Comparative analysis of the major histocompatibility complex (MHC) in mammals – Current status of research and further perspectives. 83rd Annual meeting of the German Society of Mammalogy (DGS 2009) (Dresden, Germany). September 13-17, 2009.

Hammer SE, **Ertl W**, Rütgen BC, Deutsch J, Gerner W, Ho C-S, Saalmüller A. Characterization of swine leukocyte antigen (SLA) polymorphism reveals a breed-specific constriction of SLA gene diversity in Pietrain pigs. Abstract for the 32nd Conference of the International Society of Animal Genetics (ISAG 2010) (Edinburgh, UK), 26 - 30 July, 2010.

[1]Presenting author is underlined.

Posterpresentations

Hammer SE, **Ertl W**, Rütgen BC, Groiß S, Deutsch J, Gerner W, Ho C-S, Smith DM, Saalmüller A. Characterization of swine leukocyte antigen polymorphism by sequence-based and low-resolution group-specific typing methods in Pietrain pigs. 3rd European Veterinary Immunology Workshop (EVIW 2009) (Berlin, Germany). September 10-13, 2009.

Ephraim E, Burwinkel, Palissa C, Wie Z, Simon O, Sachtleben M, Plendl J, Hammer S, **Ertl W**, Saalmüller A, Schmidt MFG. Establishment of two new lines of porcine intestinal epithelial cells for the study of anti-viral-effects of probiotics. 3rd European Veterinary Immunology Workshop (EVIW 2009) (Berlin, Germany). September 10-13, 2009.

Hammer SE, **Ertl W**, Rütgen BC, Deutsch J, Gerner W, Ho C-S, Saalmüller A. Characterization of swine leukocyte antigen (SLA) polymorphism reveals a breed-specific constriction of SLA gene diversity in Pietrain pigs. International Symposium on Animal Genomics for Animal Health (AGAH 2010) (Paris, France), 31 May - 2 June, 2010.

Conference abstracts in peer-reviewed journals

Hammer SE, **Ertl W**, Rütgen BC, Groiß S, Deutsch J, Gerner W, Ho C-S, Saalmüller A. Comparative analysis of the major histocompatibility complex (MHC) in mammals – Current status of research and further perspectives. Mammalian Biology 74 [Suppl. 1]:12.

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Hammer SE, **Ertl W**, Rütgen BC, Groiß S, Deutsch J, Gerner W, Ho C-S, Smith DM, Saalmüller A. Characterization of swine leukocyte antigen polymorphism by sequence-based and low-resolution group-specific typing methods in Pietrain pigs. Abstract book 3rd European Veterinary Immunology Workshop (EVIW 2009) (Berlin, Germany), p. 61.

Ephraim E, Burwinkel, Palissa C, Wie Z, Simon O, Sachtleben M, Plendl J, Hammer S, **Ertl W**, Saalmüller A, Schmidt MFG. Establishment of two new lines of porcine intestinal epithelial cells for the study of anti-viral-effects of probiotics. 3rd European Veterinary Immunology Workshop (EVIW 2009) (Berlin, Germany). p. 45

Hammer SE, **Ertl W**, Rütgen BC, Deutsch J, Gerner W, Ho C-S, Saalmüller A. Characterization of swine leukocyte antigen (SLA) polymorphism reveals a breed-specific constriction of SLA gene diversity in Pietrain pigs. International Symposium on Animal Genomics for Animal Health (AGAH 2010) (Paris, France), p. 57

Hammer SE, **Ertl W**, Rütgen BC, Deutsch J, Gerner W, Ho C-S, Saalmüller A. Characterization of swine leukocyte antigen (SLA) polymorphism reveals a breed-specific constriction of SLA gene diversity in Pietrain pigs. Abstract for the 32nd Conference of the International Society of Animal Genetics (ISAG 2010) (Edinburgh, UK), p. 139

Submitted Conference contributions

Hammer SE, **Ertl W**, Rütgen BC, Deutsch J, Gerner W, Ho C-S, Saalmüller A. Characterization of swine leukocyte antigen (SLA) polymorphism reveals a breed-specific constriction of SLA gene diversity in Pietrain pigs. Abstract for the 9th International Symposium of Veterinary Immunology (IVIS 2010) (Tokyo, Japan), 16 - 20 August, 2010.