Linkage Disequilibrium and Transmission Distortion Affecting Human Chromosome 6p

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Berlin, den 9. Februar 2010 Pablo Sandro Carvalho Santos

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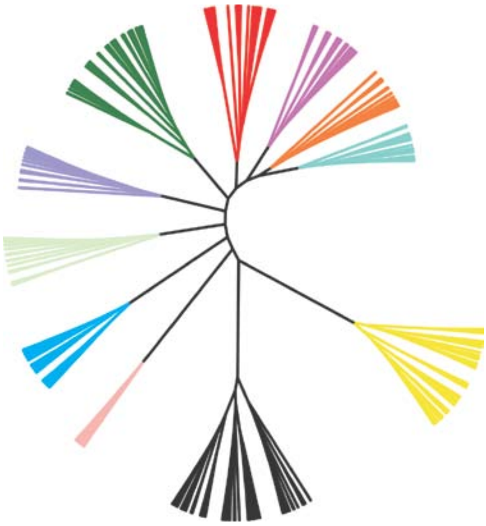
Even if this is allegedly the most widely read part of a PhD thesis, I do encourage you all to dedicate some time to the next chapters. It took me four years to write it, and it is an interesting work, in my opinion.

Thank you all.



Zusammenfassung

Sowohl die jüngste Entwicklung neuer Technologien für die Hochdurchsatzsequenzierung und -genotypisierung von Organismen als auch die Erzeugung neuer Werkzeuge für großflächige genomische Analysen, die in letzter Zeit von verschiedenen Forschungsinstituten weltweit zur Verfügung gestellt wurden, haben die Art der Fragestellungen in den verschiedenen Arbeitsfeldern der Genetik stark beeinflußt. Die vorliegende Arbeit ist ein Beispiel für die Nutzung dieser Ressourcen in Verbindung mit traditionellen Labormethoden, um sich zwei Themenfeldern in den Bereichen Immun- und Populationsgenetik zu nähern: einerseits den mit dem Haupthistokompatibilitätskomplex gekoppelten Geruchsrezeptorgenen (aus dem Englischen, *major histocompatibility complex-linked olfactory receptor genes*, MHC-linked OR), und andererseits dem Phänomen der Allelweitergabedistorsion (*transmission distortion*, TD). Das Konzept des Kopplungsungleichgewichts (*linkage disequilibrium*, LD) ist für die Diskussion beider Themenfelder grundlegend.

Mit Bezug auf die MHC-OR Gene präsentiert diese Arbeit eine detaillierte Beschreibung des LD Profils innerhalb der OR Gene sowie zwischen ihnen und dem MHC. Durch Analyse der OR Polymorphismen und konservierter MHC-Haplotypen sowie der Genotypisierung einer Kohorte von Raucherinnen und Nichtraucherinnen konnten wir den Zusammenhang zwischen einem OR Polymorphismus und dem Rauchverhalten europäischstämmiger Frauen beschreiben. Wir schilderten genotypische und phänotypische Variationen der MHC-OR Gene bei achtzehn menschlichen Zelllinien, indem wir neue Polymorphismen und OR Allele bestimmten, und damit die Grundlage für die Einschätzung der funktionalen Partizipation von MHC-linked OR bei der Partnerwahl schufen. Darüber hinaus führten wir einen chromosomalen und phylogenetischen Vergleich von MHC-OR Genen bei vierzehn Wirbeltierspezien durch und konnten Schlussfolgerungen über die phylogenetische Geschichte der Genfamilien und Tierarten ziehen.

Bezüglich TD konnten wir dieses Phänomen durch eine reine *in silico*-analyse in einer Region des menschlichen Chromosoms 6p ermitteln, welche die Transkriptionsfaktorgene *SUPT3H* (suppressor of Ty 3 homolog), *RUNX2* (runt-related transcription factor 2) sowie mikro RNA *MIR586* beherbergt. In einer anschließenden Untersuchung, in der wir eine große unabhängige südamerikanische Kohorte genotypisierten, konnten wir die Anwesenheit von TD in diesem chromosomalen Segment, zumindest bei europäischstämmigen Populationen, bestätigen. Aufgrund der hohen Bedeutung von *RUNX2* (und den entsprechenden

Proteinisoformen) haben unsere Erkenntnisse Auswirkungen auf die Interpretation einer Reihe von früheren Studien, die dieses Gen mit verschiedenen Phänotypen wie Krebs, Knochendichte und Entwicklungsstörungen in Verbindung brachten. Diese Ergebnisse wurden in elektronischen Dateien für den Internet-basierenden Genome Browser des HapMap Projekts zusammengestellt, um sie im genomischen Kontext sichtbar werden zu lassen und folglich einen ersten Schritt in Richtung einer genomweiten TD Ressource zu unternehmen. Zusätzlich fanden wir heraus, dass TD mit LD korrespondiert und stellten die Hypothese auf, TD könnte die LD „Landschaft“ des Genoms aktiv gestalten. Schließlich weisen unsere Ergebnisse auf ein potenzielles technisches Problem mit der Datenbank des HapMap Projektes hin, das wir vorschlugen bei zukünftigen Erweiterungen der Datenbank zu berücksichtigen.

Summary

The recent development of new technologies for high throughput sequencing and genotyping of organisms and individuals, as well as the generation of new tools for large-scale genomic analysis, all available through different research institutions world-wide, have changed the way questions from the different fields of genetics are addressed. This thesis is an example of the use of such resources in combination with traditional laboratory methods, in order to approach two themes belonging to immunogenetics and population genetics: on the one hand, the olfactory receptor genes linked to the major histocompatibility complex (MHC-linked OR) and, on the other hand, the allele transmission distortion (TD) phenomenon. The concept of linkage disequilibrium (LD) is regarded as a connecting subject, being fundamental for the discussion of both themes.

This thesis presents a detailed description of LD patterns both within MHC-linked OR genes, and between OR loci and the MHC. Through the analysis of OR polymorphisms and conserved MHC haplotypes and the genotyping of a known cohort of smokers and non smokers, we were able to describe the association of one OR polymorphism with smoking habits in Caucasian women. Furthermore, we analyzed genotypic and phenotypic variation of MHC-linked OR genes for 18 human cell lines based on the description of new polymorphisms and OR alleles, thereby forming a basis for the functional assessment of the participation of MHC-linked OR loci in mate choice. Moreover, we developed the most comprehensive comparison performed to date – both chromosomal and phylogenetic – of MHC-linked OR genes in fourteen vertebrate species, enabling us to derive conclusions about the phylogenetic history of gene families and species.

Considering TD, we were successful in detecting this phenomenon, through a purely *in silico* analysis of healthy family trios, in a region of human chromosome 6p that harbours the transcription factor encoding loci *SUPT3H* (suppressor of Ty 3 homolog), *RUNX2* (runt-related transcription factor 2), and the microRNA *MIR586*. In a follow-up investigation in which we genotyped a large, independent South American cohort, we were able to confirm the presence of TD in that chromosomal segment, at least for populations of Caucasian ancestry. Given the high medial relevance of *RUNX2* and the corresponding encoded protein isoforms, our findings have considerable implications for the interpretation of the many studies that found this locus to be associated with different phenotypes such as cancer, bone density and developmental disorders. These results were compiled into track files for uploading into internet-based genome browsers. These can thus be visualized in the genomic context, and provide a first step towards a genome-wide TD resource. We additionally found TD to be intimately associated to LD in the loci assessed, leading us to hypothesise that TD might be actively shaping the LD landscape of genomes. Finally, our results indicate one potential technical problem with the database of the International HapMap Project, which we suggest should be addressed in future updates of this database.

Resumo

O desenvolvimento de novas ferramentas para análises genômicas em larga escala, assim como a disponibilidade de uma grande quantidade de dados provenientes do sequenciamento e da genotipagem de vários organismos, produzidos e ofertados por diferentes instituições do mundo, tornaram possível uma nova maneira de abordar e de responder questões de diferents áreas da genética. Este trabalho é um exemplo do uso integrado de técnicas laboratoriais tradicionais com novos recursos eletrônicos disponíveis à comunidade científica, para abordar dois temas principais que se encontram entre a imunogenética e a genética de populações: de um lado, os genes de receptores olfatórios ligados ao complexo principal de histocompatibilidade (do inglês, *major histocompatibility complex-linked olfactory receptor genes*, MHC-linked OR), e de outro, o fenômeno da distorção de transmissão alélica

(*transmission distortion*, TD). O conceito de desequilíbrio de ligação (DL) fundamenta a discussão dos resultados e age assim como tema transversal.

Quanto aos genes MHC-linked OR, obtivemos evidência da associação de um haplótipo com o hábito de fumar em mulheres com ancestralidade européia. Geramos um panorama detalhado do desequilíbrio de ligação e variação genotípica/fenotípica entre haplótipos humanos, e desenvolvemos uma abrangente análise comparativa destes *loci* – tanto estrutural quanto filogenética – entre quatorze espécies de vertebrados.

Quanto a TD, tivemos sucesso em detectar evidência da presença deste fenômeno em uma região do braço curto do cromossomo 6 humano, correspondendo aos *loci SUPT3H*, *RUNX2* e *MIR586*, sendo os dois primeiros, fatores de transcrição, e o último, um microRNA. Estes resultados foram primeiramente observados em uma população já genotipada e com genótipos disponíveis através do banco de dados do projeto *HapMap*, e posteriormente confirmados em uma população independente. Os resultados mencionados têm implicações tanto para a gênese e manutenção do desequilíbrio de ligação em populações humanas, como também para a interpretação dos vários estudos que ligaram o *locus RUNX2* a diversos fenótipos, ignorando o fato de que esta região gênica está sob TD em populações de ancestralidade européia.

Esta dissertação está organizada de forma cumulativa, apresentada por meio de cinco artigos submetidos a periódicos científicos, e de um capítulo com resultados ainda não submetidos para publicação.

List of Publications that are Part of this Cumulative Thesis

1. **Santos PSC**, Füst G, Prohászka Z, Volz A, Horton R, Miretti M, Yu CY, Beck S, Uchanska-Ziegler B, and Ziegler A (2008). Association of smoking behavior with an odorant receptor allele telomeric to the human major histocompatibility complex. *Genetic Testing*, 12: 481-486.
2. **Santos PSC**, Höhne J, Schlattmann P, König IR, Ziegler A, Uchanska-Ziegler B, Ziegler A (2009). Assessment of transmission distortion on chromosome 6p in healthy individuals using tagSNPs. *European Journal of Human Genetics*, 17:1182-1189.
3. Sens-Abuázar C, **Santos PSC**, Bicalho MG, Petzl-Erler ML, Sperandio-Roxo V (2009). MHC microsatellites in a Southern Brazilian population. *International Journal of Immunogenetics*, 36:269-274.
4. **Santos PSC**, Höhne J, Schlattmann P, Poerner F, Bicalho MG, Ziegler A, and Uchanska-Ziegler B (2010). Presence of Transmission Distortion on Human Chromosome 6p Revealed by SNP Genotyping of Southern Brazilian Families. Submitted.
5. **Santos PSC**, Uehara CJS, Ziegler A, Uchanska-Ziegler B and Bicalho MG (2010). Variation and linkage disequilibrium within olfactory receptor gene clusters linked to the human major histocompatibility complex. Submitted.

List of Abbreviations

ASW	African ancestry in Southwest USA (HapMap population)
Bta	*Bos taurus* (cow)
BLAST	basic local alignment search tool
bp	base pair
cen	centromere
CEU / CEPH	Utah residents with ancestry from Northern and Western Europe (HapMap population), from the *Centre d'Etude du Polymorphisme Humain (CEPH)* panel.
Cfa	*Canis familiaris* (dog)
CHB	Han Chinese in Beijing, China (HapMap population)
Chr	chromosome
Chr6p / Hsa6p	short arm of human chromosome 6
CNV	copy number variation
CP	cytoplasmic domain
Dre	*Danio rerio* (zebra fish)
EC	extracellular domain
Eca	*Equus caballus* (horse)
F	frequency
Fca	*Felis catus* (cat)
Fig.	figure
GPCR	G-protein-coupled receptor
HapMap	international HapMap project
HLA	human leukocyte antigen
Hsa	*Homo sapiens* (human)
JPT	Japanese in Tokyo, Japan (HapMap population)
Kb	Kilobase (one thousand base pairs)
LD	linkage disequilibrium
LIGH	Laboratório de Immunogética e Histocompatibilidade (Immunogenetics and Histocompatibility Laboratory in the Federal University of Paraná)
MAF	minor allele frequency
Mamu	*Macaca mulatta* (rhesus macaque)
Mb	Megabase (one million base pairs)
Mdo	*Monodelphis domestica* (opossum)
MEX	Mexican ancestry in Los Angeles, California (HapMap population)

MHC	major histocompatibility complex
MHCHP	major histocompatibility complex haplotype project
MKK	Maasai in Kinyawa, Kenya (HapMap population)
µl	microliter (10^{-6} L)
Mumu	*Mus musculus* (mouse)
nsyn	non synonymous (nucleotide substitution)
OR	olfactory receptor / odorant receptor
PCR	polymerase chain reaction
Ppy	*Pongo pygmaeus* (orangutan)
Ptr	*Pan troglodytes* (chimpanzee)
QC	quality control
Rno	*Rattus norvegicus* (rat)
SE	shared epitope
S-M-R	genomic region including the loci *SUPT3H*, *MIR586* and *RUNX2*
SNP	single nucleotide polymorphism
Ssc	*Sus scrofa* (pig)
STR	short tandem repeat
tel	telomere
TD	transmission distortion
TDT	transmission/disequilibrium test
TM	transmembrane domain
trans	transcript specific (single nucleotide polymorphism)
Xtr	*Xenopus tropicalis* (frog)
YRI	Yoruba in Ibadan, Nigeria (HapMap population)

List of Online Resources

BioMart Project	http://www.biomart.org/
BLAST Alignment Tool	www.ncbi.nlm.nih.gov/BLAST/
Database of Drosophila Genes & Genomes	http://flybase.org/
dbSNP SNP Database	http://www.ncbi.nlm.nih.gov/snp
EMBL Nucleotide Sequence Database	http://www.ebi.ac.uk/embl/
ENSEMBL Genome Browser	http://www.ensembl.org/
ePCR	http://www.ncbi.nlm.nih.gov/sutils/e-pcr
European Bioinformatics Institute	http://www.ebi.ac.uk/
GARField Cat Genome Browser	http://lgd.abcc.ncifcrf.gov/cgi-bin/gbrowse/cat/
GenBank Sequence Database	http://www.ncbi.nlm.nih.gov/Genbank
GeneCards Human Gene Database	http://www.genecards.org/
HUGO Gene Nomenclature Committee	http://www.genenames.org/
Human Olfactory Data Explorer (HORDE)	http://genome.weizmann.ac.il/horde/
IMGT/HLA Database	http://www.ebi.ac.uk/imgt/hla/
International HapMap Project	http://hapmap.ncbi.nlm.nih.gov/
InterPro	http://www.ebi.ac.uk/interpro/
JGI Frog Genome Browser	http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=Xentr4
MUSCLE Sequence Comparison Tool	http://www.ebi.ac.uk/Tools/muscle/
NCBI’s Genome Project Resources	http://www.ncbi.nlm. nih.gov/ genomeprj
Online Mendelian Inheritance in Man (OMIM)	http://www.ncbi.nlm.nih.gov/omim/
PubMed Life Science Database	http://www.ncbi.nlm.nih.gov/sites/entrez
Reference Sequence (RefSeq) Database	http://www.ncbi.nlm.nih.gov/RefSeq/
STRING Database for Protein-Protein Interactions	http://string.embl.de/
The MHC Haplotype Project	https://www.sanger.ac.uk/HGP/Chr6/MHC/
UCSC Genome Browser	http://genome.ucsc.edu/
VEGA Genome Browser	http://vega.sanger.ac.uk/
VISTA Genome Browser	http://pipeline.lbl.gov/

Table of Contents

1. Introduction

This doctoral thesis is organized as a cumulative work presented in the form of five different manuscripts and one additional chapter with results not yet submitted for publication, having the subjects “linkage disequilibrium” and “transmission distortion” as a common focus. The five manuscripts, each of which are preceded by a short introductory summary, were written in the context of this doctoral work and submitted for publication to peer-reviewed journals within the last three years. While three of these articles have already been published and will therefore be shown here with the respective journal’s layout, two further manuscripts are currently under review by the corresponding editorial boards.

The aim of this chapter is to present general concepts that will be referred to throughout this thesis.

1.1. Linkage Disequilibrium in the Human Genome

The term “linkage disequilibrium” (LD) is used in population genetics to refer to the non-random pattern of association between alleles at different loci. LD was first described fifty years ago [Lewontin & Kojima, 1960], and the term derives from the fact that when present, LD will prevent the combination of alleles from two or more neighbouring loci on a single chromosome, also termed haplotype, to reach that expected on the basis of the frequency of each individual allele [Slatkin, 2008]. In other words, LD can be described as the difference between the observed frequency of a haplotype and the frequency it would be expected to have, based on the individual allele frequencies [Nordborg and Tavaré, 2002; Slatkin, 2008].

LD is generally denoted by the letter D:

$$D_{AB} = F_{AB} - F_A F_B \qquad (1)$$

where A and B are alleles from two different loci, and F stands for frequency.

However, because the numeric value of D is strongly dependent on the individual allele frequencies, it is often not the best way to express LD when one is interested in comparing levels of LD between different regions or different pairs of alleles. In order to make this kind of comparison possible, the concept of D′ was proposed four years after the first LD description, taking into account the highest possible value that D can reach, given the frequencies of the alleles being considered [Lewontin, 1964]:

$$D' = \frac{D}{D_{max}} \tag{2}$$

D′ is therefore a normalization of D, so that it takes values between 0 and 1 (or 0 and –1) without regard to the allele frequencies. D′ has the property that when $\mathrm{D}' = 1$, linkage of at least one of the four possible allele combinations will be absolute, and at least one of the four possible haplotypes will be absent. This way to quantify LD is often used in assessments of polyallelic loci, although many others have been proposed for these situations [Ardlie et al., 2002; Zaykin et al., 2008]. In the case of the assessment of single nucleotide polymorphisms (SNP), which are generally biallelic, an alternative way to measure LD is more commonly used, the correlation coefficient r^2 [Hill and Robertson, 1968], which is given by:

$$r^2 = \frac{D^2}{F_A(1 - F_A)F_B(1 - F_B)} \tag{3}$$

Similarly to D′, r^2 is based on D, and $r^2 = 1$ when alleles A and B are in complete LD with each other, but, differently from D’, r^2 additionally requires A and B to have similar allele frequencies in order to equal 1 [VanLiere & Rosenberg, 2008]. In summary, r^2 is a generally more stringent LD measure than D′, and it is the current standard measure of LD within genome-wide and other large scale LD assessments [Ardlie et al., 2002; Zaykin et al., 2008]. An example of an LD plot is given in Fig. 1.1.

LD is understood as a product of many interacting evolutionary forces such as natural selection, genetic drift, population bottlenecks, mixing of subpopulations, inbreeding, genomic inversions and gene conversion [Slatkin, 2008]. LD in humans in often understood from a genealogical point of view, according to which LD is the result of “remainders” of ancestral haplotypes, and tends to disappear as a function of time and recombination events. In this context, LD is traditionally believed to be stronger within population isolates (in which genetic drift and inbreeding are pronounced), but one recent study reveals that this might be a mistaken assumption [Bosch et al., 2009].

Based on LD, the human genome has been described as an assembly of more or less discrete blocks of high LD named haplotype blocks [Daly et al., 2001; Johnson et al., 2001; Patil et al., 2001; Gabriel et al., 2002]. The low LD segments separating such blocks are predicted to correspond to recombination hotspots [Reich et al., 2001, Daly et al., 2001, Patil et al., 2001; Gabriel et al., 2002]. However, it is essential to bear in mind that haplotype blocks are rather a theoretical structure than a biological phenomenon [Blomhoff et al., 2006], since the borders of a haplotype block will diverge considerably, depending on the density of assessed markers [Ke et al., 2004] and on the algorithm used to define a haplotype block [Schulze et al., 2004].

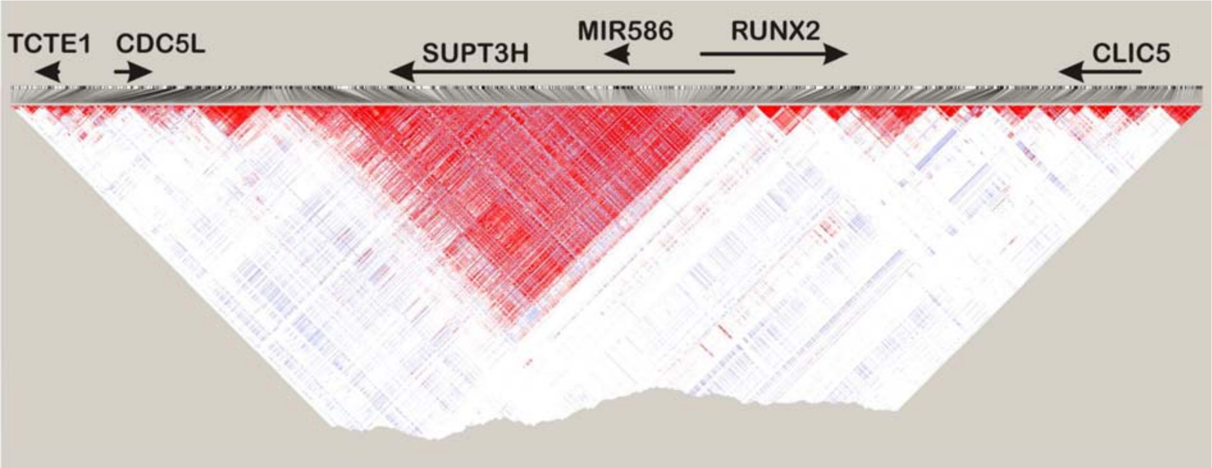


Figure 1.1: LD plot of 1170 SNPs for a region of human chromosome 6p. The positions and lengths of the arrows correspond to those of the six loci (designations and transcriptional orientation are indicated) harboured in the region. Within the plot, each diagonal represents one single SNP, and each point in the plot (the intersection of two diagonals) represents LD between two loci. Red spots indicate strong LD (statistically significant $D' = 1$ and $r^2 > 0.8$), white indicates absence of statistically significant LD, while intermediate values (high D′ but lower r^2) are indicated by the interspersed bluish spots. According to this graph, the whole reading frames of the loci *SUPT3H* and *MIR586*, as well as about one third of *RUNX2* are within one LD block. This plot was generated with genotyping data from the CEU population. (Modified from Santos et al., Eur J Hum Genet 2009).

Since LD provokes the association of alleles from different loci, it represents an obstacle for phenotype association studies, as it confounds the interpretation of results linking a region with high LD to a given phenotype. Depending on the extension of the LD block, a locus found to be linked to a disease, for instance, can be several megabases (Mb) distant from the locus responsible for the primary association [Zhang et al., 2004]. A classical example highlighting this problem is the case of the gene causing the disease haemochromatosis type 1 disease (hereditary iron overload). This medical condition was long believed to be associated with a gene-dense region on the short arm of human chromosome 6 (Chr6p) known as the human leukocyte antigen (HLA complex), characterized by the high number of genes involved in immune responses and by extreme levels of LD. The real causative locus for haemochromatosis was later identified, and located four Mb telomeric to the HLA complex [Feder et al., 1996].

Despite this "drawback", the intelligent use of LD together with the development of new computational and statistical methods as well as the genotyping and the sequencing of the genomes of a large number of individuals over the last few years has opened new possibilities for association studies and population genetics based on LD. For example, LD can be used for selection of so-called tagSNPs. These are SNPs that are informative for a whole genomic region exhibiting high LD, in which many other SNPs with similar allele frequencies could be present [Johnson et al., 2001; Miretti et al., 2005]. With this approach, one is able to assess a given genomic segment through genotyping, using only a small subset of the many polymorphisms described for that region.

1.2. The Major Histocompatibility Complex (MHC)

The major histocompatibility complex (MHC) was first described over fifty years ago [Snell, 1968; Dausset, 1981]. It is a gene dense-region present in the genomes of all vertebrates studied so far, harbouring genes that make it the most important region with regard to normal immune responses, but also for autoimmune and infectious diseases [Ryder et al., 1981; Lie et al., 2005; Trowsdale, 2005; Fernando et al., 2008; Vandiedonck and Knight, 2009]. Extreme degrees of genetic diversity and LD are also features of the MHC [Horton et al., 2004; Lie et al., 2005; Trowsdale, 2005; Vandiedonck and Knight, 2009]. The number of scientific articles published on a subject is often used as a measure of the general interest end effort dedicated to it. A current literature search on the life science online database PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) yielded 10.252 articles published with the expression "Major Histocompatibility Complex" on their titles or abstracts within the last ten years, revealing that the MHC is one of the most intensively studied areas in biomedical research today.

The classical MHC loci encode molecules that present auto- and alloantigens to T cells, building thus the immunological basis of self/nonself recognition. The human MHC is termed HLA complex. It is a ~ 3.5 Mb long region harboured on chromosome 6p (Fig. 1.2), associated with far more diseases than any other region of comparable size in the genome [Horton et al., 2004; Trowsdale, 2005; Fernando et al., 2008].

The medical relevance of the HLA is additionally enhanced due to the role played by this gene system in allogenic tissue transplantations. Especially in the cases of kidney and stem cell transplantation, the selection of a donor with HLA alleles matching those of the recipient plays a determinant role in graft survival and transplant success. However, the exuberant polymorphism of this genomic segment has the consequence that the probability of two unrelated individuals to carry the same class I and class II alleles is extremely low, and the task of finding a compatible unrelated donor is a very hard one. For the three most polymorphic loci, 965 HLA-A, 1.543 HLA-B and 762 HLA-DRB1 alleles have been described, as of January 2010 [IMGT/HLA Database, 2010].

These alleles do not appear, however, in all theoretically possible combinations. Due to the strong level of LD that characterizes the MHC, some allele combinations are more frequent than expected under free recombination. In fact, some MHC haplotypes are so remarkably conserved that LD ranges beyond the borders of the MHC [Alper et al., 1989; Yunis et al., 2003]. Based on LD, a wider area including the MHC has been designated extended MHC (xMHC), including the "core" MHC, a large telomeric neighbouring segment (extended class I), and a shorter centromeric region (extended class II) [Alper et al., 1989; 1992; Ziegler, 1997; Horton et al., 2004]. This nomenclature is based on the traditional subdivision of the core MHC into the tree subregions, class I, class III and class II (Fig. 1.2). One example of a conserved haplotype is the case of the combination HLA-A1, HLA-B8, HLA-DR3, for which LD has been reported to be extreme over the entire length of the xMHC [Alper et al., 2006], and is therefore considered one of the ancient haplotypes of the human MHC.

Another key feature of the MHC is the role played by this gene complex for reproductive patterns such as pre- and post-copulatory mate choice. The first report associating the MHC with mating patterns was observed over thirty years ago in mice [Yamazaki et al., 1976]. According to this and other related studies, females are able to distinguish, most probably through olfactory cues, males that are MHC-similar to themselves from those which are MHC-dissimilar. Mate choice may thus be driven towards maximization or an optimization of the MHC heterozygosity of the offspring [Sommer, 2005; Ziegler et al., 2005; Milinsky, 2006; Eizaguirre et al., 2009; Woelfing et al., 2009]. Similar observations have been made since the initial observation by Yamazaki and co-workers [1976] for fish [Reusch et al., 2001], lizards [Olsson et al., 2003], rats [Singh et al., 1987; Brown et al., 1989], lemurs [Schwensow et al., 2008] and humans [Wedekind et al., 1995, Ober et al., 1997; Jacob et al., 2002, Santos et al., 2005]. In this context, it is plausible that the olfactory receptor genes present within the class I region of the xMHC play an important role in odour- and MHC-dependent patterns of mate choice [Ziegler, 1997; 2000a; 2000b; 2002; Eklund et al., 2000; Thompson et al., 2010].

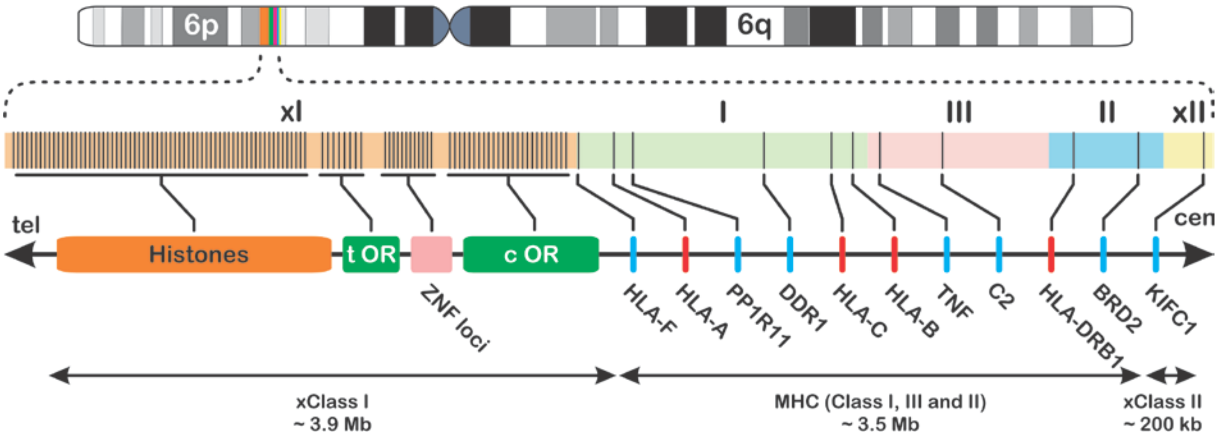


Figure 1.2: Map of the xMHC on human chromosome 6p. The localization of the region within chromosome 6 is given in the upper panel, while a representation of the five subregions: extended class I (xI, orange), class I (I, green), class III (III, pink), class II (II, blue), and extended class II (xII, yellow) is depicted in the middle panel. Selected loci or gene clusters are displayed in the lowest panel: a large histone cluster (orange box), two olfactory receptor clusters (green boxes), a zinc finger gene cluster (pink box), classical MHC genes (red ticks), and selected other selected MHC genes (blue ticks). The approximate length of each subregion is also indicated. (Generated according to genomic coordinates given by the ENSEMBL genome database).

1.3. MHC-linked Olfactory Receptor Genes

Olfactory receptors (OR) are G-protein-coupled membrane receptors that are responsible for the molecular basis of the ability to recognize odours. When expressed on the membrane of olfactory neurons present within the olfactory epithelium, these receptors are able to interact with odorant molecules dispersed in the air and provoke neuron firing and signal transduction into the olfactory bulb. OR consist of seven transmembrane domains, interspersed by four extracellular and four cytoplasmic regions (Fig. 1.3) [Lancet and Pace, 1987; Buck and Axel, 1991; Mombaerts, 1999].

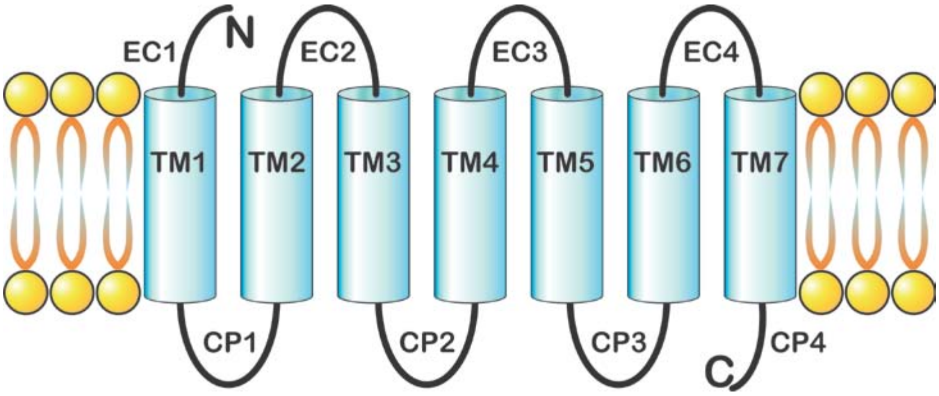


Figure 1.3: Schematic representation of an OR within the cytoplasmic membrane, with the N terminus within the extra cellular space and the C terminus within the cytoplasm. The seven transmembrane domains (TM1 to TM7) are shown as blue cylinders. The four extracellular (EC1 to EC4) and the four cytoplasmic (CP1 to CP4) loops are also indicated. (Adapted from Buck and Axel, 1991).

Olfactory receptor genes belong to the largest gene families in vertebrate genomes, with around 100 genes in fish [Ngai et al., 1993], over one thousand in mice and rats [Zhang and Firestein, 2001; Young et al., 2002], and ~ 800 in humans [Glusman et al., 2001; Zozulya et al., 2001; Malnic et al., 2004; Nei et al., 2008]. These loci are normally organized in gene clusters which, in humans, are distributed throughout the genome, over almost all chromosomes [Glusman et al., 2001; Zozulya et al., 2001; Nei et al., 2008]. Two human OR gene clusters with 34 loci are located in the extended MHC class I region, between one large histone cluster and the telomeric border of the HLA class I region (Fig. 1.4) [Younger et al., 2001].

Following the completion of the human genome project, the sequencing of genomes of other vertebrates revealed not only strong homology between members of mammalian OR gene families in different species [Lane et al., 2001; Aloni et al., 2006], but also that the presence of OR clusters linked with the MHC is remarkably conserved, as in the case of human and mouse [Amadou et al., 2003]. As previously mentioned, the biological relevance of MHC-linked OR genes has increasingly been the focus of research due to its possible role for MHC-dependent mate choice [Ziegler, 1997; 2000a; 2000b; 2002; Eklund et al., 2000; Thompson et al., 2010]. Because of the close proximity and high LD with the MHC, it is plausible that MHC variations, which have been found to correlate with odour-driven mate choice patterns or odour preferences [Yamazaki et al., 1976], correspond to variations within the linked OR clusters. Although a recent report [Thompson et al., 2010] indicates that MHC-linked OR genes do not directly drive odour preferences in humans, the suggestion that MHC-based cryptic or post-copulatory mate choice might be under the influence of these OR genes seems plausible. Several studies have shown that OR and other GPCR genes (including MHC-linked OR genes) are expressed in testis [Parmentier et al., 1992; Vanderhaeghen et al., 1997a; 1997b; Walensky et al., 1998; Tatsura et al., 2001; Volz et al., 2003; Fukuda et al., 2004], and sperm cells [Vanderhaeghen et al., 1993, Spehr et al., 2003; Fukuda et al., 2004].

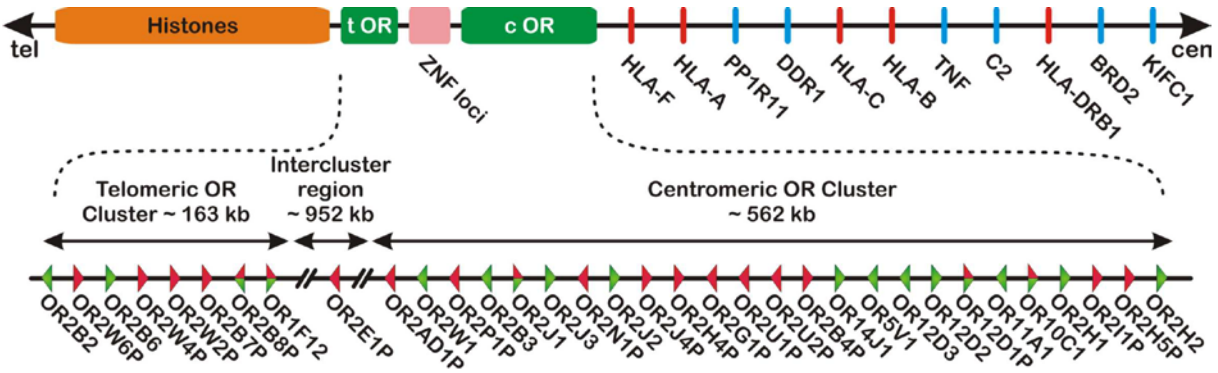


Figure 1.4: Map (not to scale) of the region encompassing the two human MHC-linked OR gene clusters on chromosome 6p. In the upper panel, the relative position of the two OR gene clusters within the xMHC is given (green boxes), while the lower part of the figure shows the schematic positions of OR genes and pseudogenes (suppressing most of the intercluster region). Triangles indicate transcriptional orientation, and are filled in green (genes), red (pseudogenes) or in both colours (segregating pseudogenes). tel: direction to the telomere; cen: direction to the centromere; tOR: telomeric OR gene cluster; cOR: centromeric OR gene cluster. (Generated according to genomic coordinates given by the ENSEMBL genome database).

1.4. Online Resources for Genomic Variation Analyses

The genomic data available through the World Wide Web developed in the last few years to an indispensable resource for genetics research. Since the sequencing of the human genome [International Human Genome Sequencing Consortium, 2001], ongoing efforts of several institutions worldwide are currently directed at the generation of sequence data from many different organisms. Once the genome assembly of one species is complete, additional sequence and genotyping data are commonly generated, in order to provide more data on the variation among individuals of that species. This huge, and continuously growing amount of data is organized in different ways through databases, genome browsers, and online resources that combine both. In this context, the ability to extract specific data out of the immense datasets available plays a critical role, and can be aided by so-called “data mining” tools.

1.4.1. Genome Browsers

Genome browsers are online resources through which the user is able to interact with databases in a visual way, being able to observe a genomic region in its genomic context. Different browsers focus on different features of genomes, while some are specialized for different species. The genome browsers used in this work were ENSEMBL (comprehensive database gathering automatic annotated genomic sequences of over 40 vertebrate species, www.ensembl.org/), VEGA (“Vertebrate Genome Annotation”, a resource based on manual annotation of genes from the genomes of human, mouse and other species, http://vega.sanger.ac.uk/), the Genome Bioinformatics Site of the University of California in Santa Cruz UCSC (http://genome.ucsc.edu/), the VISTA genome browser for comparative analyses (http://pipeline.lbl.gov/), the Genome Annotation Resource Field for *Felis catus* GARField (http://lgd.abcc.ncifcrf.gov/cgi-bin/gbrowse/cat/), the genome project resource of the National Center for Biotechnology Information (NCBI), which gathers information of over fifty vertebrate genome projects from different institutions (http://www.ncbi.nlm.nih.gov/ genomeprj), the Frog Genome Browser of the Energy Joint Genome Institute (http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=Xentr4), as well as the genome browser of the international HapMap Project (http://hapmap.ncbi.nlm.nih.gov/).

1.4.2. The International HapMap Project

The International HapMap Project (HapMap) is the result of an international venture aiming to generate a comprehensive online resource of haplotype variation in different human populations [International HapMap Consortium, 2003]. It is thus intended to promote an efficient approach for the discovery of markers associated with diseases through the use of a population-specific mapping of LD blocks and tagSNPs [International HapMap Consortium, 2003; 2005; 2007].

Until the final release of the last HapMap version (HapMap2), the project was focusing on individuals from the four following human populations: CEU (Utah residents with ancestry from Northern and Western Europe, 90 individuals from 30 family trios), YRI (Yoruba in Ibadan, Nigeria, 90 individuals from 30 family trios), CHB (Han Chinese in Beijing, 45 unrelated individuals) and JPT (Japanese in Tokyo, 45 unrelated individuals). All individuals were assessed with a high throughput SNP genotyping platform, for more than 3.1 million SNPs genome-wide [International HapMap Consortium, 2007]. This data has served as a reference for several population studies [Skelding et al., 2007; Mägi et al., 2007; Manolio et al., 2008; Gu et al., 2008], including some addressing the HLA complex, concerning its LD profile, haplotype variation, as well as genotyping efficiency [Miretti et al., 2005; de Bakker et al., 2006].

By the end of 2008, the HapMap entered a new phase (termed HapMap3). This new HapMap release, which is currently not completely established, involved an increase in the number of individuals of the "old" populations, as well as the inclusion of over one thousand new individuals from seven new populations: ASW (African ancestry in Southwest USA), CHD (Chinese in Metropolitan Denver, CO, USA), GIH (Gujarati Indians in Houston, TX, USA), LWK (Luhya in Webuye, Kenya), MEX (Mexican ancestry in Los Angeles, CA, USA), MKK (Maasai in Kinyawa, Kenya), and TSI (Tuscans in Italy). However, the panel of markers genotyped in HapMap 3 is somewhat reduced (around 50%), as compared to that of HapMap2.

1.5. Transmission Distortion

Transmission Distortion (TD) describes the situation in which the "normal" random segregation of alleles from parents to their offspring according to the Mendelian law of the independent assortment of alleles is violated [Lyttle, 1993; Pardo-Manuel de Villena and Sapienza, 2001]. The term is widely used as a synonym of "transmission ratio distortion" and "segregation distortion", although there is some dispute regarding which expression should be used in a given case of TD [Lyon, 2003].

There are several processes that can potentially distort segregation rates of alleles, LD blocks or whole chromosomes and thereby provoke TD. These include gamete selection (generally referring to the preferential fertilization of eggs depending on alleles or haplotypes carried by the sperm cells [Lyon, 2003]), meiotic drive (the asymmetric segregation of alleles during gametogenesis [Lyttle, 1993; Axelsson et al., 2010]), and embryonic selection (the genotype-dependent survival of embryos or foetuses [Murphy et al., 2008]).

The most notable case of TD has been described for the mouse t haplotypes, already over 70 years ago [Chesley and Dunn, 1936]. These are variants of the proximal one third of mouse chromosome 17, consisting generally of four non-overlapping inversions which appear with a frequency of around 15% in wild mice populations and which suppress recombination between t and wild-type haplotypes [Bennett, 1975; Willison and Lyon, 2000; Lyon, 2003]. The most important feature of t haplotypes is, however, their ability to violate Mendelian transmission in their favour. Although females segregate t- and wild-type haplotypes according to Mendelian expectations, more than 50% (in fact up to 99%) of the offspring from heterozygous t/+ males inherit the t-haplotype [Willison and Lyon, 2000; Lyon, 2003]. The balance equilibrates, keeping the locus in heterozygous state for basically two reasons: t/t homozygosity is either lethal or causes sterility [Lyon, 2003] and, as has only recently been demonstrated, t/+ heterozygous males face a strong reproductive disadvantage in comparison to wild-type homozygous males regarding their territorial behaviour [Carroll et al., 2004]. The genomic region “affected” by t haplotypes comprises up to 40 Mb of mouse chromosome 17 [Lyon et al., 1988]. This segment is largely syntenic to the short arm of human chromosome 6 and generally includes both the mouse MHC and the MHC-linked OR genes.

Other examples of TD have since been described for plants [Fishman et al., 2008], Drosophila [Ganetzky, 1977], birds [Aparicio et al., 2010; Axelsson et al., 2010], mice [Wu et al., 2005; Haston et al., 2007; Kriz et al., 2007; Purushothaman et al., 2008; Girirajan et al., 2009], cattle [Murphy et al., 2008] and also humans [Evans et al., 1994; Chakraborty et al., 1996; Naumova et al., 1998; 2001; Eaves et al., 1999; Lemire et al., 2004; Zöllner et al., 2004; Dean et al., 2006], including genes harboured within the HLA complex [Hanchard et al., 2006]. The statistical problem of multiple testing is, however, a constant obstacle preventing low levels of TD in large-scale investigations to be detected [Zöllner et al., 2004; International HapMap Consortium, 2005].

While the molecular mechanisms leading to TD in humans remain unknown, mouse t haplotypes are much better understood. In this case, the transcription of a t haplotype-associated variant of the sperm motility kinase-1 gene (*Smok1*) is enhanced specifically in t haplotype-carrying sperm cells, rescuing them from a “poisonous” effect that other t haplotype-associated genes (called t complex distorters) have caused in parallel, affecting the development of all germ cells [Herrmann et al., 1999]. An explanation of how the t complex-related *Smok1* gene exerts its specificity selectively for t-bearing sperm has only recently been obtained [Véron et al., 2009]. The consequence of this selective activity of *Smok1* is that t sperm and wild type sperm differ in flagellar motility, with an advantage for t sperm regarding egg cell fertilization [Herrmann et al., 1999; Véron et al., 2009].

In humans, however, TD is not only of interest as a biological phenomenon, but is also of critical importance for numerous disease association studies that are currently performed. As discussed elsewhere [Greenwood et al., 2000; Paterson et al., 2003; 2009], TD can, if present in a given genomic region in the general population, introduce significant bias into association studies regarding that region when TD is disregarded. According to this notion, family-based association studies that assess only the affected offspring are prone to falsely conclude that a locus is linked with the disease under investigation, while it might actually be under TD in the general population [Greenwood et al., 2000; Paterson et al., 2003; 2009].

1.6. Aims and Scope of this Work.

As shown in this chapter, the major histocompatibility complex, linkage disequilibrium, MHC-linked olfactory receptor genes and allelic transmission distortion are subjects that are intimately related.

The specific objectives of the studies assessing OR genes were the following:

1. Investigate the genetic structure and phylogenetic relationships of MHC-linked OR genes among all animals for which enough sequence data has been produced and made available through public databases.
2. Investigate, through direct DNA sequencing and the use of publicly available sequence data, the LD structure and the presence of unknown polymorphisms within human MHC-linked OR genes, in order to generate an overview of human genotypic and phenotypic variation of this genomic region. Additionally, assess the possibility that new cases of loci can be found in which intact genes and pseudogenes segregate in different haplotypes.
3. Investigate the degree of association between polymorphisms within human MHC-linked OR genes with smoking habits, focusing on HLA haplotypes that were previously described to be associated with diseases that are strongly influenced by smoking.
4. Generate a panel of LD blocks and tagSNPs for the human MHC-linked OR genes, using population data available through public databases.

The specific objective of the study assessing the MHC was the following:

1. Investigate the possibility of increasing genotyping efficiency of HLA complex alleles through the description of high levels of TD between these alleles and microsatellites.

The specific objectives of the studies assessing TD were the following:

1. Investigate the presence of TD within the extended MHC and surrounding genomic regions, using population data available through public databases.
2. Seek for confirmation of TD, trough direct genotyping of an independent population cohort.
3. Investigate possible relationships between LD and TD.

2. MHC-linked Olfactory Receptor Genes and Smoking

2.1. Summary

The work published by Füst and co-workers [Füst et al., 2004] described a correlation between a specific haplotype of the HLA complex (A1-B8-DR3) with smoking habits in Caucasian women. As the genes assessed lacked any clear link to smoking, as they were neither involved in nicotine metabolism or predisposition to addiction, etc, the primary association responsible for the findings could in fact be due to genes that are part of this haplotype, but located in the telomeric vicinity of the HLA, namely on one or both the olfactory receptor gene clusters. The work described in the following article aimed to test this hypothesis, as well as the possible association of smoking with two other haplotypes involved in smoking-related diseases that are known to be influenced by the HLA complex. The methodology integrated publicly available SNP genotyping information from 180 Caucasian haplotypes (CEU population) from the international HapMap project, as well as traditional "in house" genotyping methods. It could be determined, *in silico*, which polymorphism, among the hundreds available, should be assessed. A sample from the Füst study [Füst et al., 2004] was then genotyped, and the results revealed that one single non-synonymous SNP within the gene *OR12D3* was stronger correlated with smoking habits than the originally found association between smoking and the A1-B8-DR3 haplotype. For the first time, a human behaviour was found to be correlated to an olfactory receptor polymorphism.

2.2. Publication

Santos PS, Füst G, Prohászka Z, Volz A, Horton R, Miretti M, Yu CY, Beck S, Uchanska-Ziegler B, Ziegler A (2008): Association of smoking behavior with an odorant receptor allele telomeric to the human major histocompatibility complex. Genetic Testing 12: 481-486.

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Association of Smoking Behavior with an Odorant Receptor Allele Telomeric to the Human Major Histocompatibility Complex

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Smoking behavior has been associated in two independent European cohorts with the most common Caucasian human leukocyte antigen (HLA) haplotype (A1-B8-DR3). We aimed to test whether polymorphic members of the two odorant receptor (OR) clusters within the extended HLA complex might be responsible for the observed association, by genotyping a cohort of Hungarian women in which the mentioned association had been found. One hundred and eighty HLA haplotypes from Centre d'Etude du Polymorphisme Humain families were analyzed *in silico* to identify single-nucleotide polymorphisms (SNPs) within OR genes that are in linkage disequilibrium with the A1-B8-DR3 haplotype, as well as with two other haplotypes indirectly linked to smoking behavior. A nonsynonymous SNP within the *OR12D3* gene (rs3749971^{T}) was found to be linked to the A1-B8-DR3 haplotype. This polymorphism leads to a ^{97}Thr → Ile exchange that affects a putative ligand binding region of the OR12D3 protein. Smoking was found to be associated in the Hungarian cohort with the rs3749971^{T} allele ($p = 1.05 \times 10^{-2}$), with higher significance than with A1-B8-DR3 ($p = 2.38 \times 10^{-2}$). Our results link smoking to a distinct OR allele, and demonstrate that the rs3749971^{T} polymorphism is associated with the HLA haplotype–dependent differential recognition of cigarette smoke components, at least among Caucasian women.

Introduction

There were nearly 1.3 billion smokers worldwide in the year 2003, and this number is expected to rise to 1.7 billion (~1.2 billion males and 500 million females) by 2025, with the number of female smokers contributing most to the increase (American Cancer Society, 2003). In nearly all investigated regions of the world, the ratio of female to male smokers among young people was found to be higher than the ratio among adults, suggesting a global trend for an increase in smoking habits among female adolescents and young women (Global Youth Tobacco Survey Collaborating Group, 2003). Smoking is associated with many serious health problems, including cancer of various organs, coronary artery disease, as well as several autoimmune disorders (Hegediüs *et al.*, 2004; Klareskog *et al.*, 2006; Warren *et al.*, 2006; American Cancer Society, 2007; Koch *et al.*, 2007; Hawkes, 2007), and it is thus considered a leading cause of death and disability worldwide. Although there is general agreement that nicotine is the core addictive component of cigarette smoke (Jarvis, 2004), there are hundreds of further substances that may influence the initiation and continuation of tobacco abuse (Baker *et al.*, 2004), independent of nicotine (Franklin *et al.*, 2007). Smoking is also modulated by genetic factors, as demonstrated by epidemiological and twin studies (Sullivan and Kendler, 1999; Li *et al.*, 2003). In support, a haplotype of the major histocompatibility (human leukocyte antigen, HLA) complex was found to be associated with smoking behavior, stronger in women (odds ratio: 13.6) than in men (odds ratio: 2.79) (Füst *et al.*, 2004). This haplotype, –HLA-A1-B8-DR3–, the most common among Caucasians (Alper *et al.*, 2006), is also associated with autoimmune disorders, of which some are clearly connected with tobacco abuse, such as Graves' ophthalmopathy (Weetman, 2000; Hunt *et al.*, 2001; Hegediüs *et al.*, 2004; Holm *et al.*, 2005).

At least two further HLA haplotypes have also been linked to autoimmune diseases that are triggered or heavily influenced by tobacco smoking. The HLA-A3-B7-DR15 haplotype

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is overrepresented in individuals with multiple sclerosis (Dyment *et al.*, 2004; Herrera *et al.*, 2006), while HLA-DR "Shared Epitope" (SE) haplotypes are overrepresented in individuals who smoke, possess antibodies against citrullinated proteins, and suffer from rheumatoid arthritis (Klareskog *et al.*, 2006; Linn-Rasker *et al.*, 2006). SE haplotypes are characterized by the presence of the HLA-DRB1*01, -DRB1*04, or -DRB1*10 alleles.

The exceptionally strong linkage disequilibrium (LD) that is typical for certain haplotypes of the xHLA encompasses a chromosomal segment with a length of about 7 Mb between the gene *HFE* and loci within the HLA class II region (Horton *et al.*, 2004). In case of the A1-B8-DR3 haplotype, LD is extreme over its entire length (Alper *et al.*, 2006), suggesting that an allele of any genes within the region of LD could in principle predispose to smoking. Therefore, polymorphic members of the two odorant receptor (OR) gene clusters within the telomeric xHLA (Ehlers *et al.*, 2000; Younger *et al.*, 2001; Ziegler and Uchańska-Ziegler, 2006) must be considered as plausible candidate genes.

The Centre d'Etude du Polymorphisme Humain (CEPH) panel of families (Miretti *et al.*, 2005) offers unique opportunities for genetic association studies comprising the HLA region. The samples analyzed here comprise 180 founder chromosome 6 that have already been analyzed with regard to their single-nucleotide polymorphism (SNP) alleles and tagSNPs (representative SNPs for a genomic region exhibiting high LD) (Miretti *et al.*, 2005). Recently, the analysis has been extended to both OR gene clusters at the telomeric section of the xHLA, permitting the correlation with alleles within the HLA class I, II, and III regions (de Bakker *et al.*, 2006).

The present study was conducted in two steps: (i) *in silico*, we aimed to identify SNP alleles within the two HLA-linked OR gene clusters that are characteristic for the HLA haplotypes mentioned before; and (ii) *in vitro* we wanted to test whether their possible association with tobacco abuse is stronger or weaker than the one observed with A1-B8-DR3, by genotyping a subsample of the cohort in which an association between smoking and loci at the HLA class III region had been found before.

Materials and Methods

In silico *tagSNP selection and assessment of HLA haplotype–dependent OR SNP alleles*

All *in silico* analyses were based on xHLA high-density SNP genotyping data from 180 founder chromosome 6 from the CEPH collection (Miretti *et al.*, 2005). Details on marker selection, CEPH subjects, and on their genotypings are given elsewhere (Miretti *et al.*, 2005; de Bakker *et al.*, 2006). A total of 1170 SNPs spanning the region between *OR2B2* and *MOG* (Fig. 1) were initially considered and analyzed with regard to their allelic diversity (supplemental Fig. S1, available online at www.liebertpub.com). A list of all 1170 SNPs with their genomic coordinates is given in the supplemental Table S1 (available online at www.liebertpub.com). From this set of markers, we chose 110 tagSNPs capturing the haplotypic information from the whole genomic region. SNP tagging was performed using a pairwise tagging algorithm (de Bakker *et al.*, 2006), as implemented in HAPLOVIEW v. 4 (Barrett *et al.*, 2005), and considering a maximum intermarker distance of 650 kb and an LD coefficient (r^2) threshold of ≥0.8. Loci with a minor allele frequency of less than 1%, those not conforming to Hardy–Weinberg equilibrium, or not reported by the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) were excluded.

We tested the 110 tagSNPs characterizing the *OR2B2-MOG* segment for association with three groups of haplotypes (A1-B8-DR3, A3-B7-DR15, and SE), the aim being to determine which tagSNPs were characteristic for each of the three haplotype groups.

Genotyping of SNP rs3749971

The genotyping of the SNP *rs3749971* was performed by real-time PCR in a sample of 32 Hungarian female Caucasians (average age 46.75 years, ranging from 24 to 76 years), which

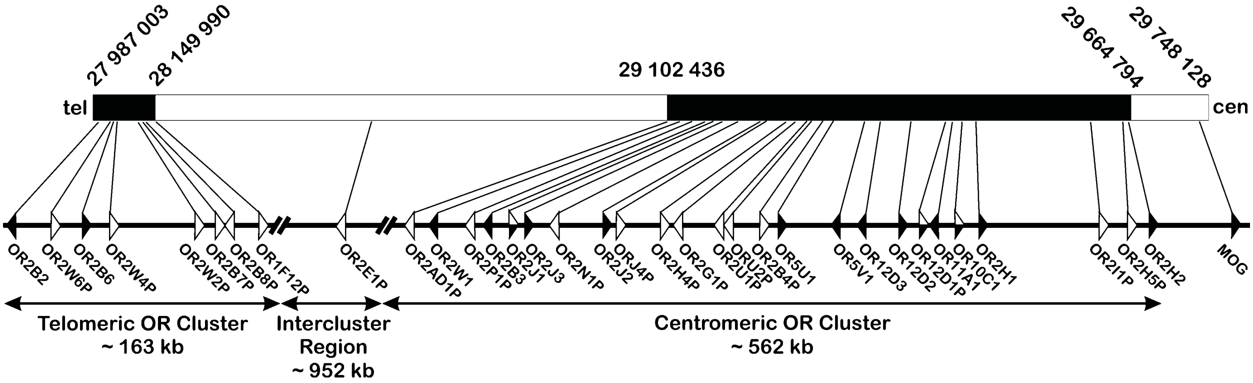

FIG. 1. Map (not to scale) of the region encompassing the two OR clusters on chromosome 6p. Above the figure, the NCBI 36 coordinates (bp) of the chromosomal segments analyzed here are depicted. The upper plot is filled in black for OR clusters and in white for regions outside the OR clusters, while the lower part of the figure shows the OR genes/pseudogenes at their relative chromosomal locations (suppressing most of the intercluster region). Triangles indicate transcriptional orientation, and are filled in black (genes), white (pseudogenes), or in both colors (haplotype-dependent genes/pseudogenes). tel: direction to the telomere; cen: direction to the centromere.

was a subsample of a cohort in which a correlation had previously been found between smoking and loci of the HLA class III region (Füst *et al.*, 2004). Based on LD, women who carried the C4A*Q0 (mono-S) genotype as well as the AGER-429C, HSPA1B-1267G, and TNF-308A alleles were considered carriers of the A1-B8-DR3 haplotype (Füst *et al.*, 2004). Other genotypings of these subjects, details on registration of smoking habits, preparation of genomic DNA, as well as informed consent from the cohort have been provided before (Füst *et al.*, 2004).

The PCR reactions were performed using a Stratagene realtime PCR instrument (Stratagene, Amsterdam, NL) with the following cycle conditions: 1 cycle of 94°C for 1 min and 80 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 30 s, and 78°C for 10 s. Fluorescence was measured during the 62°C (annealing) step. Twenty-microliter PCR reactions contained 100 ng of human genomic DNA; 1.5 pmol C- or T-specific reverse primers (details below); 3 pmol forward primer (rs3749971-For: AGCGAAGAGGATTGCAGATGGC); 2 μL Genetherm polymerase buffer; 0.7 mM each of dATP, dCTP, dGTP, and dTTP; 2 mM MgCl; and 2 U of GenTherm™ Taq Polymerase. Allele-specific primers were designed as molecular beacons (Jordens *et al.*, 2000) (rs3749971-C: Fam-atacagc CTATATCTTTTCTAGGCTGTA$\boldsymbol{T}_{\mathrm{BHQ}}$CA**C** and rs3749971-T: Hex-atacagcCTATATCTTTTCTAGGCTGTA$\boldsymbol{T}_{\mathrm{BHQ}}$CA**T**), labeled either with FAM (6-carboxyfluorescein) or Hex (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein), and quenched with Black Hole Quencher (BHQ™) attached to a ***T*** natively present within the primer binding site. Seven bases were added to the 5′ end (displayed in lower-case letters) to allow the formation of a 29-bp "hairpin" with the 10-bp complementary region to ensure fluorescence quenching of the unused primers. To assess the reproducibility of this genotyping approach, control DNA of seven individuals (including three CEPH samples) was typed 15 times independently, with 100% reproducibility.

Statistical analyses

Nucleotide diversity (π) was calculated as described by Nei (1987), using the software DNAsp (Rozas and Rozas, 1999). The two-sided Fisher's exact test was employed for all association analyses, with a 1% level of significance. The Bonferroni correction for multiple comparisons was applied for *p*-values of the *in silico* analyses only.

Results

In silico *analyses*

The degree of nucleotide diversity as assessed by 1170 SNPs (elicited in the supplemental Table S1) was found to be highest between the genes *OR12D3* and *OR10C1* (supplemental Fig. S1), including the most polymorphic loci within this cluster (Ehlers *et al.*, 2000). The SNP diversity outside of the OR clusters did not differ substantially from that within the clusters. SNP densities within the telomeric (0.388/kb) and the centromeric OR clusters (0.877/kb) were relatively high when compared with other genomic regions (Zhao *et al.*, 2003).

Within the panel of 180 xHLA haplotypes, 11 A1-B8-DR3, 5 A3-B7-DR15, and 59 HLA-DR SE haplotypes were found. No significant association between any of the tagSNPs and the 59 HLA-DR SE haplotypes was observed, as shown in Figure 2. A similar result was obtained with regard to the A3-B7-DR15 haplotypes, although this could be due to the low number of A3-B7-DR15 haplotypes (five) in the CEPH panel. In contrast, a significant correlation was found for 12 tagSNPs (representing 81 tagged SNPs displayed in supplemental Table S2, available online at www.liebertpub.com) when the 11 A1-B8-DR3 haplotypes were compared with the 169 non–A1-B8-DR3 haplotypes for association with the 110 tagSNPs (Fig. 2), and after setting a Bonferroni cutoff for multiple comparisons. Only one of the 81 captured SNPs is a coding, nonsynonymous SNP (*rs3749971*, tagged by tagSNP #51), within the gene *OR12D3*. In A1-B8-DR3 haplotypes, the respective allele (rs3749971$^{\mathrm{T}}$) is responsible for a Thr→Ile exchange at amino acid position 97 within the OR12D3 protein. The remaining 80 SNPs lead either to synonymous exchanges or are located in intergenic or in intronic regions. Because none of these are, to our knowledge, directly involved in any, so far known, biological process, they were not considered further.

Since the A1-B8-DR3 haplotype was reported in another independent European cohorts to be overrepresented in

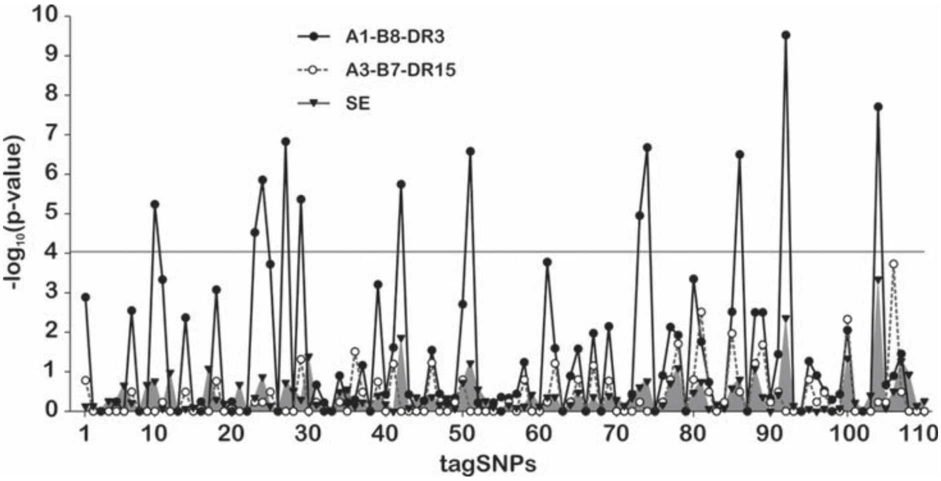

FIG. 2. Allelic association of 110 tagSNPs from the region comprising both HLA-linked OR clusters with A1-B8-DR3, A3-B7-DR15, and SE haplotypes. The marker *rs3749971* is represented by tagSNP #51. The gray horizontal line marks the Bonferroni threshold for 110 tests with a significance level of 1%.

smokers (Icelandic sample, Füst *et al.*, 2004), and is also associated with various autoimmune diseases, of which some correlate with this behavioral trait, as in Graves' disease (Hegediüs *et al.*, 2004; Holm *et al.*, 2005), this data demonstrate that these associations must also extend to the A1-B8-DR3–associated rs3749971^{T} allele.

Cohort genotyping

In order to validate this finding, comparing it to the previously found association, we genotyped *rs3749971* in 32 female individuals from the same Hungarian cohort (Füst *et al.*, 2004). These individuals differ in their smoking behavior and do not suffer from any autoimmune disease (Füst *et al.*, 2004). Here we found that the correlation between smoking and rs3749971^{T} was slightly stronger ($p = 1.05\times10^{-2}$, Fig. 3b) than the correlation between this trait and A1-B8-DR3 ($p = 2.38\times10^{-2}$, Fig. 3c). Seven and 25 subjects were rs3749971$^{C/T}$ and rs3749971$^{C/C}$ carriers, respectively. A strong association ($p = 5.87\times10^{-4}$) between the rs3749971^{T} allele and the A1-B8-DR3 haplotype was observed, as five out of seven of the rs3749971^{T} carriers but only 1/25 of the noncarriers were found to be A1-B8-DR3 positive (Fig. 3d). The group of rs3749971$^{C/C}$ carriers includes both smokers and nonsmokers, as shown in Figure 3a and b.

Discussion

HLA-A1–positive individuals, in contrast to those with HLA-A2 or HLA-A3, have been reported to exhibit a preference for the odor of bergamot (Milinski and Wedekind, 2001). The existence of a relationship between rs3749971^{T} (or OR12D3^{97Ile}) and this predilection for a perfume ingredient is supported by the strong LD between HLA-A1 and rs3749971^{T} that is described here. The fact that many tobacco brands are scented with bergamot oil components (Baker *et al.*, 2004) provides a plausible explanation for the correlation of rs3749971^{T} with smoking, and the identification of OR12D3^{97Ile} ligands will facilitate the design of volatiles that might be used as antidotes in individuals with a predisposition to tobacco abuse. The exchange of the hydrophilic amino acid threonine by isoleucine, a residue with a hydrophobic side chain, is expected to alter the physicochemical properties of the OR12D3 protein. However, as no X-ray crystallographic studies of ORs have been reported to date, the likely location of ^{97}Ile within the OR12D3 protein can only be inferred from models (Man *et al.*, 2004; Katada *et al.*, 2005; Abaffy *et al.*, 2007; Schmiedeberg *et al.*, 2007) that take the structure of rhodopsin (Palczewski *et al.*, 2000) into account. Such models locate residue 97 at the end of the first extracellular loop or at the beginning of the third transmembrane domain, close to

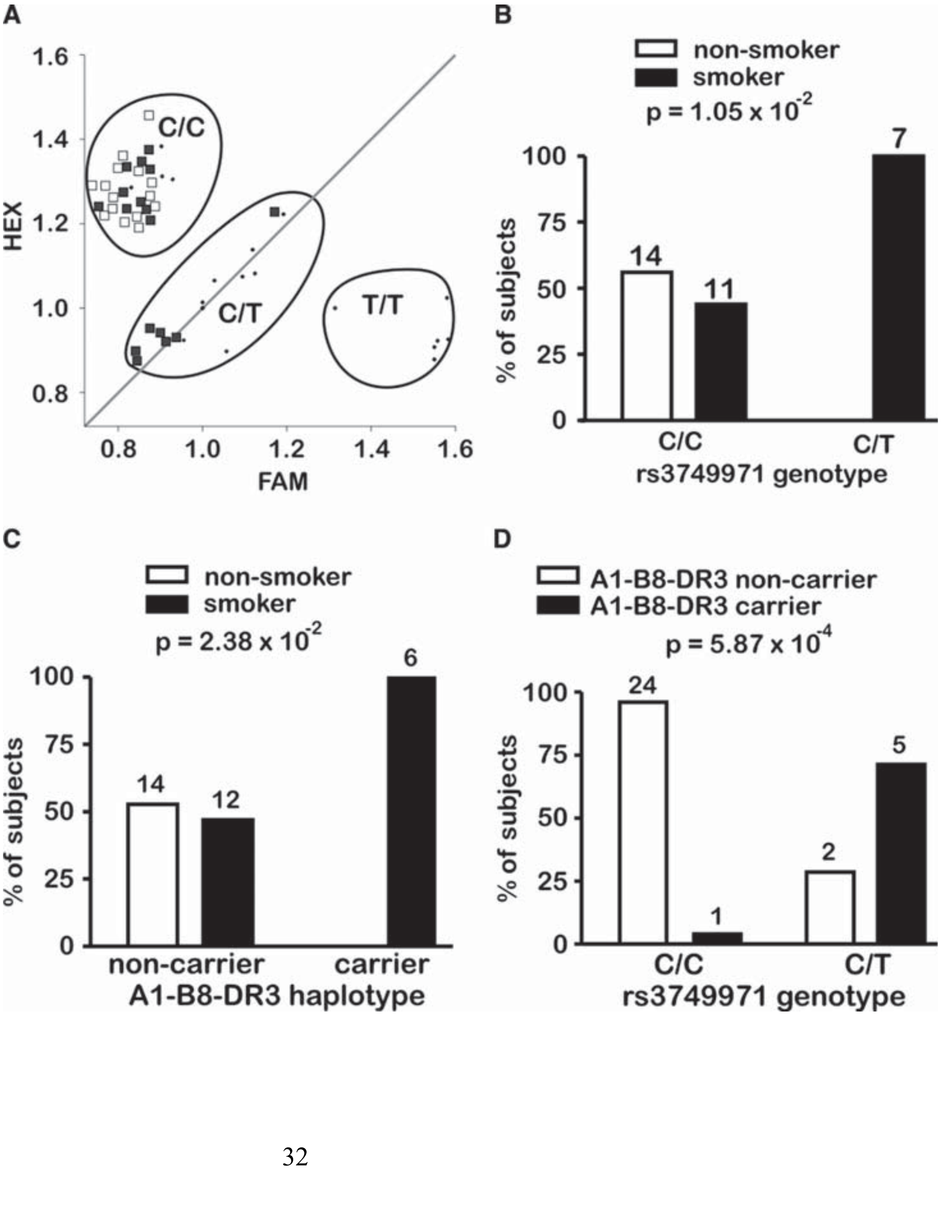

FIG. 3. Association of smoking behavior with the *rs3749971* genotype and the A1-B8-DR3 haplotype. (**A**) HEX and FAM are primer fluorophores marking the *rs3749971* T and the C allele primers, respectively. Values indicate the normalized relative number of PCR cycles necessary to reach the genotyping threshold for each allele. HEX values around 1 (or below) are indicative for the presence of the T allele, and the same is true for FAM values, indicating the presence of the C allele. Filled squares: 17 smokers; open squares: 15 nonsmokers; dots: controls. (**B**) Association of smoking behavior with *rs3749971* genotypes. (**C**) Association of smoking behavior with the carrier state of A1-B8-DR3. (**D**) Association of A1-B8-DR3 with *rs3749971* genotypes. (**B–D**) The number of individuals in each category is indicated above the corresponding bars, as well as the *p*-values, as determined by Fisher's exact test.

the ligand binding site. Keller *et al*. (2007) have recently demonstrated that a variant of an OR gene can substantially influence sensitivity (in both intensity and pleasantness) to specific odors in humans. They showed that a mutant allele of the *OR7D4* gene encoding an OR with two amino acid substitutions (residues 88 and 133) as compared to the most common allele causes functional impairment of the receptor *in vitro*. These substitutions also alter the perception of the smell of androstenone and androstadienone in a significant manner. Residues 88 and 133 are located within the first extracellular and the second intracellular loop. At least residue 88 is close to the beginning of the third transmembrane domain, suggesting that the region in the vicinity of residue 97 in the OR12D3 protein might indeed be involved in ligand binding. Further support for a role of amino acids close to the residue 97 in ligand binding is provided by the recently published crystal structure of the human β_2 adrenergic G-protein–coupled receptor (Rasmussen *et al.*, 2007).

Apart from social and psychological factors (Pomerleau *et al.*, 1992; Barman *et al.*, 2004; Lerer *et al.*, 2006), it has been shown that the individual genetic constitution plays a role in initiating and continuing tobacco consumption (Sullivan and Kendler, 1999; Li *et al.*, 2003). Genes within the xHLA contribute as well, as suggested by the finding of an HLA haplotype–dependent association of smoking in two independent European cohorts, with a clear-cut gender bias toward females (Füst *et al.*, 2004). The present study confirms these results by identifying an HLA-linked OR allele that is associated with tobacco abuse. In addition, our work implies that the rs3749971^T allele is involved in the smoking-induced aggravation of certain autoimmune diseases that can be observed in patients carrying A1-B8-DR3 (Hegediüs *et al.*, 2004; Holm *et al.*, 2005). Given the fact that the A1-B8-DR3 haplotype occurs with a frequency of 5–10% in Caucasians (Alper *et al.*, 2006), the *rs3749971* polymorphism should be suitable for large-scale screening tests, particularly among young people. Studies of the isolated OR12D3 protein will be indispensable to evaluate the consequences of the Thr97Ile exchange within this receptor on its ligand specificity.

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Disclosure Statement

The authors declare that no competing interests exist.

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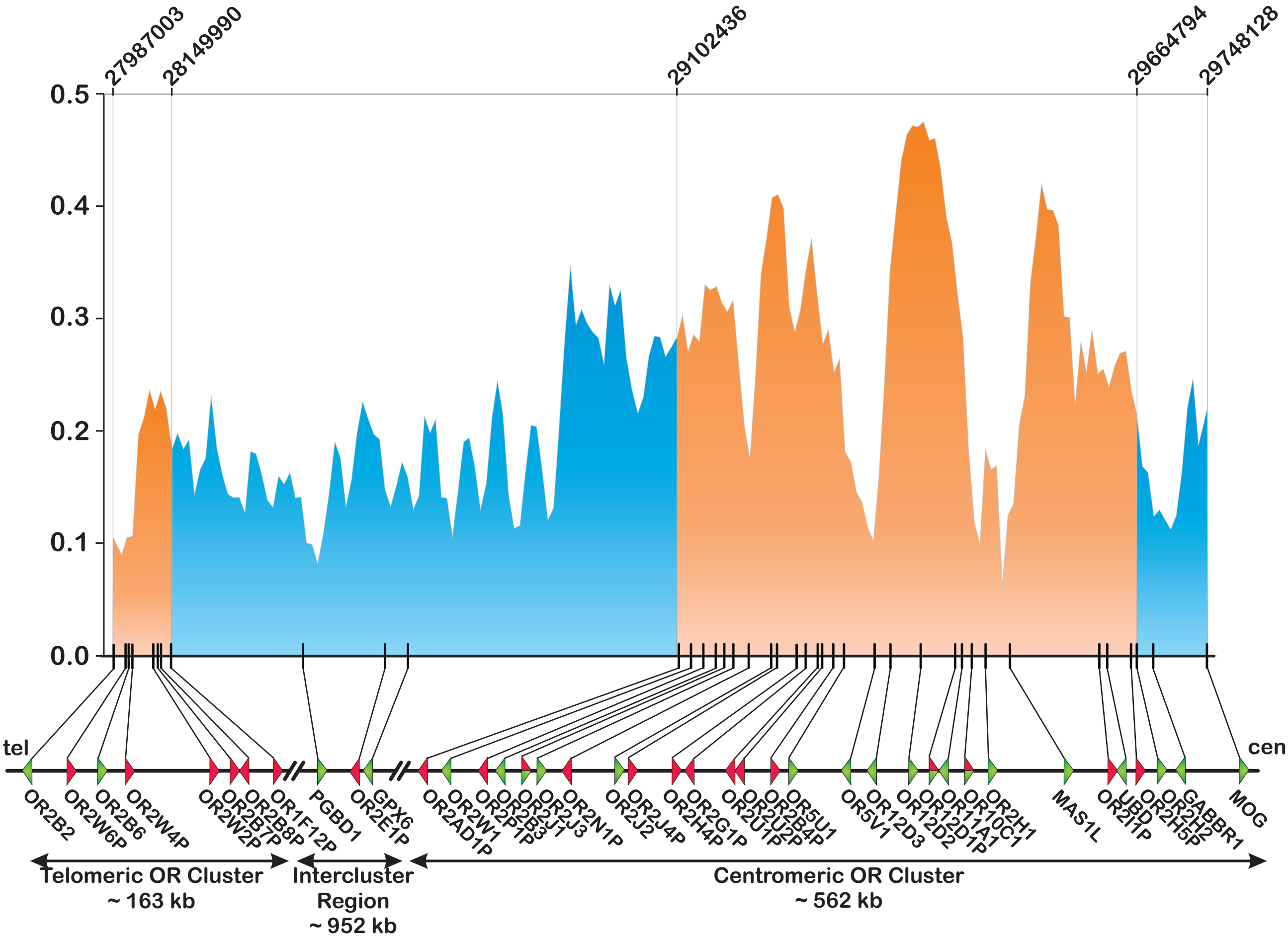

Supplementary Figure 1: Diversity of 1170 SNPs in a region of the xHLA complex
This region encompasses the two odorant receptor (OR) clusters on chromosome 6p, between the genes *OR2B2* and *MOG*). The average number of nucleotide differences per site ( ) was determined based on sliding windows (18 bases wide, stepping 6 bases after each measurement). The regions of both OR clusters are plotted in orange, and blue is used for the regions outside them. The ticks under the graph mark the position of OR genes and pseudogenes in relation to the plot. Above the graph, the NCBI 36 coordinates (bp) of selected loci are depicted. The line underneath the graph shows OR genes and pseudogenes at their approximate chromosomal locations. Triangles indicate transcriptional orientation, and are filled in green (genes), red (pseudogenes) or in both colours (haplotype-dependent genes/pseudogenes).
tel: directon to the telomere; cen: direction to the centromere

Supplementary Table 1

Compilation of all 1170 SNPs assessed in the genomic region between *OR2B2* and *MOG*, with indication of ID at the dbSNP database, and genomic coordinates according to NCBI36. SNPs chosen as tagSNPs are indicated.

Order	dbSNP ID	Coordinate (NCBI 36)	tagSNP
1	*rs2394015*	27978337	
2	*rs200945*	27989883	
3	*rs201910*	27991536	
4	*rs13218875*	27991991	
5	*rs9468254*	27992895	
6	*rs1015075*	27995254	
7	*rs6927241*	27997313	
8	*rs6933825*	27998610	
9	*rs156737*	28003192	tagSNP #1
10	*rs9468256*	28003483	
11	*rs12663899*	28007558	
12	*rs7760871*	28009020	tagSNP #2
13	*rs7742529*	28010848	
14	*rs7742858*	28010971	
15	*rs10456357*	28011446	
16	*rs6910968*	28012160	
17	*rs2893937*	28018029	
18	*rs276366*	28035460	
19	*rs9295753*	28036424	tagSNP #3
20	*rs276364*	28039599	
21	*rs276363*	28041280	
22	*rs7769416*	28056553	
23	*rs9380031*	28062677	tagSNP #4
24	*rs7748445*	28069926	tagSNP #5
25	*rs1143887*	28072802	
26	*rs149901*	28073482	
27	*rs156743*	28075068	
28	*rs12333142*	28076983	
29	*rs149946*	28078010	
30	*rs6939591*	28087157	
31	*rs10484402*	28087604	tagSNP #6
32	*rs9368540*	28089652	
33	*rs149971*	28090131	
34	*rs149972*	28091206	
35	*rs149973*	28091592	
36	*rs149974*	28093075	
37	*rs6456804*	28093367	
38	*rs2840222*	28093541	
39	*rs2893947*	28094129	
40	*rs149975*	28094319	
41	*rs9368545*	28107023	
42	*rs1529749*	28108340	
43	*rs3926997*	28108508	
44	*rs149941*	28109012	
45	*rs149942*	28109589	
46	*rs149943*	28110367	
47	*rs185741*	28111250	
48	*rs149897*	28114629	tagSNP #7
49	*rs149896*	28116503	
50	*rs175954*	28119564	
51	*rs149900*	28122576	
52	*rs149962*	28123897	
53	*rs149965*	28126668	
54	*rs9393879*	28126923	
55	*rs203888*	28129568	
56	*rs9468274*	28134056	
57	*rs6903723*	28135789	
58	*rs13198131*	28137003	
59	*rs9380046*	28138478	
60	*rs7741570*	28141484	
61	*rs149952*	28141864	
62	*rs149957*	28144999	
63	*rs9380047*	28145872	
64	*rs203881*	28146096	
65	*rs4713135*	28147565	
66	*rs5021186*	28149070	
67	*rs6913038*	28152735	tagSNP #8
68	*rs3956922*	28153995	
69	*rs172164*	28154457	
70	*rs203876*	28154652	
71	*rs12211199*	28155049	
72	*rs203877*	28156603	
73	*rs9393885*	28157988	
74	*rs427348*	28166344	tagSNP #9
75	*rs1853097*	28166614	
76	*rs175955*	28167713	
77	*rs13197574*	28168218	
78	*rs3823180*	28169723	
79	*rs203893*	28170045	
80	*rs9380051*	28172070	
81	*rs169433*	28172715	
82	*rs203891*	28175777	
83	*rs10946952*	28181443	
84	*rs203885*	28184865	
85	*rs203884*	28185353	
86	*rs6924324*	28190345	
87	*rs9368554*	28190690	
88	*rs1654775*	28191404	
89	*rs4713137*	28191500	
90	*rs6456807*	28194055	
91	*rs1770132*	28194644	
92	*rs478616*	28196359	
93	*rs7747772*	28198641	
94	*rs539474*	28200285	
95	*rs6922063*	28202345	
96	*rs1770129*	28203125	
97	*rs4713140*	28205172	tagSNP #10
98	*rs1383394*	28205474	
99	*rs6914592*	28208091	
100	*rs4713141*	28209657	
101	*rs6941992*	28214120	
102	*rs4713142*	28214326	tagSNP #11
103	*rs4713145*	28214806	
104	*rs3757188*	28215336	
105	*rs3757186*	28215641	
106	*rs1904841*	28216064	
107	*rs1904840*	28216211	
108	*rs2791331*	28216691	
109	*rs2277103*	28217612	
110	*rs868987*	28218127	
111	*rs1890809*	28218457	
112	*rs2791333*	28219093	
113	*rs1225715*	28221352	
114	*rs4713149*	28227777	
115	*rs2622318*	28227865	
116	*rs6908414*	28229807	
117	*rs6932313*	28230328	
118	*rs1225604*	28230544	
119	*rs1150666*	28231907	
120	*rs1150667*	28232042	
121	*rs6928131*	28233428	
122	*rs1225603*	28233606	
123	*rs9393897*	28235689	
124	*rs1225618*	28237692	
125	*rs1150671*	28239022	
126	*rs1564442*	28240405	tagSNP #12
127	*rs1225713*	28241091	
128	*rs9283884*	28243639	
129	*rs1144707*	28243978	
130	*rs3173443*	28245006	
131	*rs4713152*	28245433	
132	*rs9393902*	28245709	tagSNP #13
133	*rs1150675*	28245996	
134	*rs1150677*	28248113	
135	*rs1150678*	28250255	
136	*rs2840214*	28250520	
137	*rs6904045*	28252208	
138	*rs6904277*	28252423	
139	*rs1233667*	28254653	
140	*rs9295761*	28255966	
141	*rs1225591*	28256731	
142	*rs1225593*	28258498	
143	*rs1150683*	28263293	
144	*rs12205680*	28265083	
145	*rs1237873*	28266933	
146	*rs1233704*	28274902	
147	*rs1233702*	28275869	
148	*rs1233701*	28276705	
149	*rs768484*	28277094	
150	*rs1233698*	28277602	
151	*rs735765*	28278276	
152	*rs1233708*	28281198	tagSNP #14
153	*rs1150696*	28283528	
154	*rs1150697*	28283615	
155	*rs1233666*	28284394	
156	*rs1150700*	28290349	
157	*rs1150701*	28291865	
158	*rs1233664*	28293705	tagSNP #15
159	*rs1736894*	28294533	
160	*rs1736892*	28294957	
161	*rs1736891*	28295080	
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759	rs3130829	29343893	
760	rs6942318	29346010	
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763	rs2394550	29356453	
764	rs5875188	29356556	tagSNP #68
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771	rs1033568	29365978	
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773	rs4446587	29367634	
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775	rs4398768	29369046	
776	rs7383161	29372666	
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782	rs7774255	29382366	
783	rs9257694	29382496	
784	rs1474859	29383595	
785	rs9257697	29384190	
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788	rs9257711	29391455	
789	rs1535151	29392363	
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806	*rs9257748*	29417780	
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808	*rs9257751*	29419450	
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810	*rs7745768*	29422975	tagSNP #74
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813	*rs9257768*	29430097	tagSNP #75
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873	*rs1028411*	29475276	
874	*rs4713220*	29475375	
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876	*rs4713221*	29475619	
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879	*rs2394604*	29477135	
880	*rs2394605*	29477187	
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882	*rs2394607*	29477386	
883	*rs1419634*	29477539	
884	*rs2894099*	29478724	
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886	*rs1362060*	29479282	
887	*rs429479*	29480190	
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889	*rs1419633*	29481080	
890	*rs1419632*	29481116	
891	*rs7453752*	29484933	
892	*rs2097771*	29486306	
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895	*rs720497*	29490300	tagSNP #80
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900	*rs3131024*	29493104	
901	*rs1011985*	29493639	
902	*rs12207410*	29494473	
903	*rs6456947*	29496173	
904	*rs2523421*	29498307	
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907	*rs2074470*	29502292	
908	*rs7770592*	29503612	tagSNP #81
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912	*rs2394609*	29505865	
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971	*rs6932526*	29572837	
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973	*rs7766082*	29575874	
974	*rs1233487*	29576677	tagSNP #86
975	*rs9393967*	29577357	
976	*rs1233486*	29577520	
977	*rs4711192*	29578654	
978	*rs3131019*	29578853	
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980	*rs757256*	29580784	
981	*rs3130858*	29581369	tagSNP #87
982	*rs3130860*	29582253	
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984	*rs1002187*	29583335	
985	*rs1233482*	29583550	
986	*rs3131020*	29583770	
987	*rs3131021*	29584010	
988	*rs3131022*	29584552	
989	*rs1557820*	29585108	
990	*rs2158281*	29585651	
991	*rs1233478*	29585689	
992	*rs2285791*	29586169	
993	*rs1010408*	29587401	tagSNP #88
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997	*rs2523433*	29590300	
998	*rs1592410*	29591842	
999	*rs1015869*	29592541	
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1065	*rs2076484*	29631899	

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1069	*rs64036*	29635163	
1070	*rs2272991*	29635495	
1071	*rs362513*	29636214	
1072	*rs995185*	29637033	
1073	*rs1233405*	29637650	
1074	*rs362531*	29638351	
1075	*rs362512*	29638524	
1076	*rs422878*	29638840	
1077	*rs362527*	29639714	
1078	*rs2523447*	29640631	tagSNP #93
1079	*rs388234*	29641187	
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1081	*rs1119080*	29644577	
1082	*rs1235162*	29645116	
1083	*rs1003582*	29646295	
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1085	*rs1233399*	29647374	
1086	*rs1003581*	29648096	tagSNP #94
1087	*rs362509*	29648753	tagSNP #95
1088	*rs6912437*	29649286	
1089	*rs362525*	29651538	
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1091	*rs362522*	29653100	tagSNP #96
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1093	*rs4568477*	29654804	
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1098	*rs362521*	29664651	tagSNP #98
1099	*rs1233386*	29666082	
1100	*rs1233384*	29667187	tagSNP #99
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1103	*rs3025644*	29675815	
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1106	*rs2076483*	29679437	tagSNP #101
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1114	*rs881284*	29683939	
1115	*rs29230*	29684285	tagSNP #102
1116	*rs2076489*	29684329	
1117	*rs29263*	29684482	
1118	*rs29262*	29684484	
1119	*rs29258*	29685509	
1120	*rs29257*	29685922	
1121	*rs2076488*	29686421	
1122	*rs29255*	29687436	
1123	*rs29253*	29688328	
1124	*rs29227*	29688480	
1125	*rs29226*	29688903	tagSNP #103
1126	*rs29225*	29688933	
1127	*rs29251*	29689649	
1128	*rs6927867*	29690417	
1129	*rs6919973*	29695767	
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1144	*rs3095267*	29714937	tagSNP #104
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1149	*rs29273*	29718882	tagSNP #105
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1152	*rs3131854*	29719778	tagSNP #106
1153	*rs3129073*	29723708	
1154	*rs439812*	29724493	tagSNP #107
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1160	*rs2535267*	29733641	
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1162	*rs2535260*	29736767	
1163	*rs2256266*	29740203	
1164	*rs6905408*	29741707	tagSNP #109
1165	*rs2071652*	29743249	
1166	*rs2071653*	29743860	
1167	*rs2273192*	29746111	
1168	*rs9357083*	29747576	
1169	*rs2535242*	29748614	tagSNP #110
1170	*rs9461544*	29749235	

Supplementary Table 2

List of SNPs captured by the 12 tagSNPs significantly associated with the haplotype A1-B8-DR3. Their number in supplementary Table 1 and their genomic position in relation to known or predicted genes are indicated.

tagSNP #10 - Representative for 9 SNPs:
97: intronic region of *ZSCAN16*
104: between *ZSCAN16* and *ZNF192*
151: between the pseudogenes *LOC222701* and *LOC222699*
181: intronic region of *ZSCAN4*
182: between *ZSCAN4* and the predicted gene *C6orf194*
183: between *ZSCAN4* and the predicted gene *C6orf194*
190: between the predicted gene *C6orf194* and *ZNF187*
196: telomeric, within 2 kb of a mRNA transcript for *ZNF187*
198: intronic region of *ZNF187*
tagSNP #23 - Representative for 2 SNPs:
226: intronic region of *ZNF323*
231: intronic region of *ZNF323*
tagSNP #24 - Representative for 3 SNPs:
232: intronic region of *ZNF323*
237: within the 5’ UTR of *ZSCAN3*
280: between *ZSCAN12* and the pseudogene *COX11P*
tagSNP #27 - Representative for 6 SNPs:
259: intronic region of *ZSCAN12*
205: intronic region of *PGBD1*
77: centromeric, within 2 kb of a mRNA transcript for *ZNF165*
46: in the pseudogene *OR2W2P*
27: between the pseudogene *OR2W4P* and *LOC340192*
4: centromeric, within 2 kb of a mRNA transcript for *OR2B2*
tagSNP #29 - Representative for 2 SNPs:
283: between *ZSCAN12* and *COX11P*
287: centromeric, within 2 kb of a mRNA transcript for *COX11P*
tagSNP #42 - Representative for 2 SNPs:
382: between *LOC646160* and the pseudogene *LOC442181*
396: in the pseudogene *LOC442181*
tagSNP #51 - Representative for 34 SNPs:
378: between *LOC646160* and *LOC442181*
392: between *LOC646160* and *LOC442181*
398: between *LOC442181* and *LOC646192*
417: between *LOC442181* and *LOC646192*
423: between *LOC442181* and *LOC646192*
430: intronic region of *LOC646192*
443: between *LOC646192* and hypothetical gene *LOC401242*
444: between *LOC646192* and hypothetical gene *LOC401242*
459: between *LOC646192* and hypothetical gene *LOC401242*
461: between *LOC646192* and hypothetical gene *LOC401242*
464: between *LOC646192* and hypothetical gene *LOC401242*
470: in the hypothetical gene *LOC401242*
473: in the hypothetical gene *LOC401242*
474: centromeric, within 2 kb of a mRNA transcript for the hypothetical gene *LOC401242*
476: between the hypothetical gene *LOC401242* and *TRIM27*
479: between the hypothetical gene *LOC401242* and *TRIM27*
480: between the hypothetical gene *LOC401242* and *TRIM27*
494: between the hypothetical gene *LOC401242* and *TRIM27*

504: between the hypothetical gene *LOC401242* and *TRIM27*
514: intronic region of *TRIM27*
523: coding region of *TRIM27*: synonymous substitution
532: between *TRIM27* and *KRT18P1*
539: between *TRIM27* and *KRT18P1*
552: between *TRIM27* and *KRT18P1*
555: between *TRIM27* and *KRT18P1*
564: between *KRT18P1* and *ZNF311*
570: between *KRT18P1* and *ZNF311*
590: between *ZNF311* and *OR2AD1P*
592: between *ZNF311* and *OR2AD1P*
638: in *LOC646260* (*SAR1P1*)
716: between *OR2J4P* and *OR2H4P*
762: between *OR2U2P* and *OR2B4P*
774: between *OR2B4P* and *OR5U1*
834: coding region of *OR12D3*: non synonymous substitution in residue 97 (Thr > Ile)
tagSNP #73 - Representative for 3 SNPs:
800: between *DDX6P* and *OR5V1*
801: between *DDX6P* and *OR5V1*
807: between *DDX6P* and *OR5V1*
tagSNP #74 - Representative for 4 SNPs:
445: between *LOC646192* and *LOC401242*
455: between *LOC646192* and *LOC401242*
804: between *DDX6P* and *OR5V1*
810: between *DDX6P* and *OR5V1*
tagSNP #86 - Representative for 2 SNPs:
949: centromeric, within 2 kb of a mRNA transcript for *LOC646366*
974: between *LOC442195* and *GPR53P*
tagSNP #92 - Representative for 10 SNPs:
851: intronic region of *OR5V1* and *OR12D3*
887: intronic region of *OR5V1* and *OR12D3*
942: in pseudogene *UBDP1*
948: telomeric, within 2 kb of a mRNA transcript for *LOC646366*
963: centromeric to *RPS17P1*, in the hypothetical transcript for *LOC442195*
966: between *RPS17P1* and *GPR53P*
969: between *RPS17P1* and *GPR53P*
1055: in pseudogene *OR2I1P*
1064: coding region of *UBD*: synonymous substitution
1082: intronic region of *GABBR1*
tagSNP #104 - Representative for 4 SNPs:
1144: between *MOG* and *GABBR1*
1148: between *MOG* and *GABBR1*
1153: between *MOG* and *GABBR1*
1157: telomeric, within 2 kb of a mRNA transcript for *GABBR1*

3. Variation and Linkage Disequilibrium within Olfactory Receptor Gene Clusters Linked to the Human Major Histocompatibility Complex

3.1. Summary

The study published by Ehlers and co-workers [Ehlers et al., 2000] established, using a group of 10 human cell lines, a milestone for the MHC-linked OR research by sequencing 13 of its 34 loci and assessing their polymorphism. The following study aimed at completing the initiated investigation by sequencing all three remaining functional loci, in addition to further nine genes known so far only as pseudogenes. Moreover, integration with publicly available genomic information was performed through the inclusion of sequence data from the recently completed human MHC haplotype project [Horton et al., 2008], for which eight further (homozygous) human cell lines were sequenced. The gene *OR2B8P*, previously known only as a pseudogene, was found to have functional alleles, while *OR1F12*, previously known as a potentially functional gene, was found to present a truncated allele.

Questions about LD dynamics within the OR polymorphisms, as well as between the latter and the HLA complex, could be answered and discussed in this study. Moreover, based on phylogenetic and comparative analyses of the 18 cell lines, a comprehensive picture of the genotypic and phenotypic variability of both MHC-linked OR gene clusters was generated. Beyond its population genetics relevance, these results build the basis for the functional assessment of the participation of HLA-linked OR loci in reproduction.

3.2. Manuscript

Santos PSC, Uehara CJS, Ziegler A, Uchanska-Ziegler B, Bicalho MG: Variation and linkage disequilibrium within odorant receptor gene clusters linked to the human major histocompatibility complex. Hum Immunol. 2010, 71(9):843-50.

DOI:10.1016/j.humimm.2010.06.011

Pages 44-64

4. Comparative Genomic Analysis of MHC-linked Olfactory Receptor Repertoires among 14 Vertebrate Species

Comparative genomic analyses belong to the most powerful tools in evolutionary genetics. The recent sequencing of the genomes of different organisms has shown that almost all vertebrates studied in detail so far have one or more clusters of genes encoding olfactory receptors (OR) in close linkage to the MHC. A systematic comparison of the sequences of these receptors from different organisms has been, however, limited to humans and rodents. Additionally, a comparative analysis focusing on genomic morphology (i.e. linkage, position and transcriptional orientation of loci or gene clusters) of MHC-linked OR genes is, to the best of my knowledge, still absent. In this chapter, I present the so far most comprehensive comparison of protein sequence and genomic morphology of MHC-linked OR genes performed to date, among 14 vertebrates for which enough sequence information has been generated by different sequencing projects, and made available through internet-based databanks. 464 peptide sequences (Table 1) from human, chimpanzee, orangutan, rhesus macaque, dog, cat, cow, pig, horse, mouse, rat, opossum, frog and zebra fish were assessed.

Table 4.1: List of organisms analyzed for their MHC-linked OR genes, with indication of number of protein sequences assessed, the respective chromosome, as well as the approximate total length of the OR clusters

Organism	Short	# Protein Seqs	Chr.	Length (Mb)
Homo sapiens (Human)	Hsa	34	6p	2,3
Pan troglodytes (Chimpanzee)	Ptr	13	6p	3,0
Pongo pygmaeus (Orangutan)	Ppy	13	6	3,0
Macaca mulatta (Rhesus macaque)	Mamu	17	4	3,0
Canis familiaris (Dog)	Cfa	10	35	1,5
Felis catus (Cat)	Fca	8	B2	1,5
Bos taurus (Cow)	Bta	57	23	1,5
Sus scrofa (Pig)	Ssc	77	7	3,4
Equus caballus (Horse)	Eca	71	20	4,0
Mus musculus (Mouse)	Mumu	44	17	1,2
Rattus norvegicus (Rat)	Rno	59	20p	1,6
Monodelphis domestica (Opossum)	Mdo	38	2	9,0
Xenopus tropicalis (Frog)	Xtr	3	Scf_396	0,02
Danio rerio (Zebrafish)	Dre	20	15	0,3
Total		**464**		

Phylogenetic analyses demonstrate that most OR families present in the two human MHC-linked clusters are also present in the other species, although some are specifically expanded in certain taxa (Fig. 4.1). A map of the respective genomic regions was generated for each species, through which several morphological features such as the number of loci, the gene order and localization, as well as the presence of particular OR gene families could be identified as typical for each of the taxa assessed: primates, ungulates, carnivores, rodents, marsupial, amphibian and teleost (Fig. 4.2).

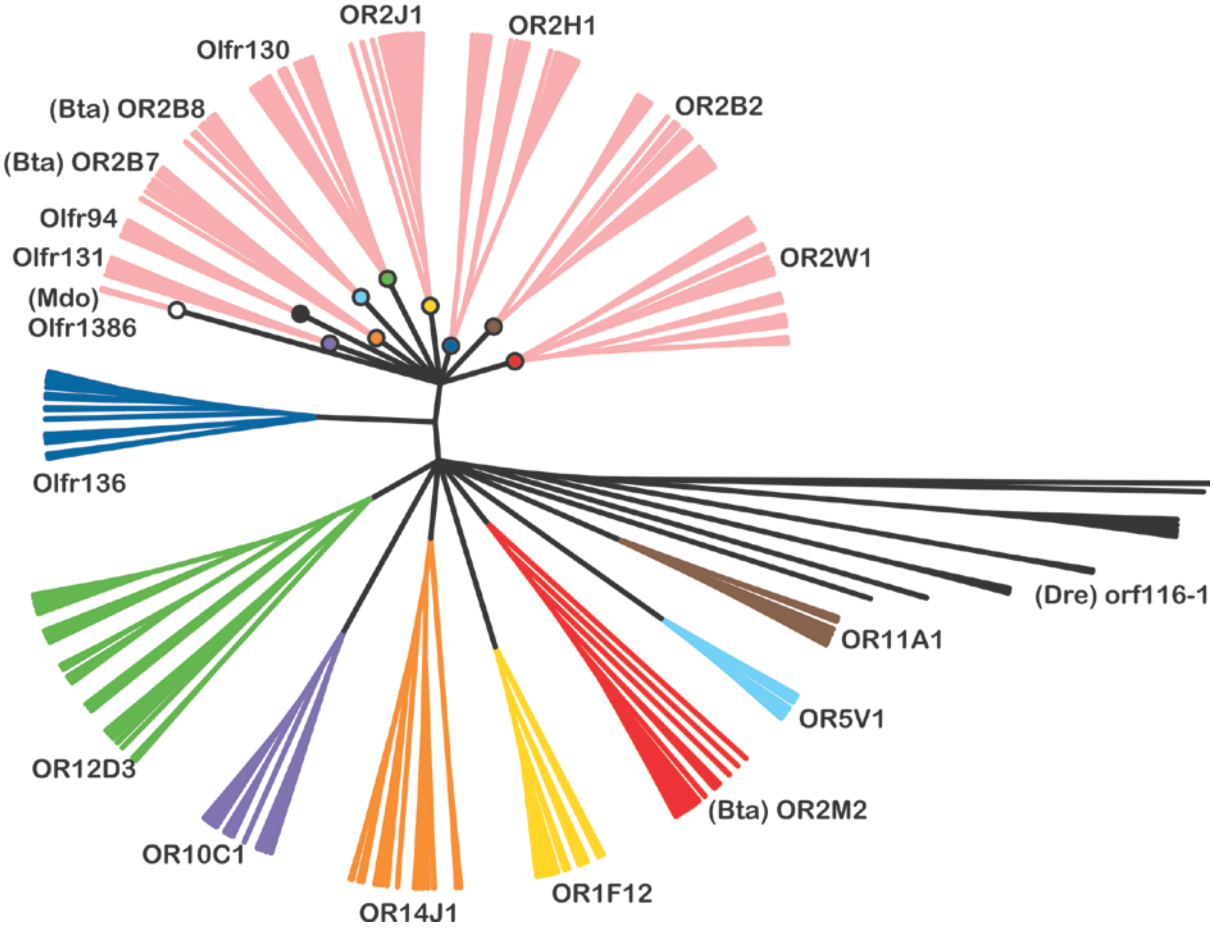


Figure 4.1: Phylogenetic tree depicting the evolutionary relationships of 464 MHC-linked OR amino acid sequences from 14 vertebrates. The colours used to identify each branch of sequences on the tree are the same used in the corresponding loci of all panels of Fig. 4.2. For each branch the name of one locus is given as representative of the group. The tree was inferred using the neighbour-joining method.

The genomes of humans, mouse, rat and zebra fish are the best established vertebrate genome assemblies available. Genome browsers like ENSEMBL (www.ensembl.org/) and VEGA (http://vega.sanger.ac.uk/) gather information from different genome projects worldwide and make them available with official gene nomenclatures and coordinates. Obtaining their sequences involved finding the MHC through the literature or an electronic BLAST search against the whole genome, scanning its vicinity for OR genes, and downloading them manually.

In contrast, most genes of the remaining ten vertebrate genome assemblies still lack a nomenclature for OR genes, and the MHC cannot easily be identified through the respective genome browsers. Data mining in these cases involved, besides of literature research, several runs of BLAST searches for unequivocal localization of the MHC within a chromosome, with following BLAST searches for OR loci in a range of 10 Mb around the MHC. Additionally, InterPro (http://www.ebi.ac.uk/interpro/) protein signatures were used to locate OR genes. InterPro is an online database that integrates data from several institutions aiming at the classification and automatic annotation of proteins and genomes [Hunter et al., 2009]. It uses information from known amino acid sequence motifs in order to recognize the structure and predict the function or a large number of proteins. After testing the strategy of sequence capturing based on InterPro signatures on four well established genomes (human, mouse, rat and zebra fish) with 100% concordance, I used InterPro in order to obtain sequences from the remaining vertebrates. Signatures used were “IPR000725” (olfactory receptor), and in some cases “IPR000276” (seven transmembrane g-protein-coupled receptor, rhodopsin-like). Nucleotide or amino acid sequences were obtained from the web servers of the following institutions: NCBI (www.ncbi.nlm.nih.gov, pig and horse), LGD (http://home.ncifcrf.gov/ccr/lgd/, cat), JGI (http://genome.jgi-psf.org/, frog) and ENSEMBL (www.ensembl.org, all other organisms). In most cases, the electronic tool BioMart (www.biomart.org) could be used in order to identify InterPro OR signatures within the sequences.

Primates (Fig. 4.2, panel a) exhibit two OR clusters telomeric to the MHC, with a large histone cluster flanking the distal OR cluster, and well conserved sub-organization of both clusters. The proximal OR cluster includes the framework genes gamma-aminobutyric acid (GABA) B receptor 1 (*GABBR1*), myelin oligodendrocyte glycoprotein (*MOG*) and ubiquitin D (*UBD*). As discussed in the article shown in chapter 4, apparently functional orthologs of the human gene *OR2W6P* are present within the MHC distal OR cluster, in the same position and transcriptional orientation. In the case of the macaque, the *OR2W6P* ortholog is the only locus that remained functional in that cluster. Orangutan is the only primate that seems to have "lost", through pseudogenization, all members of the OR12D family (discussed in chapters 2 and 3). Although the human loci *OR2B8P* and *OR12D1P* are widely considered as pseudogenes, they were included in Fig. 4.2 due to segregating functional alleles found in the study presented in the chapter 3.

Rodents (mouse and rat, Fig. 4.2, panel b) exhibit a single OR cluster telomeric to the MHC, homologous to the centromeric MHC-linked OR cluster of primates. In both organisms, the homologous histone clusters are located on other chromosomes, which possess a syntenic region harbouring OR loci that are evolutionarily related to those of the telomeric OR cluster on human Chr6p. The basic organization is remarkably conserved between the two murine species, which have, as compared to the other organisms, the number of loci being homologous to the human families OR2N1, OR14J, OR1F and OR10C strongly expanded. The apparently obligatory framework genes are also present, close to the MHC.

In comparison to primates, ungulates (horse, cow and pig, Fig. 4.2, panel c) were found to have a third cluster of MHC-linked OR, with all loci being similar to human gene *OR2M3* (which is harboured within another large OR cluster on human Chr. 1q44). The histone genes of ungulates are split in three relatively short clusters, which have conserved positions between horse and cow, but not in pig. Another peculiarity of *Sus scrofa* refers to the MHC-proximal OR cluster: horse and cow have the three framework genes close to the MHC, whereas the pig has them neighbouring the telomeric side of the same OR cluster, in opposite transcriptional direction. This fact, as well as the positions and transcriptional orientations of the pig orthologs of *OR2J3* and neighbours, are suggestive of a chromosomal inversion that has probably taken place after the divergence of pigs from the ancestral ungulate.

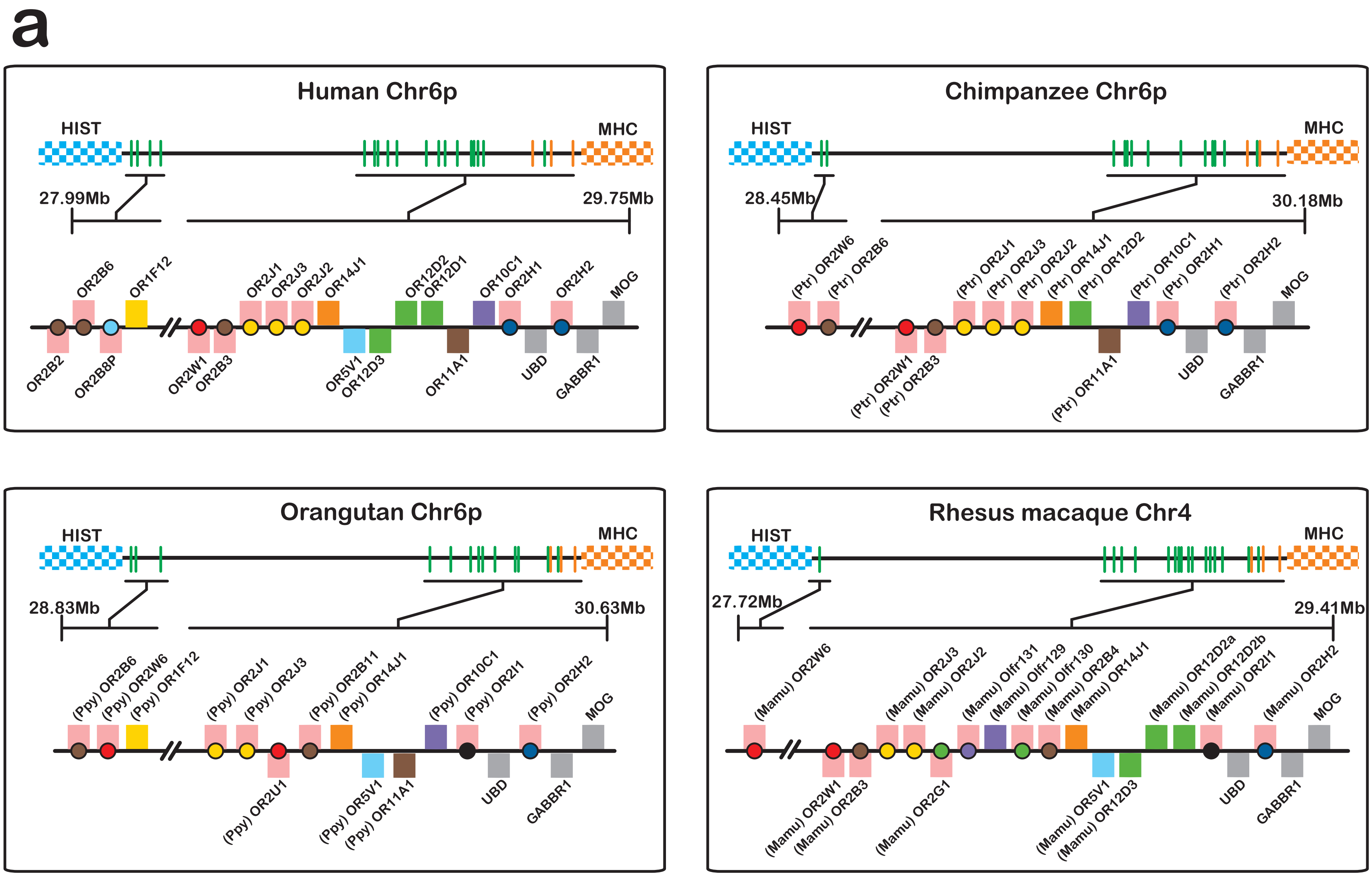
Carnivores (cat and dog, Fig. 4.2, panel d) were the only vertebrates in this analysis not found to have OR linked to the MHC, but only to the MHC framework genes *GABBR1* and *MOG*. As discussed elsewhere [Yuhki et al., 2007], a chromosomal break between the MHC and OR seems to have taken place before the evolutionary split of canine and feline groups. In both cases, the OR clusters are very close to the telomeres of ChrB2 (cat) and Chr35 (dog), and a large histone cluster is present linked to the OR clusters. Apart from chicken, these carnivores are so far the only vertebrates known to lack MHC-linked OR genes.

Opossum, the only marsupial that could be studied (Fig. 4.2, panel e), presents two relatively distant MHC-linked OR clusters, with the MHC-distal cluster harbouring an interspersed histone cluster. Similarly to ungulates, the opossum possesses also OR that cluster with members of the OR2M subfamily (Fig. 4.1) within the MHC-distal cluster. The framework genes are present in the MHC-proximal OR cluster. This species lacks loci of some gene families present in almost all other mammals (as OR5V and OR14J). On the other hand, the opossum is so far the only species in which homologs of the mouse Olfr1386 are MHC-linked. In all other organisms, including the mouse, this gene is not linked to the MHC.

The genomic assembly of the frog (Fig. 4.2, panel f) is still incomplete for the MHC region and its vicinities. However, at least part of the MHC class II, three OR genes with sequence similarity to human *OR1F12*, and the framework gene *GABBR1* were confirmed to be linked so far, in a segment provisionally called “scaffold 396”. It remains unclear whether further OR, framework or MHC loci are linked to this region.

Although all terrestrial vertebrates analyzed are characterized by MHC-linked OR loci belonging to a defined subgroup of families (Fig. 4.2, panels a to f), this is different in the only teleost included in the analysis: in the zebra fish, a distinct group of OR are linked to genes that in mammals are part of the MHC class I and III regions (Fig. 4.2, panel g).

Figure 4.2 (next six pages): Gene maps of the MHC-linked OR genes in 14 vertebrates grouped in seven taxa: primates (a), rodentia (b), ungulata (c), carnivora (d), marsupialia (e), amphibia (f) and teleostei (g). Within each species map, an overview of the region is given above and a detailed view is given below. In the overview, the MHC complex and histone clusters are shown when present (in orange and blue checked boxes, respectively), as well as the relative positions of each OR gene (green vertical ticks) and selected framework genes (orange vertical ticks). The names of each OR locus are depicted in the detailed view below, where only human, mouse, rat and zebra fish have widely accepted nomenclatures for these loci, and have the official gene symbols shown. For all other species, I assigned a name for each locus based on sequence similarity to known genes, having the human and the mouse genomes as references. A gene was considered homolog of the human or mouse locus with which its amino acid sequence had the highest identity, considering 75% identity as a minimum threshold. In these cases, the gene designation of the locus is preceded by the species code, and has an additional letter in cases of multiple homologs (“Bta_OR2M2a” for instance, meaning the *Bos taurus* homolog of human *OR2M2*, fist copy). The colours of the boxes correspond to the clustering of the respective amino acid sequence in the phylogenetic tree (Fig. 4.1), and these are placed above or below the base line in order to indicate transcriptional orientation to the right or to the left, respectively. MHC framework genes are depicted in grey.


a
Human Chr6p
HIST
MHC
27.99Mb
29.75Mb
OR2B6
OR1F12
OR2J1
OR2J3
OR2J2
OR14J1
OR12D2
OR12D1
OR10C1
OR2H1
OR2H2
MOG
OR2B2
OR2B8P
OR2W1
OR2B3
OR5V1
OR12D3
OR11A1
UBD
GABBR1
Chimpanzee Chr6p
HIST
MHC
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30.18Mb
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(Ptr) OR2B6
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(Ptr) OR2J3
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MOG
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(Ptr) OR2B3
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UBD
GABBR1
Orangutan Chr6p
HIST
MHC
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30.63Mb
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(Ppy) OR2W6
(Ppy) OR1F12
(Ppy) OR2J1
(Ppy) OR2J3
(Ppy) OR2B11
(Ppy) OR14J1
(Ppy) OR10C1
(Ppy) OR2I1
(Ppy) OR2H2
MOG
(Ppy) OR2U1
(Ppy) OR5V1
(Ppy) OR11A1
UBD
GABBR1
Rhesus macaque Chr4
HIST
MHC
27.72Mb
29.41Mb
(Mamu) OR2W6
(Mamu) OR2J3
(Mamu) OR2J2
(Mamu) Olfr131
(Mamu) Olfr129
(Mamu) Olfr130
(Mamu) OR2B4
(Mamu) OR14J1
(Mamu) OR12D2a
(Mamu) OR12D2b
(Mamu) OR2I1
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MOG
(Mamu) OR2W1
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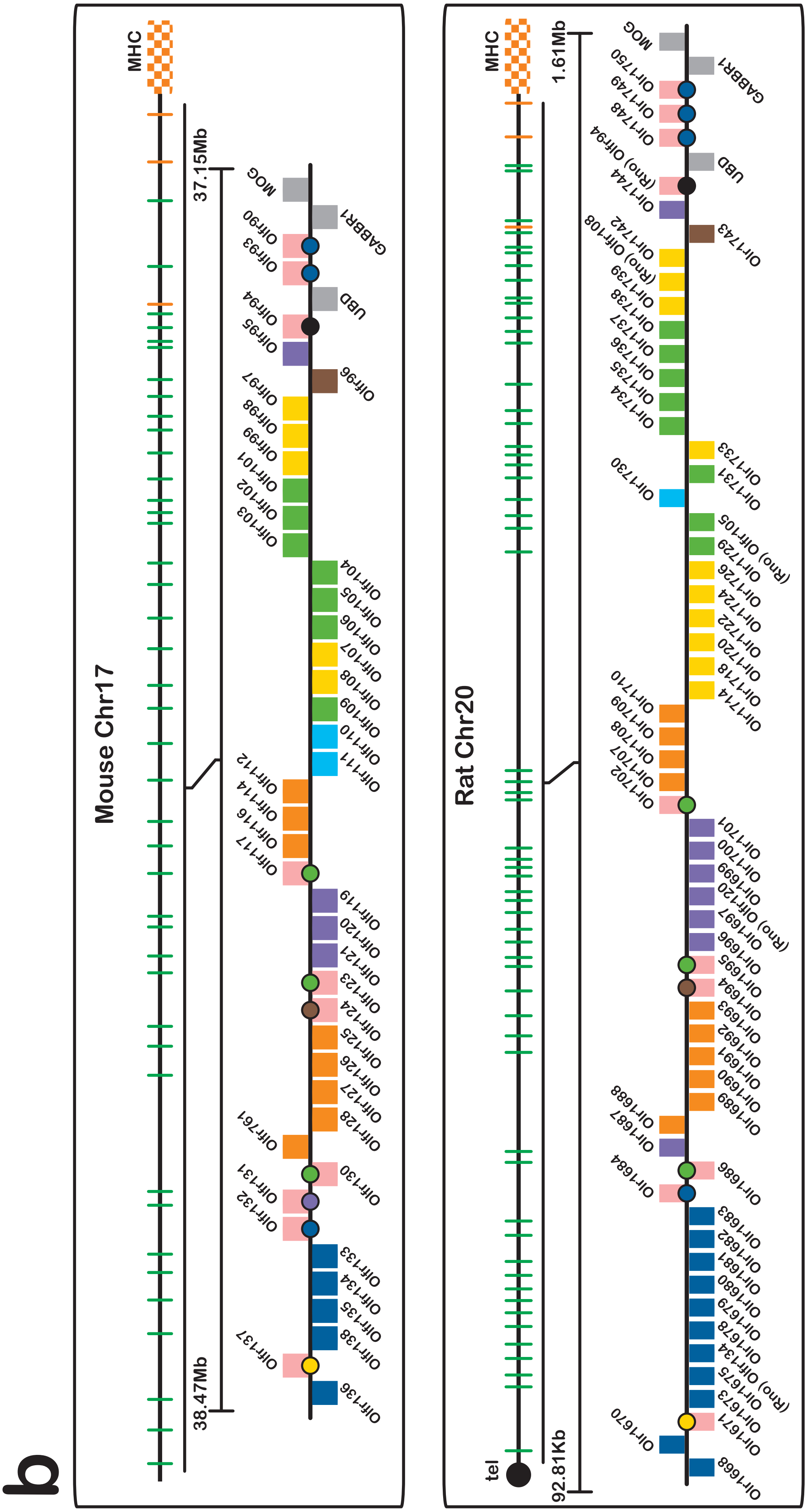
(Mamu) OR12D3
UBD
GABBR1


b
Mouse Chr17
MHC
37.15Mb
38.47Mb
MOG
GABBR1
Olfr90
Olfr93
UBD
Olfr94
Olfr95
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Olfr97
Olfr98
Olfr99
Olfr101
Olfr102
Olfr103
Olfr104
Olfr105
Olfr106
Olfr107
Olfr108
Olfr109
Olfr110
Olfr111
Olfr112
Olfr114
Olfr116
Olfr117
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Olfr120
Olfr121
Olfr123
Olfr124
Olfr125
Olfr126
Olfr127
Olfr128
Olfr761
Olfr130
Olfr131
Olfr132
Olfr133
Olfr134
Olfr135
Olfr138
Olfr137
Olfr136
Rat Chr20
MHC
1.61Mb
tel
92.81Kb
MOG
GABBR1
Olr1750
Olr1749
Olr1748
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UBD
Olr1744
Olr1743
Olr1742
(Rno) Olfr108
Olr1739
Olr1738
Olr1737
Olr1736
Olr1735
Olr1734
Olr1733
Olr1731
Olr1730
Olr1729
(Rno) Olfr105
Olr1726
Olr1724
Olr1722
Olr1720
Olr1718
Olr1714
Olr1710
Olr1709
Olr1708
Olr1707
Olr1702
Olr1701
Olr1700
Olr1699
(Rno) Olfr120
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Olr1683
Olr1682
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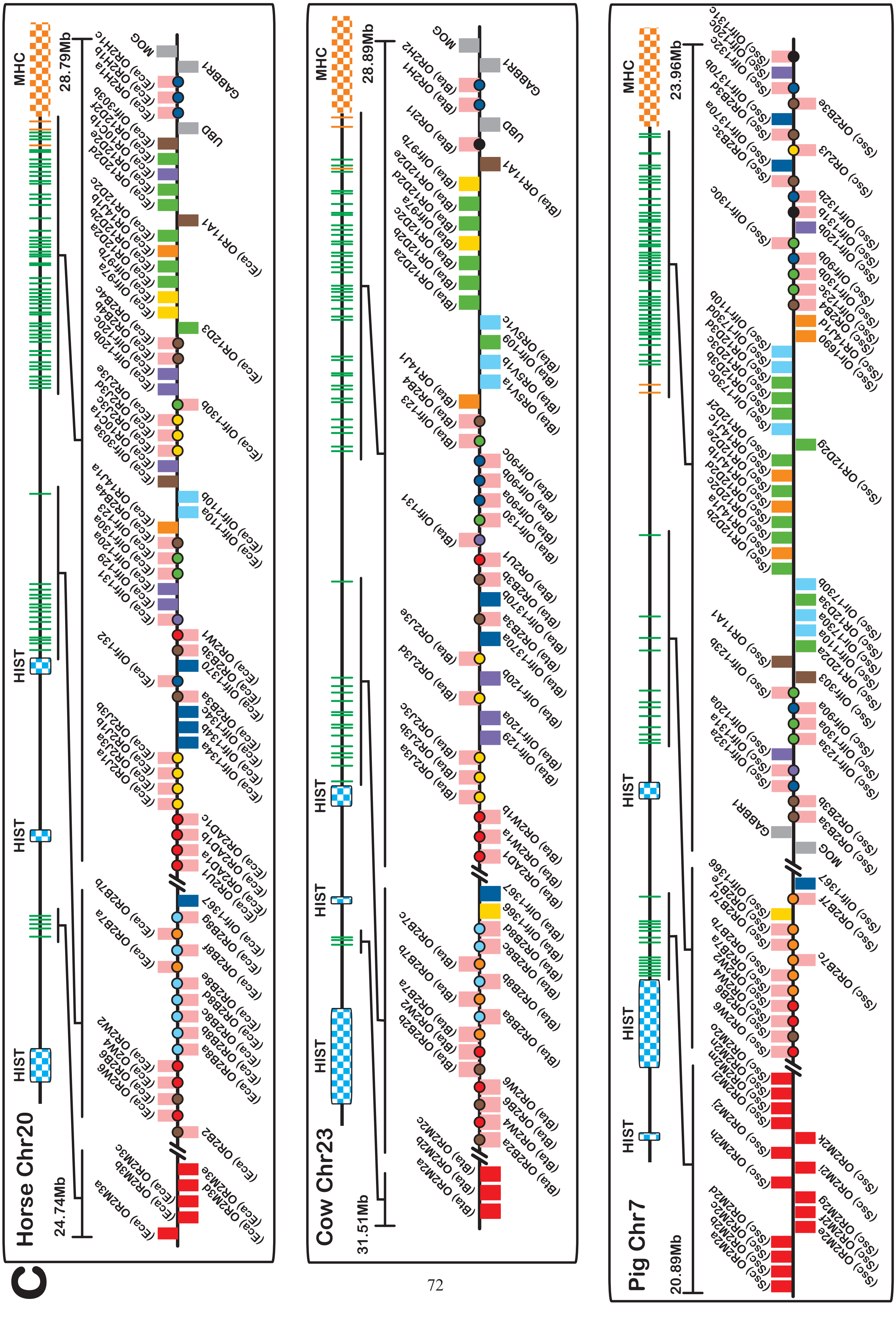
Olr1671
Olr1670
Olr1668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Horse Chr20
MHC
HIST
HIST
HIST
28.79Mb
24.74Mb
MOG
GABBR1
UBD
(Eca) OR2H1c
(Eca) OR2H1b
(Eca) OR2H1a
(Eca) Olfr303b
(Eca) OR12D1b
(Eca) OR10C1b
(Eca) OR12D2e
(Eca) OR12D2d
(Eca) OR12D2c
(Eca) OR14J1b
(Eca) OR12D2b
(Eca) OR12D2a
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(Eca) Olfr97a
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(Eca) OR2B4b
(Eca) Olfr130c
(Eca) Olfr120b
(Eca) OR2J3e
(Eca) OR2J3d
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(Eca) OR2M3d
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MHC
HIST
HIST
HIST
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31.51Mb
MOG
GABBR1
UBD
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(Bta) Olfr97a
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(Bta) OR5V1a
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(Bta) OR2B3b
(Bta) Olfr1370b
(Bta) OR2B3a
(Bta) Olfr1370a
(Bta) Olfr120b
(Bta) Olfr120a
(Bta) Olfr129
(Bta) OR2W1b
(Bta) OR2W1a
(Bta) OR2AD1
(Bta) Olfr1367
(Bta) Olfr1366
(Bta) OR2B8d
(Bta) OR2B8c
(Bta) OR2B8b
(Bta) OR2B8a
(Bta) OR2W6
(Bta) OR2B6
(Bta) OR2W4
(Bta) OR2B2a
Pig Chr7
MHC
HIST
HIST
HIST
23.96Mb
20.89Mb
GABBR1
(Ssc) Olfr131c
(Ssc) Olfr120c
(Ssc) Olfr132c
(Ssc) Olfr1370b
(Ssc) OR2B3d
(Ssc) Olfr1370a
(Ssc) OR2B3c
(Ssc) Olfr130c
(Ssc) Olfr110b
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(Ssc) OR12D3b
(Ssc) Olfr1730c
(Ssc) OR12D2f
(Ssc) OR14J1c
(Ssc) OR12D2e
(Ssc) OR14J1b
(Ssc) OR12D2d
(Ssc) OR12D2c
(Ssc) OR14J1a
(Ssc) OR12D2b
(Ssc) OR11A1
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(Ssc) OR2M2m
(Ssc) OR2M2l
(Ssc) OR2M2j
(Ssc) OR2M2h
(Ssc) OR2M2d
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(Ssc) OR2J3
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(Ssc) Olfr131b
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(Ssc) Olfr130b
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(Ssc) Olfr1730b
(Ssc) OR12D3a
(Ssc) Olfr1730a
(Ssc) Olfr110a
(Ssc) OR12D2a
(Ssc) Olfr303
(Ssc) Olfr90a
(Ssc) Olfr130a
(Ssc) Olfr133a
(Ssc) OR2B3b
(Ssc) OR2B3a
(Ssc) MOG
(Ssc) Olfr1367
(Ssc) OR2B7f
(Ssc) OR2B7c
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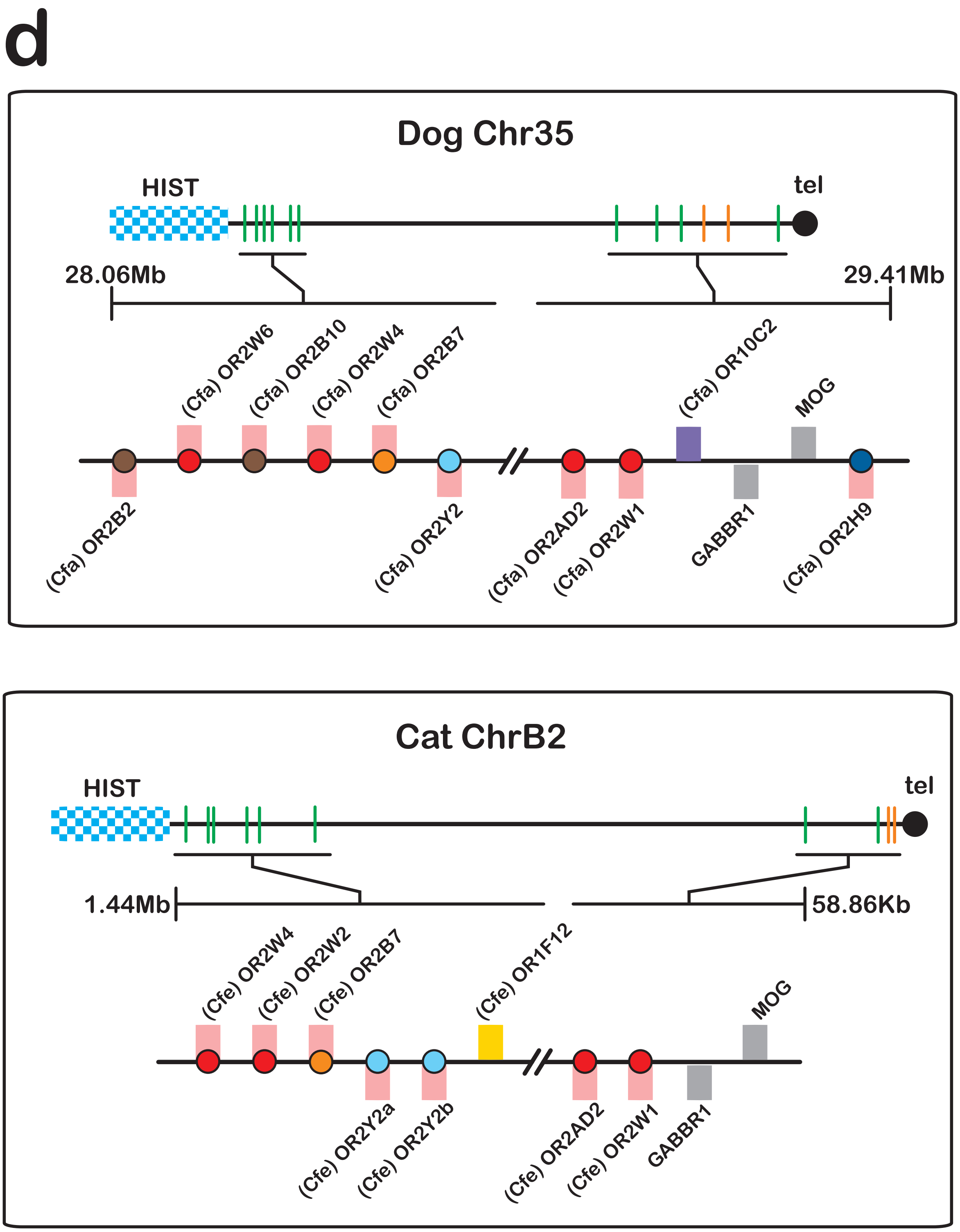
(Ssc) OR2M2e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Dog Chr35
HIST
tel
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29.41Mb
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(Cfa) OR2B7
(Cfa) OR10C2
MOG
(Cfa) OR2B2
(Cfa) OR2Y2
(Cfa) OR2AD2
(Cfa) OR2W1
GABBR1
(Cfa) OR2H9
Cat ChrB2
HIST
tel
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58.86Kb
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(Cfe) OR2B7
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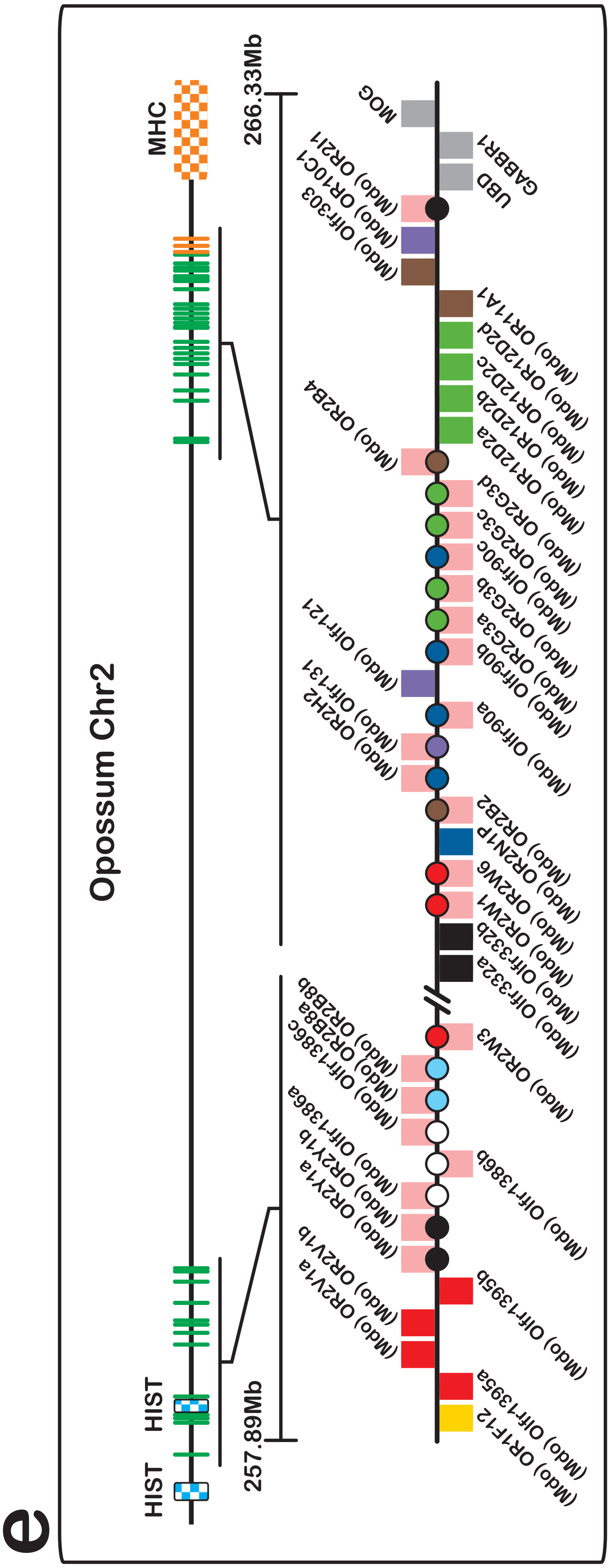
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Opossum Chr2
MHC
HIST
HIST
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257.89Mb
MOG
GABBR1
UBD
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(Mdo) Olfr303
(Mdo) OR11A1
(Mdo) OR12D2d
(Mdo) OR12D2c
(Mdo) OR12D2b
(Mdo) OR12D2a
(Mdo) OR2B4
(Mdo) OR2G3d
(Mdo) OR2G3c
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(Mdo) OR2G3a
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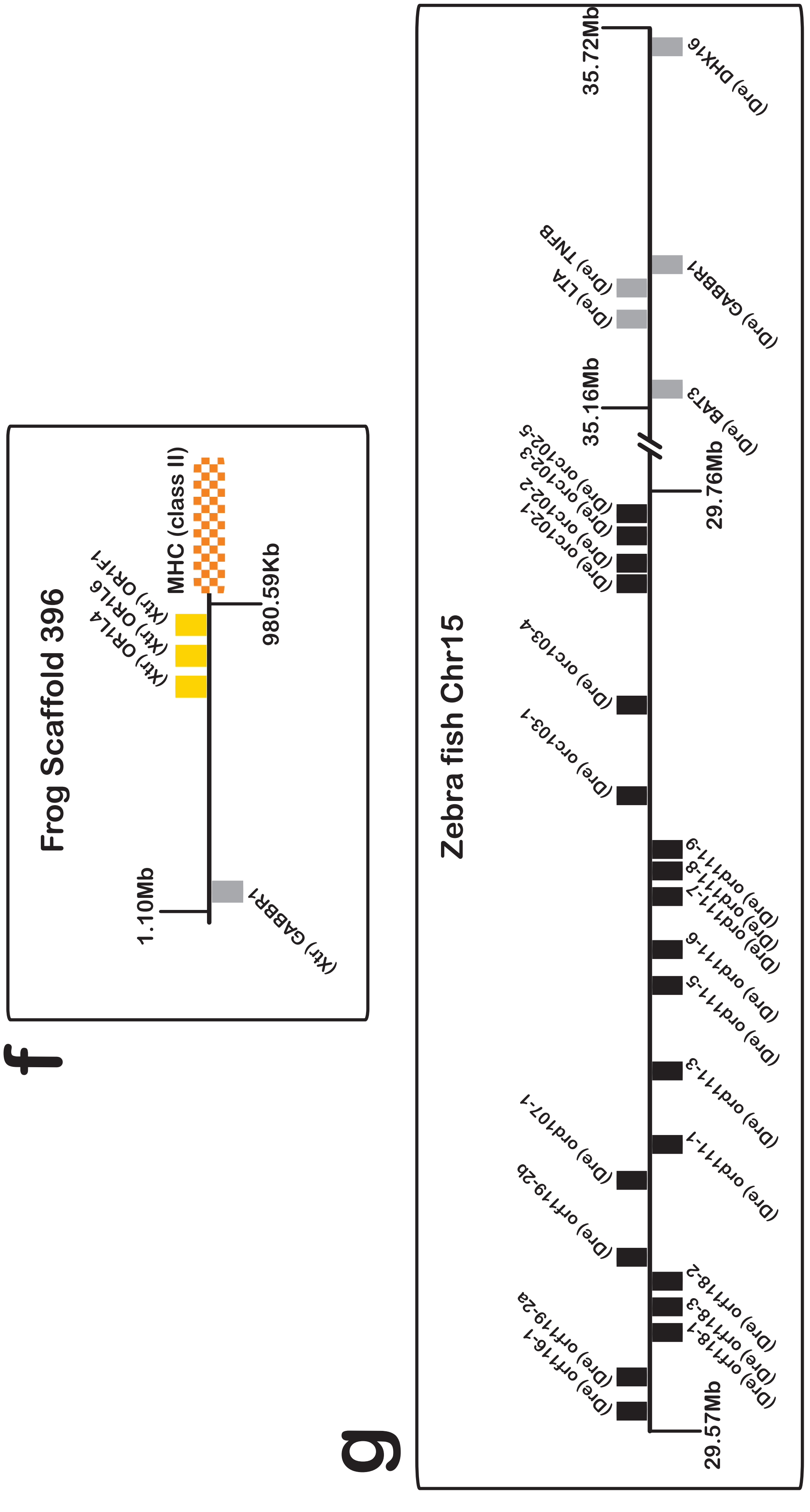
f
Frog Scaffold 396
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(Xtr) OR1L4
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MHC (class II)
980.59Kb
g
Zebra fish Chr15
29.57Mb
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(Dre) orf118-2
(Dre) orf119-2b
(Dre) ord107-1
(Dre) ord111-1
(Dre) ord111-3
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(Dre) orc103-4
(Dre) orc102-1
(Dre) orc102-2
(Dre) orc102-3
(Dre) orc102-5
29.76Mb
35.16Mb
(Dre) BAT3
(Dre) LTA
(Dre) TNFB
(Dre) GABBR1
35.72Mb
(Dre) DHX16

5. Transmission Distortion in the Human Genome

In this chapter, I will present three manuscripts that describe the work performed focusing the phenomenon of allelic transmission distortion in humans. While two of them (5.1 and 5.2) address the TD problematic in phenomenon itself both in publicly available and in independently sampled populations, the third manuscript (5.3) addresses a technical problem associated with one of the most important online resources for human sequence variation today: the international HapMap project.

5.1. Transmission Distortion in the Human Chromosome 6p

5.1.1. Summary

The fact that the mouse t haplotypes are under extreme transmission distortion (TD, a departure from Mendel's law of independent segregation of alleles in a given heterozygous locus or region) affect a region syntenic to large parts of the short arm human chromosome 6 (Chr6p) – including the HLA complex and linked olfactory receptors – led us to investigate the same phenomenon in the populations genotyped through the international haplotype mapping consortium (HapMap). Beyond the 180 founder haplotypes of the CEU population assessed for the study described on chapter 2, the HapMap had, at that time, SNP data from 60 family trios (father-mother-adult child) available – 30 from the CEU and 30 from the YRI population. The study presented in the following article aimed at testing, *in silico*, the possibility that alleles of SNPs on Chr6p could be under TD. Both OR clusters and the MHC were found to segregate conforming to Mendelian expectation. However, a centromeric region of Chr6p harbouring, among others, the transcription-factor encoding locus *RUNX2* was found to be under strong TD. Besides of discussing the importance of TD studies in healthy cohorts, and of giving examples of studies that could be biased because TD was not considered, the article describes a way to use linkage disequilibrium data in order to choose subsets of SNPs carrying haplotypic information and thus circumvent the statistical problem of multiple testing.

One additional result of this work concerns the data resource property of the international HapMap project. Scientists world-wide use the HapMap as a guide for study design and interpretation, as an overview of genomic diversity can be interactively browsed by the user through the internet. The results of the following work were compiled in a code file (Supplemental File S3) that can be uploaded by readers into the HapMap genome browser and viewed within the genomic context. This is a first step towards a future genome-wide TD map, which should ideally be available for browsing by HapMap users investigating a genomic region. As we point out in the paper, TD is a feature that has to be taken into account during interpretation of results from phenotype association studies. In this sense, the ability to check, through an internet browser, if one given region of interest shows evidence of TD or not, would be extremely helpful.

5.1.2. Publication

Santos PS, Höhne J, Schlattmann P, König IR, Ziegler A, Uchanska-Ziegler B, Ziegler A (2009): Assessment of transmission distortion on chromosome 6p in healthy individuals using tagSNPs. European Journal of Human Genetics 17:1182-1189

DOI:10.1038/ejhg.2009.16

Pages 78-90

5.2. Transmission Distortion in Southern Brazilian Families

5.2.1. Summary

The work described in this chapter aimed at verifying, with a larger and independent cohort, the results obtained with the TD assessment of Chr6p using the HapMap families from the CEU and YRI populations (see 5.1). Even though results of that study were statistically significant, the fact that TD was observed among CEU but not among YRI trios left several questions open. Is TD an ethnicity-dependent feature? Is the YRI cohort an exception to the rule? Or is the TD observed just an artefact resulting from sample stratification within the CEU sample of families and inflated by linkage disequilibrium in the S-M-R region? The study shown in this section aimed at answering these questions. Genomic DNA from 239 individuals from 141 family trios from Curitiba, Southern Brazil, were obtained through a cooperation with a Brazilian immunogenetics laboratory that recruits volunteer bone marrow donors, which, in some cases, concur in participating in population genetics studies like the one presented here. All individuals were genotyped for SNPs covering the S-M-R region. An additional genome-wide search for SNPs showing evidence of TD, based on criteria from the International HapMap project was performed as well. The results of the first study (Section 5.1) were confirmed in Southern Brazilian trios, and TD could be established as a general feature of the S-M-R region among Caucasian populations. However, TD was not found for other SNPs in the genome, although suggestive results were obtained for the follistatin-like 1 (*FSTL1*) locus on chromosome 2.

5.2.2. Manuscript

Santos PS, Höhne J, Schlattmann P, Poerner F, Bicalho MG, Ziegler A, Uchanska-Ziegler B: Presence of Transmission Distortion on Human Chromosome 6p Revealed by SNP Genotyping of Southern Brazilian Families. Submitted.

Pages 92-112

Presence of Transmission Distortion on Human Chromosome 6p Revealed by SNP Genotyping of Southern Brazilian Families

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Running Head: Transmission Distortion in Chr6p among Southern Brazilian Families

Abstract

Background. Transmission distortion (TD) is a statistically significant violation of Mendel's expected 1:1 allelic transmission ratio from heterozygous parents to their offspring. We have previously reported evidence for TD of single nucleotide polymorphisms (SNPs) within human chromosome 6p around the loci *SUPT3H, MIR586* and *RUNX2* (*S-M-R* region) among 30 HapMap family trios of European ancestry through a systematic *in silico* investigation. Several questions however, regarding the relatively small sample size, the differences between investigated populations, the haplotype phasing and the possibility of genotyping errors remained unanswered.

Results. We now report a search for TD in a fully independent cohort of 141 Southern Brazilian family trios of predominantly European ancestry by SNP genotyping. We primarily focused on the *S-M-R* region, but performed, in addition, a genome-wide search within the 30 CEU trios for markers for which paternal and maternal TD were reciprocally compensated within each family trio. The over four-fold increased sample size allowed us to dispense with the use of software-based haplotype phasing, and thus consider undisputable transmissions only, without losing statistical power. Strong evidence of TD however, was found only for the *S-M-R* region.

Conclusions. Although it still remains enigmatic why TD is present, and how a considerable degree of heterozygosity is retained in the corresponding loci, TD of the *S-M-R* region is clearly a phenomenon present in the general, "unaffected" population. As a consequence, TD has to be taken into account when assessing the outcome of linkage studies, especially in the case of the intensively investigated *RUNX2* locus.

Background

Transmission Distortion (TD) refers to the contravention of Mendel's law of independent assortment of characteristics. The best known example of TD, first described over 70 years ago, concerns the mouse t haplotypes, which are an extreme – yet not completely understood – example of distorted segregation of large segments of mouse chromosome 17. [1] In the present study, we will refer to TD in a broad sense, denoting a statistically significant deviation from the generally expected 1:1 transmission ratio of alleles from heterozygous parents to their offspring, without regard to its molecular causes or mechanisms.

TD has already been described for different loci in many species, [1–4] including humans. [5–7] In humans, TD has traditionally been investigated among families selected for different diseases or phenotypic traits, but the focus of these studies has recently been extended also to families without a disease phenotype. [5,8–14] TD is not only interesting as a biological phenomenon, but the detailed identification of loci under TD in the general, healthy population, is a requirement for the correct interpretation of linkage and association studies based on allelic transmission within families. If ignored, the presence of TD can introduce serious bias into such studies. [5,9,12,15,16]

We recently reported evidence of TD in a region of the short arm of human chromosome 6 (Chr6p) harboring three genes: the microRNA locus *MIR586* and the two transcription factor-encoding *SUPT3H* and *RUNX2*. [14] The close physical linkage of these two transcription factor loci is apparently an extreme case of phylogenetic conservation, which can be observed not only in all mammals, birds and reptiles studied so far, [17] but also amphibians, [18] fish, [19] and even in the demosponge *Amphimedon queenslandica*, indicating the existence of linkage between *SUPT3H* and *RUNX2* for at least 700 million years. [20] Whereas little information is currently available for *MIR586* and *SUPT3H*, this is not the case for *RUNX2*. The protein encoded by this gene is a master transcription factor crucially related to bone, cartilage and tooth morphogenesis. [21–24] It was also shown to be expressed in non-skeletal tissues, [25] and to be associated with many central processes such as cell growth, determination of cell fate, epigenetic regulation, [26,27] and hematopoiesis. [28,29] A recent query to the STRING database yielded 119 human proteins as functional partners of Runx2, with high confidence prediction. [30] Additionally, its role on clinical conditions as osteoporosis, [31,32] cancers of breast, [33] prostate, [34] bone, [35] and lymphoid tissues [36,37] makes Runx2 a promising target also for therapeutic interventions. [38] It seems therefore essential to assess the possibility that the linkage disequilibrium (LD) block around *RUNX2*, *MIR586* and *SUPT3H* (the "*S-M-R* region") is under TD in the general, healthy population. Likewise, since TD in this chromosomal segment appears to be an ethnicity-dependent phenomenon, [14] an investigation of trios belonging to further ethnic groups is desirable.

The main limitation of many investigations addressing TD – including our own previous analysis [14] – was the small number of trios, and consequently the reduced statistical power. In the present investigation we analyzed the occurrence of TD in a sample of family trios over four times as large as previously. These trios belong to a completely independent population which is, however, ethnically related to the CEU sample. Additionally, we investigated whether it would be possible to detect TD among single nucleotide polymorphisms (SNPs) from the International HapMap Project using a group of criteria that considered not only a sex-compensatory version of the transmission/disequilibrium test (TDT), [39] but also the LD shape of the respective region, and the number of SNPs involved. Following this rationale, we performed a genome-wide search for markers fulfilling those criteria, and genotyped a second group of SNPs in the larger, independent cohort mentioned above, looking for evidence of TD.

Results and Discussion

The analysis of SNPs from the *S-M-R* region allowed us to assess the existence of TD for that region in the Brazilian population. Considering only Euro-Brazilian trios (Fig. 1), TD was observed for rs12530016 and rs2038765 (parent-unspecific p-value = 0.0052 and 0.0086 respectively). While mothers and fathers seem to equally contribute to the effect observed for rs2038765, this is not the case for rs12530016. For this marker, mothers are driving the observed distortion (mother-specific p-value = 0.0236). The same analysis, considering all analyzed trios, is depicted in figure 2. TD is still observed for rs2038765 (parent-unspecific p-value = 0.0024), but is only marginal for rs12530016 (parent-unspecific p-value = 0.0347).

Regarding the sex-compensatory analysis, the only locus with a marker showing relatively strong TD was rs4676781, in the follistatin-like 1 (*FSTL1*) locus. When only Euro-Brazilians were considered, the parent-unspecific p-value was 0.0072, and the mother specific p-value was 0.0186, whereas for all analyzed trios the parent-unspecific p-value was 0.0085, and the mother specific p-value was 0.0133. However, these results did not withstand correction for multiple comparisons (Bonferroni).

Although all markers were genotyped together, the present study addressed two independent problems: (i) Is TD present in the *S-M-R* region of an unrelated population? and (ii) Are loci picked out of a genome-wide search from the HapMap CEU population according to a series of criteria including TD, sex-compensatory effects and LD, under TD in an independent population? While we could demonstrate the presence of TD in the S-M-R region also among Brazilian trios, we did not find unequivocal evidence for TD with regard to further loci.

The six markers analyzed for the *S-M-R* region were chosen as tagSNPs for a genomic segment spanning ~ 1.7 Mb, for which we had previously obtained evidence supporting the existence of TD in the CEU HapMap population. [14] Two of these SNPs showed evidence of TD: one telomeric (rs12530016), in high LD with markers mapping to the *SUPT3H* and *MIR586* loci, and one central SNP (rs2038765), in complete LD with at least 62 markers distributed over *SUPT3H* and the telomeric half of *RUNX2*, which is consistent with our previous findings. A list of SNPs tagged by rs12530016 and rs2038765, as well as a visual representation of their relative positions, are given in figure 3 and table 2. The sexes of the parents responsible for the observed TD seem to imply a difference between our present findings and those from the HapMap: while TD in the CEU population was largely a paternal effect, the distortion observed in the Brazilian population is an added effect from both sexes, even with mothers of European ancestry dominating in the case of rs12530016 (Fig. 2). The only explanation for this discrepancy which we regard as likely is the lack of statistical power in the previous HapMap analyses, [14] in which only thirty trios had been tested. Moreover, the relatively high number of trios available for the present study allowed us to dispense software-based phasing of haplotypes, which was necessary in the HapMap trios and is a possible source of bias. This means that we considered only undisputable transmissions (e.g. in the case of a heterozygous father and a homozygous mother) yet keeping high statistical power.

With the second analysis, we addressed a problem emerging from a genome-wide search for loci in the HapMap CEU population possessing several features (see methods) that can be taken as evidence of TD. Although some SNPs seemed to show evidence of TD (in particular, the loci *FSTL1*, neuroligin 1 (*NLGN1)*, huntingtin (*HTT*) and the zinc finger protein 667 (*ZNF667*) deserve attention in follow-up studies), none withstood correction for multiple testing. Having genotyped 48 markers, TD in any given SNP would have to yield a p-value lower than 10^{-3} in order to "survive" correction. Therefore, we cannot exclude the possibility that results are due to chance alone, even in suggestive cases as *FSTL1*: as in the case of the product of the *RUNX2* gene, the FSTL1 protein is involved with carcinogenesis, [45] and is predicted to interact with the bone morphogenetic protein 2 and the noggin precursor, [30] both essential for several developmental processes. Additionally, the *FSTL1* locus is associated with two sharp LD blocks in all populations (www.hapmap.org).

Whereas for most families of German, Polish, Ukrainian and Japanese ancestries the immigration to Southern Brazil is a relatively recent event (around 100 years ago), this is different for families with African, Portuguese, Italian and, of course, Amerindian ancestries. [41] A certain degree of ethnic admixture is therefore expected to be present in many families assessed here (Euro-Brazilians or not). Even so, genotyping of the *S-M-R* region still revealed evidence of TD for rs2038765 among all Brazilian families (Fig. 1, 75% of Euro-Brazilians). As expected, the restriction of the sample only to Euro-Brazilians (Fig. 2) yielded a picture more similar to the HapMap CEU population, with the emergence of one additional TD signal due to rs12530016. The fact that we are still able to detect TD, despite the assumed genetic admixture, reinforces its presence.

After performance of the genome-wide search for SNPs under TD that supported our sex-compensatory analysis, the HapMap entered a new phase with the release of HapMap3. This was an extreme improvement compared to HapMap2, as seven new populations were added to the project, and a new set of markers was genotyped, making inter-population comparisons easier to perform. However, it seems that many markers under TD have been preferentially excluded from the genotyping plates of HapMap3, possibly because of irregularities in Hardy-Weinberg equilibrium. As a result, TD is hardly detectable in HapMap3. Even so, mothers of a Maasai (MKK) population still show evidence of TD in the *S-M-R* region, while (HapMap3) CEU mothers exhibit strong evidence of TD on the rs4676781 marker within the *FSTL1* locus (results not shown).

The present study further substantiates the evidence that the *S-M-R* region is under TD not only in disease families, [7] but also in healthy individuals. [14] It remains unclear, however, what forces keep this region in a heterozygous state, and also what the biological significance of this phenomenon might be. As TD has sometimes been shown to relate to genotypes of maternal or paternal grandparents, [8,10] we believe that this kind of assessment is desirable for future studies that address TD within the *S-M-R* region. The 1000 genomes project (www.1000genomes.org), once concluded for family trios, is expected to shed new light on these questions. Whereas a whole-genome search for loci under TD is expected to face strong statistical obstacles due to the principal problem of multiple testing (see Santos et *al.* [14] for an extended discussion), the *S-M-R* region undoubtedly represents a set of genetic markers that are suitable to investigate not only the relationship between TD and LD, but also the reasons for continued TD despite the apparent absence of sex-compensatory effects.

Subjects and Methods

Study population

Our cohort was composed of 141 Southern Brazilian Family trios, from 49 unrelated families (49 mothers, 49 fathers and 141 children, totaling 239 individuals) with an average of 2.88 children per family. All subjects belonged to families recruited in the context of searching for compatible bone marrow donors for patients with a transplant indication. All family members were aged 18 or above, signed an informed consent allowing population studies and were residents of the city (or surroundings) of Curitiba, State of Paraná, Southern Brazil. Curitiba's ethnic composition is a result of immigration waves in the nineteenth Century from Europe (mainly from areas corresponding to today's Germany, Poland, Ukraine and Italy), as well as migration movements within Brazil (through which people of Portuguese, West African, Lebanese and Japanese ancestry settled in the region). [40-42] All individuals were asked to reveal information about their ethnic origin and, accordingly, 36 families were of European origin (Portuguese, Italian, German or Polish backgrounds). The other families were of African (3), Japanese (1), Amerindian (3), or unknown (6) ancestry. There was no conflicting self-classification within family members. We excluded subjects for which the genotyping of more than 50% of markers failed, as well as those showing more than 1 Mendelian error (pointing at the possibility of an individual not being a biological child of both parents in the trio), which led to the exclusion of 19 individuals (11 for the former and 8 for the latter reason). DNA was extracted from 250 µl of fresh buffy coat by a salting out method. [43]

Candidate SNPs

In order to assess the Brazilian sample for TD in the *S-M-R* region, we selected 10 SNPs from this segment for genotyping. Markers were selected as tagSNPs, and covered the area reported to be under TD [14]: the entire reading frames of *SUPT3H* and *MIR586*, as well as the telomeric half of *RUNX2*. The tagging procedure was performed as previously described. [14] Additionally, we selected a second group of SNPs from throughout the genome, in order to shed light on a further problem: if a locus is under TD, one would expect it to be within a region of strong and well defined LD (strong LD block with sharp borders), and the reciprocal compensation of paternal and maternal TD could be an explanation why loci under TD remain heterozygous in a population. With this in mind, we performed a genome-wide search for markers under TD in the HapMap CEU population (results not shown) fulfilling the mentioned criteria, and selected fifty SNPs, from which twelve were coding for a non-synonymous amino acid exchange, and five had been reported as transcript-specific. [44]

All candidate SNPs were genotyped using the SNPlex® Genotyping System (experiments outsourced to Geneservice™, www.geneservice.co.uk). After genotyping, SNPs had to fulfill the following criteria in order to be kept for further analysis: less than two Mendelian errors, minor allele frequency of at least 10%, lack of complete LD with a neighboring SNP (in order to avoid redundancy), and successful genotyping in at least 50% of individuals. 49 SNPs (six from the *S-M-R* region and 43 from the genome-wide approach) passed all inclusion criteria and were considered further. Since only six SNPs (that do not segregate independently from each other) were tested in the first part of the study, p-values were not corrected for multiple testing. The second set of SNPs consisted of 43 SNPs, mostly independent from each other, and these tests underwent therefore statistical multiple testing correction. A list of the 49 SNP IDs with other details is given in table 1.

Statistical analyses

To investigate the parental specific TD in the *S-M-R* region, we computed the standard TDT [39] for each SNP 3 times: We tested the father-specific, the mother-specific and the parent-unspecific components. Under the null hypothesis (Mendelian transmission, $\theta = 0.5$) the TDT statistic follows a χ_1^2 distribution. The test statistic is computed in analogy to the McNemar test with $(b-c)^2 / (b+c)$, where b and c are given in the contingency table described by Spielman and colleagues. [39] A modification of this test was used to quantify sex-compensatory effects in the genome-wide approach of this study. Sex-compensatory TD occurs if allelic transmission from both the father and the mother are non-Mendelian and compensate each other. The test statistic is computed with

$$\frac{\left((b_F + c_M) - \ (c_F + \ b_M)\right)^2}{b_F + c_F + b_M + c_M}$$

Thereby we test if $\theta_F < 0.5 < \theta_M$ or $\theta_M < 0.5 < \theta_F$. Indices stand for father ('F') and mother ('M'). The derivation of this test is shown in the appendix.

List of abbreviations

CEU: Utah residents with ancestry from Northern and Western Europe (HapMap population), LD: linkage disequilibrium, MKK: Maasai in Kinyawa, Kenya (HapMap population), nsyn: non synonymous, *S-M-R* region: region on chromosome 6p including the loci *SUPT3H*, *MIR586* and *RUNX2*, SNP: single nucleotide polymorphism, TD: transmission distortion, TDT: transmission/disequilibrium test, trans: transcript specific.

Acknowledgements

We are grateful to C. Backhaus for excellent technical assistance. The Berliner Krebsgesellschaft e.V. supported this study (*Ernst-von-Leyden* fellowship to PSCS and a grant to BU-Z).

Conflict of interest

The authors declare that no competing interests exist.

Appendix

For a biallelic marker with alleles X and Y, heterozygote fathers transmit X more likely and heterozygous mothers transmit Y more likely – or vice versa. According to the contingency table described by Spielman and colleagues, [39] b_F specifies the number of heterozygous fathers that transmit X, and c_F heterozygous fathers transmit Y. The same holds for mothers with b_M and c_M.

In terms of the Chi-square test, we have two observations:

$$O_1 : b_F + c_M$$
$$O_2 : c_F + b_M.$$

If there is no sex-compensatory effect, we expect to observe

$$E_1 = E_2 = \frac{b_F + c_F + b_M + c_M}{2}$$

We can apply the χ^2 test

$$\chi^2 = \sum_i \frac{(O_i - E_i)^2}{E_i}$$

$$= \frac{\left(b_F + c_M - \frac{b_F + c_F + b_M + c_M}{2}\right)^2}{\frac{b_F + c_F + b_M + c_M}{2}} + \frac{\left(c_F + b_M - \frac{b_F + c_F + b_M + c_M}{2}\right)^2}{\frac{b_F + c_F + b_M + c_M}{2}}$$

$$= \frac{\left((b_F + c_M) - (c_F + b_M)\right)^2}{b_F + c_F + b_M + c_M}$$

.

Since we have two observations, the test statistic follows a χ^2 distribution with one degree of freedom.

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Table 1. List of the 49 analyzed SNPs, the loci or region they belong to, and the sums of transmission events of both alleles from heterozygous parents to their offspring. nsyn: non synonymous coding SNPs; trans: transcript specific SNPs. Region 1 includes loci *ARHGAP29* and *ABCD3*; region 2 includes loci *GHR* and *SEPP1*; region 3 includes loci *GDF9*, *UQCRQ*, *LEAP2*, and *AFF4*; region 4 includes loci *PLAC9* and *ANXA11*; and region 5 includes the loci *KIF21A* and *ABCD2*.

***S-M-R* analysis**					
SNP ID	**Locus**	**All Brazilians**		**Euro-Brazilians**	
		Fathers	**Mothers**	**Fathers**	**Mothers**
rs12530016	*S-M-R*	12:19	19:31	10:19	10:23
rs13215618	*S-M-R*	21:24	20:24	19:21	19:17
rs2038765	*S-M-R*	11:23	20:37	7:16	17:30
rs6458419	*S-M-R*	17:20	14:21	15:17	13:15
rs1555681	*S-M-R*	23:19	20:11	20:18	15:10
rs6901786	*S-M-R*	19:25	15:20	14:17	15:17
Genome-wide approach					
SNP ID	**Locus**	**All Brazilians**		**Euro-Brazilians**	
		Fathers	**Mothers**	**Fathers**	**Mothers**
rs6426724	*USP48*	23:18	15:14	13:15	11:9
rs16825896 (nsyn)	*USP48*	23:26	14:12	19:16	9:10
rs6667223	*USP48*	26:27	19:13	22:16	12:11
rs11749 (nsyn)	*DNALI1*	19:23	19:17	11:17	15:12
rs12126162	*NEGR1*	19:14	22:20	15:12	16:16
rs6699841	*NEGR1*	16:22	24:22	14:16	23:19
rs1929132	Region 1	27:24	13:14	25:21	9:9
rs11165122	Region 1	24:23	10:7	20:19	9:7
rs12057415	*ABCD3*	24:26	10:9	21:22	10:8
rs1356424	*TSGA10*	20:14	25:23	16:11	21:18
rs1581249	*TSGA10*	21:17	26:20	13:11	23:17
rs12712041	*TSGA10*	16:20	25:21	13:13	22:19
rs2053724 (nsyn)	*DPP10*	22:20	21:20	16:15	15:17
rs1259294	*FSTL1*	18:26	23:21	14:23	17:19
rs4676781	*FSTL1*	15:23	9:23	13:22	7:19
rs11705889	*NLGN1*	25:24	16:21	24:23	12:16
rs11713253	*NLGN1*	24:32	18:28	21:24	14:22
rs1983060	*NLGN1*	28:19	30:20	25:16	26:14
rs362331 (nsyn)	*HTT*	28:17	21:11	20:14	18:10
rs2910864	Region 2	23:29	23:15	17:20	19:11
rs230819	Region 2	24:26	25:15	17:18	22:11
rs24705	Region 3	32:28	26:16	22:13	24:15
rs4705874	Region 3	28:26	28:19	19:12	26:18

rs2074506 (nsyn)	*VARS2*	5:3	4:12	4:3	4:10
rs9449444 (nsyn)	*IBTK*	18:21	19:19	11:14	14:13
rs10869500 (trans)	*OSTF1*	17:25	21:19	15:14	15:9
rs1054402	*PAPPA*	14:18	21:10	12:16	18:8
rs17302884	*PAPPA*	24:16	21:24	19:12	14:17
rs4837520	*PAPPA*	18:25	18:12	13:20	15:10
rs7071579	Region 4	20:16	37:21	15:11	24:15
rs1556897	Region 4	20:18	28:15	16:12	23:15
rs10769716 (nsyn)	*GVIN1*	24:21	21:9	16:12	18:9
rs17121881 (nsyn)	*AMICA1*	12:16	23:22	11:15	21:20
rs7968837	Region 5	22:13	20:14	18:10	19:11
rs7301705 (nsyn)	*OR6C74*	20:21	18:19	17:18	12:12
rs10083789 (nsyn)	*USP31*	17:15	13:16	14:11	11:16
rs9898390	*SMG6*	10:18	16:21	5:14	10:13
rs8074850	*SMG6*	28:28	14:15	23:24	9:8
rs4986764 (nsyn)	*BRIP1*	24:22	14:17	14:13	13:16
rs6505780 (trans)	*CEP192*	20:21	27:22	16:12	21:20
rs527839 (trans)	*CEP192*	24:20	24:22	18:13	20:20
rs3760849 (nsyn)	*ZNF667*	20:19	21:28	11:16	12:25
rs17738540 (nsyn)	*SEC14L4*	5:12	6:9	5:12	4:9

Table 2. List of markers tagged by each of the two SNPs found to be under TD among Southern Brazilian family trios (*rs2038765* and *rs12530016*).

SNPs tagged by rs2038765		**SNPs tagged by rs12530016**	
Marker ID	**Coordinate**	**Marker ID**	**Coordinate**
rs2023311	44786284	rs12530016	44974300
rs12205657	44788593	rs13206526	44924159
rs6924185	45125206	rs16869119	44922870
rs11961316	45137576	rs3799972	44931251
rs11970412	45145494	rs3799974	44932926
rs2038765	45184472	rs3823252	44918396
rs12198982	45185892	rs9296450	45061764
rs12193720	45211340	rs9472414	45054484
rs12209161	45212299		
rs10456542	45215557		
rs1324536	45224239		
rs12193812	45248739		
rs11965706	45249419		
rs17209636	45250495		
rs12206568	45260725		
rs12213735	45260826		
rs12198376	45261659		
rs10948212	45264911		
rs12205860	45265301		

rs10948213	45266493
rs6919813	45267155
rs6919998	45267267
rs6919873	45267314
rs6920046	45267409
rs12191566	45267980
rs12211519	45268508
rs10456122	45271035
rs17209678	45271579
rs12212745	45274236
rs10948214	45278444
rs12193030	45283188
rs2093900	45284661
rs10456543	45286885
rs12192890	45290412
rs12206561	45290787
rs17288250	45300000
rs17288257	45301054
rs6927213	45314261
rs10456549	45333739
rs12191262	45334994
rs4443508	45343930
rs10948220	45344632
rs4400216	45345933
rs4479922	45356349
rs11964690	45366046
rs10948223	45381899
rs12191751	45390089
rs17209741	45400416
rs12201555	45402378
rs12205523	45404596
rs12201899	45406182
rs10948226	45407152
rs12199256	45411471
rs17209769	45429183
rs11966878	45436243
rs12194628	45437354
rs12203466	45438241
rs10807321	45438952
rs17288320	45446681
rs12216308	45450283
rs12210230	45452832
rs17288327	45457855

Figure Titles and Legends:

Figure 1. TD plot of the 49 genotyped SNPs among all Brazilian family trios analyzed in this study (N = 122). For each marker, the pink, green and blue bars contain information regarding the corresponding loci, rs number and chromosome, respectively. Yellow vertical bars are used to indicate those for which TD was found with a p-value below 10^{-2}, while two horizontal lines indicate p-value thresholds of 10^{-2} and $10^{-2.5}$. Green circles correspond to TD measured among fathers, red for mothers, while black circles indicate parent-unspecific TD. Regions 1 to 5 contain more than one locus, and their codes are given in table 1.

Figure 2. TD plot of the 49 genotyped SNPs among Euro-Brazilian family trios (N = 98). For each marker, the pink, green and blue bars contain information regarding the corresponding loci, rs number and chromosome, respectively. Yellow vertical bars are used to indicate those for which TD was found with a p-value below 10^{-2}, while two horizontal lines indicate p-value thresholds of 10^{-2} and $10^{-2.5}$. Green circles correspond to TD measured among fathers, red for mothers, while black circles indicate parent-unspecific TD. Regions 1 to 5 contain more than one locus, and their codes are given in table 1.

Figure 3. Screen shot of the HapMap genome browser displaying the positions of each group of tagged SNPs within the *S-M-R* region. Red ticks are used for SNPs tagged by rs2038765 and blue for those tagged by rs12530016. The reading frames of *SUPT3H* and *RUNX2* can be seen under the tagSNP tracks. Relative exon and intron positions of alternative transcripts, as well as transcriptional orientations are indicated.

Fig 1

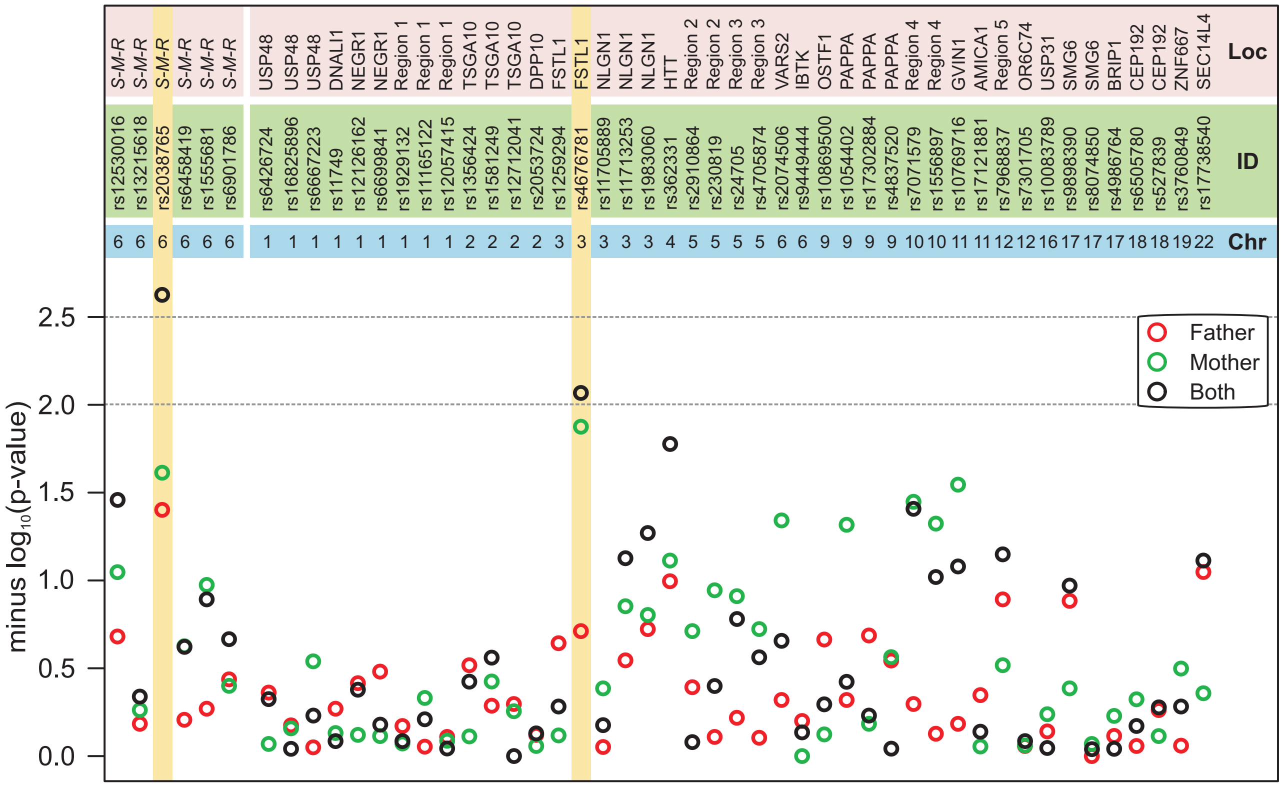

Loc
S-M-R S-M-R S-M-R S-M-R S-M-R S-M-R USP48 USP48 USP48 DNALI1 NEGR1 NEGR1 Region 1 Region 1 Region 1 TSGA10 TSGA10 TSGA10 DPP10 FSTL1 FSTL1 NLGN1 NLGN1 NLGN1 HTT Region 2 Region 2 Region 3 Region 3 VARS2 IBTK OSTF1 PAPPA PAPPA PAPPA Region 4 Region 4 GVIN1 AMICA1 Region 5 OR6C74 USP31 SMG6 SMG6 BRIP1 CEP192 CEP192 ZNF667 SEC14L4
ID
rs12530016 rs13215618 rs2038765 rs6458419 rs1555681 rs6901786 rs6426724 rs16825896 rs6667223 rs11749 rs12126162 rs6699841 rs1929132 rs11165122 rs12057415 rs1356424 rs1581249 rs12712041 rs2053724 rs1259294 rs4676781 rs11705889 rs11713253 rs1983060 rs362331 rs2910864 rs230819 rs24705 rs4705874 rs2074506 rs9449444 rs10869500 rs1054402 rs17302884 rs4837520 rs7071579 rs1556897 rs10769716 rs17121881 rs7968837 rs7301705 rs10083789 rs9898390 rs8074850 rs4986764 rs6505780 rs527839 rs3760849 rs17738540
Chr
6 6 6 6 6 6 1 1 1 1 1 1 1 1 1 2 2 2 2 3 3 3 3 3 4 5 5 5 5 6 6 9 9 9 9 10 10 11 11 12 12 16 17 17 17 18 18 19 22
minus log10(p-value)
2.5
2.0
1.5
1.0
0.5
0.0
Father
Mother
Both

Fig 2

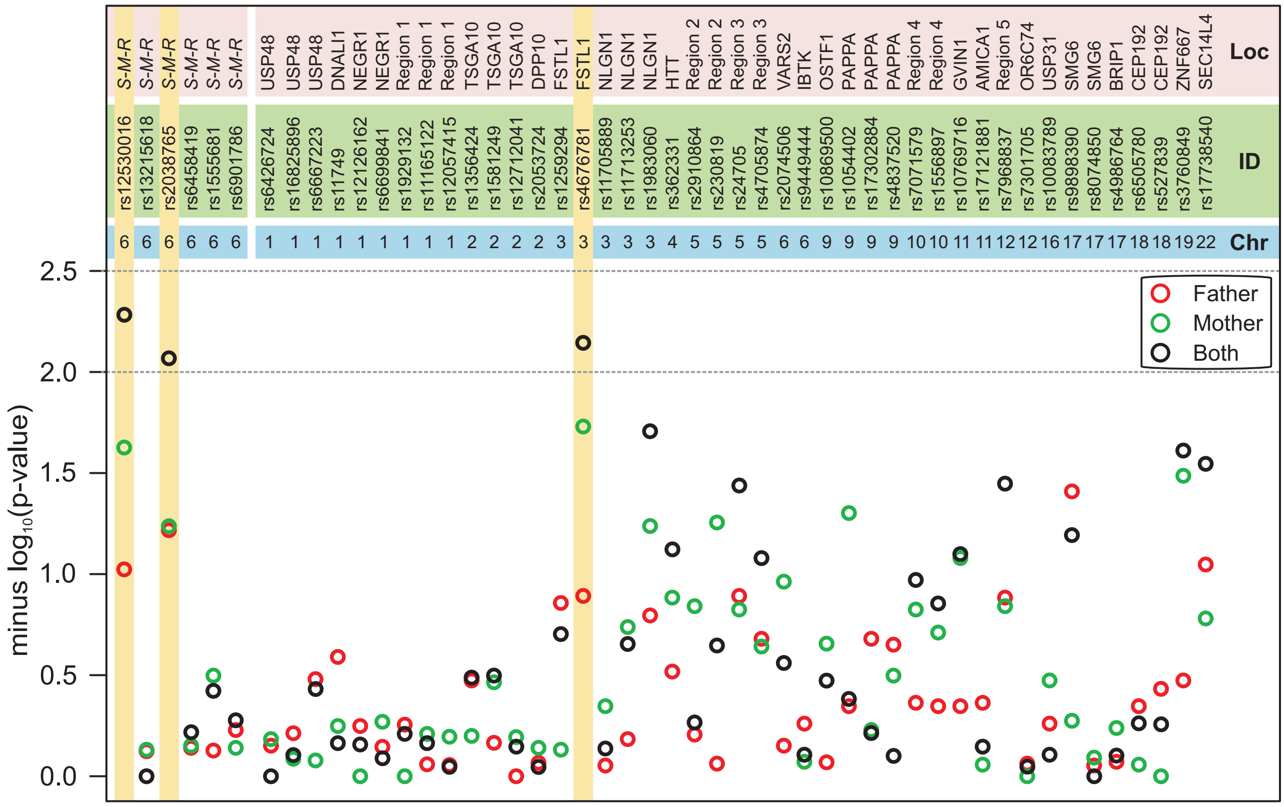
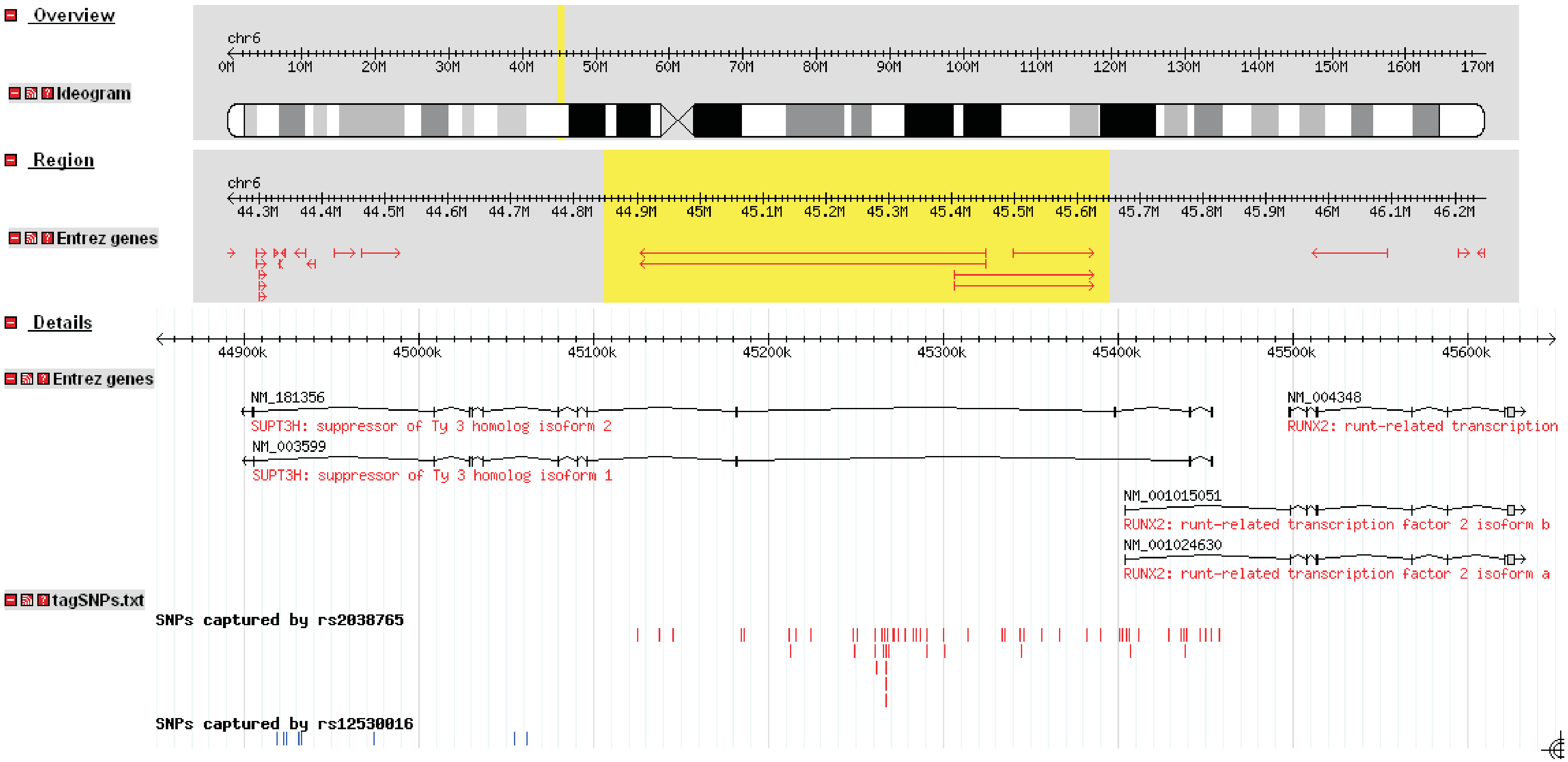

Loc
S-M-R S-M-R S-M-R S-M-R S-M-R S-M-R USP48 USP48 USP48 DNALI1 NEGR1 NEGR1 Region 1 Region 1 Region 1 TSGA10 TSGA10 TSGA10 DPP10 FSTL1 FSTL1 NLGN1 NLGN1 NLGN1 HTT Region 2 Region 2 Region 3 Region 3 VARS2 IBTK OSTF1 PAPPA PAPPA PAPPA Region 4 Region 4 GVIN1 AMICA1 Region 5 OR6C74 USP31 SMG6 SMG6 BRIP1 CEP192 CEP192 ZNF667 SEC14L4
ID
rs12530016 rs13215618 rs2038765 rs6458419 rs1555681 rs6901786 rs6426724 rs16825896 rs6667223 rs11749 rs12126162 rs6699841 rs1929132 rs11165122 rs12057415 rs1356424 rs1581249 rs12712041 rs2053724 rs1259294 rs4676781 rs11705889 rs11713253 rs1983060 rs362331 rs2910864 rs230819 rs24705 rs4705874 rs2074506 rs9449444 rs10869500 rs1054402 rs17302884 rs4837520 rs7071579 rs1556897 rs10769716 rs17121881 rs7968837 rs7301705 rs10083789 rs9898390 rs8074850 rs4986764 rs6505780 rs527839 rs3760849 rs17738540
Chr
6 6 6 6 6 6 1 1 1 1 1 1 1 1 1 2 2 2 2 3 3 3 3 3 4 5 5 5 5 6 6 9 9 9 9 10 10 11 11 12 12 16 17 17 17 18 18 19 22
minus log10(p-value)
2.5
2.0
1.5
1.0
0.5
0.0
Father
Mother
Both

Fig 3


Overview
chr6
0M 10M 20M 30M 40M 50M 60M 70M 80M 90M 100M 110M 120M 130M 140M 150M 160M 170M
Ideogram
Region
chr6
44.3M 44.4M 44.5M 44.6M 44.7M 44.8M 44.9M 45M 45.1M 45.2M 45.3M 45.4M 45.5M 45.6M 45.7M 45.8M 45.9M 46M 46.1M 46.2M
Entrez genes
Details
44900k 45000k 45100k 45200k 45300k 45400k 45500k 45600k
Entrez genes
NM_181356
SUPT3H: suppressor of Ty 3 homolog isoform 2
NM_003599
SUPT3H: suppressor of Ty 3 homolog isoform 1
NM_004348
RUNX2: runt-related transcription
NM_001015051
RUNX2: runt-related transcription factor 2 isoform b
NM_001024630
RUNX2: runt-related transcription factor 2 isoform a
tagSNPs.txt
SNPs captured by rs2038765
SNPs captured by rs12530016

6. Linkage Disequilibrium between Microsatellites and MHC loci

6.1. Summary

The HLA complex of a large number of individuals is routinely genotyped in forensic institutes and for tissue transplantation purposes. In these cases, a detailed knowledge of the LD patterns of the MHC in different human populations has not only a scientific or medical, yet also economic relevance. Because microsatellite markers are relatively easy (and also cheaper) to genotype than entire HLA alleles, a detailed knowledge of the LD profile between HLA alleles and microsatellites of the HLA complex seems very opportune. The study shown in the following article aimed at describing the LD patterns between three generally genotyped HLA loci (HLA-B, HLA-DQB1 and HLA-DRB1) and four known microsatellite markers harboured in the HLA complex. The results point at high confidence LD values for some allele combinations. While microsatellite genotyping cannot substitute direct MHC genotyping, it can clearly be used as an alternative to test repetitions in the case of incomplete genotyping or as a guide for ambiguity solving.

6.2. Publication

Sens-Abuázar C, Santos PS, Bicalho MG, Petzl-Erler ML, Sperandio-Roxo V (2009): MHC microsatellites in a Southern Brazilian population. International Journal of Immunogenetics 36:269-274.

DOI:10.1111/j.1744-313X.2009.00864.x

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7. Final Discussion and Conclusions

Apart from the results presented in chapter 4, all other analyses are part of manuscripts which have either been published or are currently under review. For this reason, the results of the analyses provided in chapters 2, 3, 5 and 6 have already been discussed within the manuscripts and will not be repeated here. In this chapter, I aim to outline the implications of the results from this thesis, and discuss those from chapter 4.

7.1. MHC-linked Olfactory Receptor Genes

All three studies addressing MHC-linked OR genes in this thesis (Chapters 2, 3 and 4) take advantage of online resources in order to complete the analyzed data and serve their argumentation. While the comparison of MHC-linked OR genes among different species (Chapter 4) was performed exclusively based on publicly available data, the two other studies focusing on these genes in humans were based on a combination of the use of electronic data (HapMap genotypings for chapter 2 and MHCHP resequencing for chapter 3) with laboratory-generated data (genotyping of Hungarian cohort in chapter 2, and sequencing of ten cell lines in chapter 3).

The main result of the study shown in chapter 2 was the linkage of a polymorphism in an OR gene to smoking, which is, to my knowledge, the first time a human behaviour was associated to an OR gene. One amino acid exchange on the *OR12D3* gene – found to be in strong LD with the ancient HLA haplotype HLA A1-B8-DR3 – was found to correlate with smoking habits in Hungarian women. This finding has possible implications for the early identification of individuals with increased risk to become smokers. However, the ethical aspects of such testing, and the risk that people carrying the relevant allele become subject of genetic discrimination (in cases of health or life insurances for instance), are apparently still unresolved [Mould et al., 2003; Hall et al., 2008; Aymé et al., 2008]. In addition, this study also provided a panel of 110 tagSNPs covering both MHC-linked OR gene clusters (Chapter 2, supplementary tables 1 and 2). These markers capture almost all of the variation present in the region for the HapMap CEU population with high confidence ($r^2 \geq 0.8$), and can be utilized as a resource in future studies. As shown before [Mueller et al., 2005; Xing et al., 2008], tagSNPs are expected to be transferrable among cohorts of related ancestry.

The analyses from chapters 2 and 3 intersect each other at the LD assessment of the region encompassing both MHC-linked OR gene clusters. While the first study (Chapter 2) builds on the high levels of LD in order to define tagSNPs and polymorphisms typical for different HLA haplotypes, the results of the second study (Chapter 3) indicate the presence of recombination between OR clusters and the MHC, as well as limited LD among functional OR gene polymorphisms. However, these results are not contradictory, since the HapMap cohort (ninety unrelated persons were included in the first study) is widely considered a population sample, with the corresponding high frequencies of common HLA haplotypes (eleven times A1-B8-DR3 and 5 times A3-B7-DR15, for instance), while this is radically different for the 18 cell lines assessed in the second study. Both, the ten Berlin cell lines as well as the eight MHCHP cell lines, were specifically chosen based on their MHC diversity [Ziegler et al., 1985; Volz et al., 1992; Ehlers et al., 2000; Horton et al., 2008].

Another intersection among analyses from chapters 2 and 3 regards the *OR12D3* polymorphism found to be in high LD with the haplotype HLA-A1-B8-DR3 and associated with smoking habits in the first study. Assessing the cell lines, the mentioned polymorphism was shown, for the first time, to be also linked to a different HLA haplotype: A32-B44-DR4, from the Caucasian cell line SSTO [Horton et al., 2008], indicating one probable recombination event between *OR12D3* and the HLA complex. Also here, this finding has no implications for population genetics, as the sample in which it was found is especially prone to unexpected combinations.

The work presented in chapter 4 was dedicated to the description and phylogenetic analysis of OR gene families linked to the MHC in all vertebrate species that have been sufficiently sequenced to date, and from which genomic data is available through internet-based databanks. The comparison of the structure and sequences from the MHC-linked OR gene clusters among fourteen vertebrates described here is, so far, the most comprehensive study regarding this region among different organisms.

The phylogenetic tree (Chapter 4, Fig. 4.1) suggests a common ancestry of all these loci among terrestrial vertebrates, as most gene families (branches with different colours in the tree) contain at least one gene from almost each species. This is different only for zebra fish, for which the products of these OR genes present sequences that are more similar to each other than to any other MHC-linked OR sequence from any other vertebrates, and therefore cluster in the phylogenetic analysis (Chapter 4, Fig. 4.1, black branches), suggesting an independent origin. If this is correct, at least two independent evolutionary events must have led to the linkage between OR genes and MHC genes, a fact that reinforces the biological relevance of the linkage between MHC and OR genes that is seen in all vertebrates so far, except dog and cat.

The apparently obligatory linkage of certain MHC framework genes with MHC-linked OR genes is a remarkable finding of this investigation. Even in the case of carnivores, the genes *GABBR1* and *MOG* "kept" linked to the OR genes after the presumed chromosomal segmentation that split these from the MHC [Yuhki et al., 2007]. In this context, the fact that the split between MHC and OR clusters coincides with a strong reduction in the number of functional OR loci, may be seen as suggestive of a functional relationship that may have lost importance after the split. Moreover, possible effects of domestication and high levels of inbreeding of these two species on the genomic "architecture" of the MHC and surrounding regions still need to be studied. This aspect is also relevant for the other domesticated animals that were assessed here: cow, pig and horse.

The framework gene *GABBR1* encodes the subunit 1 of the gamma-aminobutyric acid (GABA) B receptor (GABA$_B$), a seven transmembrane receptor of GABA, which is one of the main inhibitory neurotransmitters in the vertebrate central nervous system [Grifa et al., 1998; Goei et al., 1998]. This tissue is a probable background of interaction between the products of OR genes and *GABBR1*: an influence of GABA$_B$ in the behaviour of olfactory receptor neurons of zebra fish [Tabor et al., 2008], turtle [Wachowiak et al., 1999], frog [Duchamp-Viret et al., 2000], rat [Panzanelli et al., 2004], and mouse [Vucinić et al., 2006] has been reported.

Apart from being an inhibitory neurotransmitter, GABA (also through its receptor GABA$_B$) plays a central role in the sperm acrosome reaction [Hu et al., 2002; Burrello et al., 2004]. The known sperm specific expression of OR genes (discussed in chapter 3) indicates an additional probable background for interaction between OR genes and *GABBR1* products.

It must remain an open question whether the interaction of gene products, common regulation or epistatic effects of either OR gene or framework genes can explain the apparently obligatory genomic linkage observed between these loci. Interestingly, a search within the Drosophila genome browser (http://flybase.org/) revealed that even in this arthropod, which diverged from vertebrates approximately 990 million years ago [Blair Hedges & Kumar, 2003], the ortholog of human *GABBR1* (named "GABA-B-R1") is linked to at least four OR genes (Or33a, Or33b, Or33c and Or35a) on Drosophila chromosome 2L. Since OR genes from Drosophila and vertebrates are evolutionarily unrelated [Bargmann, 2006; Nozawa and Nei, 2007], apart from the fact that both are GPCR, this linkage is additionally suggestive of an important functional relationship between OR and the GABA receptor genes, which, for some as yet unknown reason, requires these genes to be physically close to each other. Considering that there are only ~ 60 OR genes in the Drosophila genome [Robertson et al., 2003], it is rather improbable that the linkage discussed here is due to chance alone.

The phylogenetic tree of the MHC-linked OR genes (Chapter 4, Fig. 4.1) indicates a common ancestry of all these loci, at least in terrestrial vertebrates, while the genomic structure depicted on the gene maps (Chapter 4, Fig. 4.2) reflects the close relationship among the different species within each assessed taxon. The case of the horse is, in this context, of special interest: Although they lack members of most mammal orders, the gene maps presented here corroborate the common understanding that horse, pig and cow (ungulates) are closely related, building a group that is apart from the one including cat and dog (Carnivora). In fact, the phylogenetic relationships between the orders Cetartiodactila (even-toed ungulates, including pigs, cows and whales), Perissodactila (odd-toed ungulates, including horses, tapirs and rhinoceroses) and Carnivora (including cats, dogs and hyenas) have long been subject of debate with contradictory results [Novacek, 1992; Graur et al., 1997; Cao et al., 2000; Murphy et al., 2001; Nishihara et al., 2006; Kitazoe et al., 2007], and have apparently not yet been resolved, although the monophyly of each of these three taxa is undisputed [Murphy et al., 2004].

According to one view [Murphy et al., 2001; Nishihara et al., 2006], Perissodactila and Carnivora are closely related to each other, building a sister group of Cetartiodactila. A phylogenetic tree based on these results is given in Fig. 7.1, panel a. In line with this notion, one recent study based on sequence analysis of genomic retroposon insertions suggests the name “Pegasoferae” for designating the alleged monophyletic clade that incorporates odd-toed ungulates, Carnivora and Chiroptera (bats) [Nishihara et al., 2006].

The competing view [Kitazoe et al., 2007] coincides with the classical “family trees” of mammals, which are based on the morphology of fossils and extant species, and suggests that Perissodactila and Cetartiodactila belong to one monophyletic taxon (here called Ungulata), a clade apart from Carnivora [Archibald, 1998; Shoshani and McKenna, 1998]. Fig. 7.1, panel b presents a phylogenetic tree based on these results. A recent molecular analysis reinforcing this notion [Kitazoe et al., 2007] considered abrupt changes of the rate of molecular evolution that are likely to have taken place at the time of the divergence of these three orders, around the Cretaceous-Paleogene extinction event [Rohde and Muller, 2005; Kitazoe et al., 2007]. This geological "moment", also known as the K-T (Cretaceous to Tertiary) boundary, is associated with abrupt climate changes and the mass extinction of plants and animals, with the sudden extinction of land dinosaurs as its best known feature [Labandeira et al., 2002; Rohde and Muller, 2005; Krug et al., 2009; Keller et al., 2009]. The Cretaceous-Paleogene extinction event is also associated with an accelerated rate of molecular evolution that had, so far, not been taken into consideration by other phylogenetic reconstructions based on molecular data [Kitazoe et al., 2007].

The recent studies supporting the two competing models for the phylogenetic history among the clades Cetartiodactila, Perissodactila, and Carnivora are based on diverging methodologies, but have the fact in common that they are built on sequence comparisons (nuclear DNA, mitochondrial DNA, amino acid, or retroposons). In contrast, the analysis presented here (Chapter 4) uses an alternative approach in order to provide strong support for the second view (horse, pig and cow belonging to a group apart of cat and dog): instead of considering many individual sequences of the different organisms, I compare the "genomic anatomy" of relatively long, syntenic genomic regions, considering the presence or absence of gene classes (OR, histones, MHC, framework), their positions relative to each other, as well as their transcriptional orientations. The presence of three orthologous histone clusters interspersed by three orthologous OR clusters in which the families, positions and transcriptional orientations are widely conserved, provides evidence for the stronger similarity of horses to Cetartiodactila than to Carnivora (Fig. 4.2, panels c and d, and Fig. 7.1, panel c).

Phylogenetic inferences are known to be more prone to bias related to convergent evolution when they are based on classical anatomic analyses, as compared to studies based on nucleic acid or amino acid sequences comparisons [Shoshani and McKenna, 1998]. On the other side, sequences are also susceptible to convergent evolution, as recently shown specifically for odorant receptor amino acid sequences [Hayden et al., 2010]. In that investigation, Hayden and collaborators analyzed around 50 thousand individual OR gene sequences in the genomes of fifty aquatic, semi-aquatic, terrestrial and flying mammals, and observed that sequence similarities between OR genes tended to depend more on the habitat and other ecological traits of the assessed animals than on the phylogenetic relationships between the species.

Since there is currently no genomic assembly available for most of the vertebrates assessed in that study, no assumption can be made about the role of MHC-linked OR genes. It is additionally interesting to observe that Hayden and co-workers [Hayden et al., 2010] assumes the notion that groups Perissodactila, Carnivora and Chiroptera under one single clade, apart from Cetartiodactila, as proposed by Nishihara and colleagues [Nishihara et al., 2006], to be part of the "consensus" phylogenetic tree of mammals.

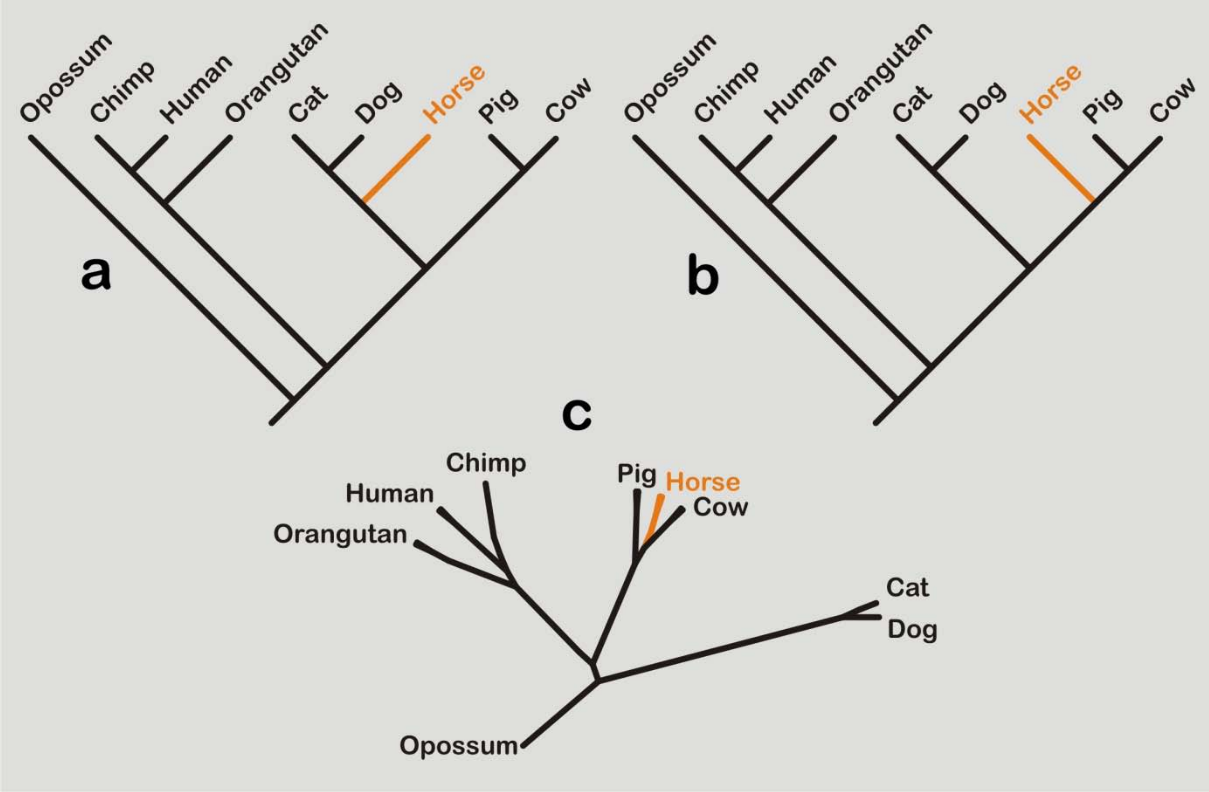

Fig. 7.1: Three phylogenetic trees of nine organisms which had their MHC-linked OR genes assessed (see Chapter 4). The tree on panel a represents the phylogenetic history of these clades as found by Murphy, Nishihara and colleagues [Murphy et al., 2001; Nishihara et al., 2006]. The tree on panel b represents the phylogenetic history of these clades as found by Kitazoe and colleagues [Kitazoe et al., 2007]. The tree on panel c represents the phylogenetic history of these clades as it can be inferred from the results regarding the genomic structure of organisms from Chapter 4. Since no assumptions of relationships between the four groups could be made, an unrooted tree was generated. The horse branches are highlighted, as they are subject to debate (see text).

In contrast both to classical anatomy and to sequence analysis, the approach described here represents a view into the genome from an intermediate distance. The size of the genomic region analyzed here, as well as the number of assessed species are certainly small, in order to provide steady evidence for resolving the debate around the evolutionary history of eutherians. For example, the series of chromosomal inversions as well as the expansion of the genes belonging to the OR2M family, which seem to have taken place specifically in the pig genome, make horse and cow seem more similar to each other than to pig (Fig. 4.2, panels c and d, and Fig. 7.1, panel c). This artificial similarity is probably related to the insufficient number of species and genomic regions assessed. It remains an interesting question whether the genomic architecture of segments as the one assessed here is also prone to convergent evolution. As for today, there is, to the best of my knowledge, no reason to believe so.

7.2. Linkage Disequilibrium and Transmission Distortion

In human genetics, LD is generally understood from a genealogical point of view: ancestral haplotypes have been broken and shuffled by means of recombination events throughout evolutionary history [Ardlie et al., 2002; Slatkin, 2008]. Because of population constraints such as bottlenecks and genomic drift, the linkage of some segments was maintained and, according to this view, also these will be shuffled through meiotic recombination with time.

Nevertheless, the assessment of TD described here (Chapter 5), can shed new light on the traditional understanding of LD creation and maintenance. Besides its possible biological implications that are discussed in the manuscripts, TD can be interpreted as one of the evolutionary forces shaping the LD landscape of the human genome, although it has apparently been widely ignored. If present in a given genomic region, TD will not only protect LD blocks from the “erosive” work of recombination by conserving LD blocks through the prevention of recombination events, but also by actively generating such LD blocks. If TD is an ethnically-specific phenomenon, as described in the two manuscripts of chapter 5, it should be expected to be an additional force responsible for the distinct LD profiles observed in different populations. Moreover, recombination hotspots are expected to be found flanking the LD block (which could be, in this case, called a TD block). The observed correspondence between LD and TD from the results of this thesis (see section 5.1, Fig. 1), as well as from other reports (see section 5.1, supplementary Fig. S5) support this point of view. This proposition remains a hypothesis which needs extensive further testing in order to be proven, but this idea has, to the best of my knowledge, not been suggested before.

Based on HapMap data, several studies found a high correspondence between the LD profiles among ethnically related populations [Mueller et al., 2005; Xing et al., 2008; Hu et al., 2008].

A detailed knowledge of LD data has practical consequences that include haplotype tagging (shown in chapter 2), lowering the number of markers in order to avoid statistical burdens linked to multiple testing (chapter 5), and increasing the efficiency of genotyping (chapter 6). The work with microsatellites presented in chapter 6 describes an approach that takes advantage of the LD present between relevant HLA alleles and neighbouring microsatellite loci within the MHC in order optimize the genotyping of HLA alleles (for example through resolving ambiguities and avoiding repetition of experiments). According to studies suggesting the transferability of tagSNPs among related populations [Mueller et al., 2005; Xing et al., 2008; Hu et al., 2008], the results shown in this article (Chapter 6) should also be valid for other populations of European ancestry.

Finally, taking the existence of several new internet-based resources into account, and the increased relevance that these databases and tools for data mining have reached for life sciences research within the last years [Buckingham, 2004; Krallinger et al., 2008; Hubbard et al., 2009; Sayers et al., 2010], the implementation of a web-based visual databank for integrating results from different studies relevant to TD on the human genome seems very appropriate. The works presented in the chapter 5 are initial steps towards this goal. In fact, one recently published study [Deng et al., 2009] builds on the published suggestion of the first of the two LD investigations presented here (Section 5.1), describing a first large scale map of transmission distortion (for all human autosomes), based on HapMap data. The article by Deng and colleagues [Deng et al., 2009] cites the paper presented in section 5.1 several times, and reports the use of a similar strategy to detect SNPs with evidence of TD.

The studies presented in the chapter 5 had two of the HapMap populations as a reference – CEU and YRI – because these consisted, in contrast to the others, of family trios and were therefore suitable also for investigating TD (see also sections 1.4.2 and 1.5). As briefly mentioned in section 1.4.2, the international HapMap project has recently reached a historical milestone with the release of genotyping data from the Phase III samples in November 2008. With this release (Public Release #26), the HapMap increased data richness through the inclusion of seven new populations to the original four genotyping panels, totalling eleven populations that can now be compared to each other in the context of genotypic and haplotypic variation. Three of the new populations (ASW, MEX and MKK) are structured in the form of family trios, as originally only the CEU and YRI panels. This allows scientists to perform a very reliable phasing of the genotypings, as well as to assess allelic and haplotypic transmission within families in a way that has never been possible before. Moreover, a series of new quality control (QC) checks for both samples and markers were introduced.

Nevertheless, when the investigation reported in section 5.1 was repeated using data from the latest HapMap release, we observed that TD was now completely absent in Chr6p, and generally lower in the rest of the genome. At first glance, this would suggest that the section 5.1 analyses are flawed, disappearing with the new HapMap data. However, three important facts suggest an alternative explanation:

1. Most SNPs exhibiting TD in the article (section 5.1) were not included in the Phase III genotyping panels. While around 50% of the SNPs from phase II "survived" QC and were kept in the Phase III release (taking the whole chromosome 6 as an example), this was the case for only ~25% of the SNPs from genomic areas with evidence of TD. As discussed in both TD studies (sections 5.1 and 5.2), TD seems to be an ethnicity-related property. Therefore, the stringency of QC may have led, unintentionally, to the systematic exclusion of markers that show skewed allele segregation rates, as they are less likely to fulfil Hardy-Weinberg expectancy thresholds at least in one of the eleven populations analyzed. As currently given in the HapMap page from the Sanger Centre (http://www.sanger.ac.uk/humgen/hapmap3/), SNPs had to pass QC apparently in all populations in order to be included in the Phase III. Another recent report [Fardo et al., 2009] discusses and reinforces the fact that the exclusion of SNPs not in Hardy-Weinberg equilibrium might be counterproductive (or unnecessary) in the context of disease association studies.

2. TD for markers residing in the *SUPT3H* area could undoubtedly be confirmed, in an independent population, through the results from the second TD study (section 5.2). Although all twelve *SUPT3H* markers assessed in that study had been taken from the HapMap Phase II SNP set, only four of these were kept and genotyped in the Phase III release.

3. The possibility that the reason for exclusion of markers shown to be under TD could have been duplicated identification numbers, mapping problems or other inconsistencies was checked and found not to be the case for any of the investigated markers, at least on Chr6p. The possibility that the fathers responsible for TD observed using the Phase II data were those excluded due to QC proceedings was also investigated, and again this

was not the case: TD can still be observed around *SUPT3H* among the CEU fathers belonging to family trios kept in the Phase III release.

It seems therefore that the criteria for SNP inclusion in the latest HapMap Phase III data set were too stringent, as SNPs with deviations from the expected transmission ratio like the one we reported (section 5.1) were preferentially excluded from the data set. As a consequence, it becomes very difficult to compare data using the Phase II release with those from Phase III in the context of allelic segregation distortion, since investigations focusing on TD with the Phase III data are expected to have their results artificially distorted. The current status of the HapMap website (http://hapmap.ncbi.nlm.nih.gov/) lists HapMap3 as the newest update of the HapMap resources, and unadvised users have no reason to prefer the earlier release, which is still available.

7.3. Concluding Remarks

The results from the analyses focusing on the MHC-linked OR genes carried out as part of this work contribute to the understanding of human variation regarding this genomic region, and have implications for the conservation of genomic structure among vertebrates. The region is characterized by high LD with the MHC, a feature used in the other studies as the basis for the selection of tagSNPs, genotyping optimization and the first TD analysis.

While most results obtained and discussed represent the descriptive answers to a the series of questions raised throughout this work, two new ideas (both originated from unexpected “side effects” of the analyses) that were suggested here remained without further testing, and will be subject of future work: the use of genomic anatomy for phylogenetic inferences, and the role of TD as a force shaping the LD landscape of the human genome.

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9. Oral Communications and Posters

1. **Santos PSC**, Beck S, Füst G, Horton R, Miretti M, Uchanska-Ziegler B, Ziegler A. Analysis of the Genomic Variation at the two HLA-linked Odorant Receptor Clusters in different extended HLA Haplotypes. 8th International Meeting on Human Genome Variation and Complex Genome Analysis. Hong Kong, China, Sep 2006 (Poster).

2. **Santos, PSC.** A Map of Transmission Distortion in the Human extended MHC (xMHC). 2nd Göttingen Workshop on Immunogenetics. Göttingen, Germany, Feb 2007 (Talk).

3. **Santos PSC**, Uchanska-Ziegler B, Ziegler A. A Map of Transmission Ratio Distortion on chromosome 6p. 12th Human Genome Organisation Meeting. Montreal, Canada, May 2007 (Talk and Poster).

4. **Santos, PSC.** Assessment of Transmission Distortion on Human Chromosome 6p in healthy individuals using tagSNPs. 16. Jahrestagung der Deutschen Gesellschaft für Immungenetik. Essen, Germany, Sep 2008 (Talk).

5. **Santos PSC**, Höhne J, Schlattmann P, König IR, Ziegler A, Uchanska-Ziegler B, Ziegler A. Assessment of transmission distortion on chromosome 6p in healthy individuals using tagSNPs. 10th Human Genome Variation Meeting 2008. Toronto, Canada, October 2008 (Poster).

6. **Santos PSC**, Kellermann T, Uchanska-Ziegler B, Ziegler A. Comparison of MHC-linked odorant receptor repertoires among 14 vertebrates. 23rd European Immunogenetics and Histocompatibility Conference (EFI). Ulm, Germany, May 2009 (Poster)

7. **Santos PSC.** Confirmation of Transmission Distortion in the Human Genome through SNP Genotyping. 11th Human Genome Variation Meeting 2009. Tallinn, Estonia, Sep 2009 (Talk)