University of South Bohemia

Faculty of science

Specification of the pheromone receptor repertoire of the domestic dog (*Canis familiaris*) at DNA level

Master thesis

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Annotation:
The aim of this study was to identify the complete genetic repertoire of potentially functional pheromone receptors in the genome of the domestic dog (*Canis familiaris*). Receptor sequences were identified on basis of similarity with already published sequences of vertebrate pheromone receptors. DNA of the identified V1R class genes was amplified from genomic DNA of Boxer breed and cloned into plasmid vectors suitable for subsequent research. Part of the work was also dedicated to implementing new cloning methods.

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České Budějovice, 20th April 2012

Bc. Petr Prášil

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1 Introduction

Chemical signaling is the oldest method of communication. It has evolved, from an extension of the chemical mechanisms that primitive organisms used to identify food and locate mates, to sophisticated signaling systems in higher animals. Molecules of biogenic chemicals serve as signals that are spread by direct contact with the recipient, diffusion or current flow mediating interspecific (allomones) or intraspecific (pheromone) communication. Since the sender's ability to manage the creation and transmission of chemical signals in time is limited, systems combining several chemicals in a specific ratio, called pheromone blends, mosaics or odor signatures evolved in higher animals.

Vertebrates produce signal chemicals in two basic ways: as waste metabolites in urine, faeces, saliva, or sweat formed by organs associated with digestion and reproduction, often providing useful information about gender, condition, reproductive phase and dominance status, or as products of secretory glands, whose excretion is usually associated with specific behaviors and social conditions mediating a clear signal. Odors can be released into the environment or on the sender's body in three basic ways: by passive exposure of secretory tissue to medium, as a side effect of other activities, or by specialized release behaviors (spraying, dispersal in hair or self-anointing, sender-generated flow of medium or deposition of marks). The purpose of odor signals is to attract or repel the recipient.

For the detection of chemicals, divergent olfactory systems with separate organs have evolved in vertebrates. One contains ciliary sensory cells for detection of airborne signals (olfactory organ) and the other contains ciliary or microvillous sensory cells for detection of waterborne signals (vomeronasal organ). Those organs are linked to different parts of the brain and serve for different purposes. Olfactory organ mainly detects a huge array of environmental chemicals, vomeronasal organ detects specific biogenic compounds, usually pheromones.

1.1 Vertebrate dual olfactory system

Odorant sensing of most amphibians, reptiles and mammals depends on two separate olfactory systems (see Figure 1.1). The main olfactory system (MOS) consists of receptors in the main olfactory epithelium (MOE), neurally associated with main olfactory bulb (MOB). The accessory olfactory system (AOS) consists of vomeronasal organ (VNO), neurally associated with separate accessory olfactory bulb (AOB) (Munger et.al., 2009). MOS responds to general odors and is mainly thought to detect food, predators and other ambient odors. AOS responds to narrow range of specific odors such as pheromones and is considered to mainly function in intraspecific communication, although it has been shown that some pheromones are detected only through the MOS. Also, some species are able to track prey using AOS input only (snakes). Behaviors relying on both systems are also known (Bradbury and Vehrencamp, 2011).

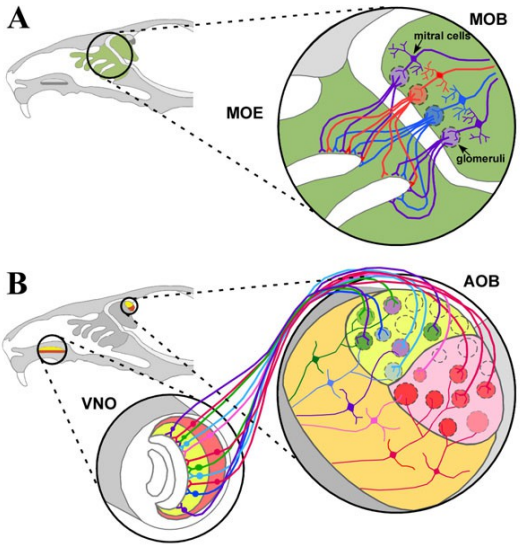


Figure 1.1: ***The mouse dual olfactory system:***
A *– neural connections from separate receptor types in MOE to dedicated mitral cells in MOB*
B *– neural connections from V1Rs* ***(yellow)*** *to multiple glomeruli in the anterior half of the AOB, from V2Rs* ***(red)*** *to multiple glomeruli in the posterior half of the AOB*
Adapted from web-book (Bradbury and Vehrencamp, 2011).

1.1.1 Olfactory epithelium and receptor proteins

Detection of chemical signals takes place in the olfactory epithelium. This epithelium contains chemosensory cells, and is covered by a mucus layer containing binding proteins important for transport of odorants as well as substances that protect the sensitive tissue and neurons against toxins and pathogens. The chemosensory cells are bipolar neurons expressing chemosensory receptor proteins, which belongs mostly to G protein-coupled receptors (GPCRs) (Bradbury and Vehrencamp, 2011).

Families of receptors in the MOE

Most of the receptors in MOE (~1000 different) is responsible for the perception of wide range of environmental chemical compounds and belongs to family of the olfactory receptor proteins (ORs) (Mombaerts, 2004). A second, much smaller family of receptors

present in the MOE is called trace-amine-associated receptors (TAARs) and encodes pheromone receptors sensitive to biogenic amines (Lindemann et al., 2005), (Liberles and Buck, 2006). Both ORs and TAARs are GPCRs. A third class of receptors sparsely distributed throughout the MOE of most vertebrates belongs to the guanylyl-cyclase-D (GC-D) system and detects several peptides and CO_2 (Young et al., 2007).

Families of receptors in the VNO

In the VNO there are mainly receptors of two classes, known as vomeronasal class 1 and class 2 receptors (V1Rs (Herrada and Dulac, 1997) and V2Rs (Matsunami and Buck, 1997)), which belong to the GPCR superfamily. These two classes differ in their amino acid sequences, in localization of their expression within the VNO, and also in neural pathways linking them with two different regions within the AOB. The last type of chemosensory receptors present in the VNO is called formyl peptide receptors (FPRs). They are present in restricted area of the basal VNO and are thought to detect pathogens or pathogenic states (Liberles et al., 2009).

1.1.2 Functional differences in main receptor proteins

As shown in Figure 1.2 all ORs, TAARs, V1Rs, and V2Rs are seven-transmembrane GPCRs embedded in the membranes of the cilia (ORs, TAARs) or the microvilli (V1Rs, V2Rs) of bipolar sensory cells. ORs and TAARs couple to $G\alpha_{olf}$ subunit, the V1Rs couple to $G\alpha_{i2}$ subunit and V2Rs couple to $G\alpha_{o}$ subunit (Spehr and Munger, 2009). Unlike other vertebrate chemosensoric cells, each V2R cell usually expresses two different V2R genes and some may also co-occur with a non-classical major histocompatibility complex (MHC) M10 or M1 class molecule and an associated molecule of β2-microglobulin, as was reported in rodents (Loconto et al., 2003).

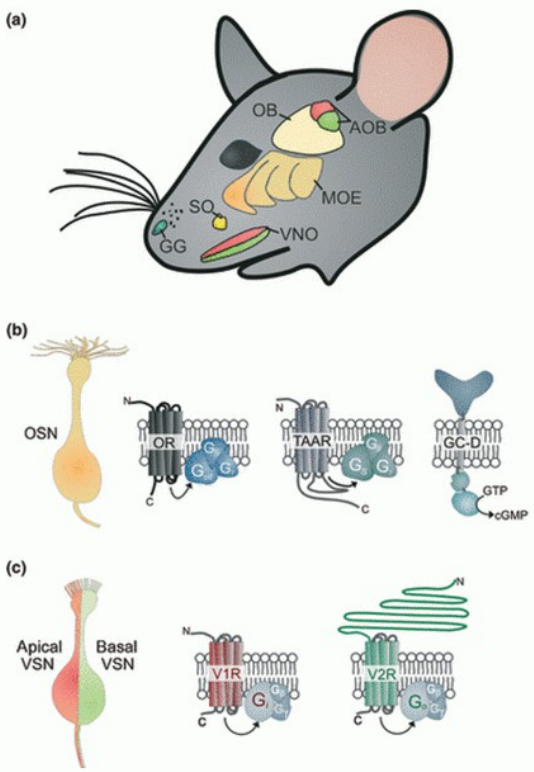


Figure 1.2: ***Olfactory receptors located in vertebrate MOE: OR, TAAR*** *– GPCRs with $G\alpha_{olf}$ subunit,* ***GC-D*** *– receptors with guanylyl-cyclace-D system*
Olfactory receptors located in vertebrate VNO: V1R *– GPCRs with $G\alpha_{i2}$ subunit,* ***V2Rs*** *– GPCRs with $G\alpha_{o}$ subunit,* ***FPR*** *– not shown but similar to V1R*
Adapted from review (Spehr and Munger, 2009).

1.1.3 Signal transduction

Each olfactory receptor interacts with certain chemical ligand. In GPCRs, ligand binding activates G protein-mediated signaling pathways that differ depending on the type of Gα subunit. All G protein signaling pathways have cyclic adenosine monophosphate (cAMP) as an effector, generated by adenylate cyclase (AC). AC catalyze the conversion of cytosolic adenosine triphosphate (ATP) to cAMP, and is directly stimulated by G-proteins of the $G\alpha_{olf}$ class in OR and TAAR receptors. In contrast, interaction with $G\alpha_{i2}/G\alpha_{o}$ subunits, present in V1R/V2R receptors, inhibits AC from generating cAMP. The level of cytosolic cAMP then determines the activity of cyclic nucleotide gated ion channels (in OR, TAAR expressing cells) as well as transient receptor potential (TRP) channels (in V1R, V2R expressing cells) (Munger et.al., 2009). Activation of these channels results in generating a nerve impulse, which is transduced by axons to the spherical glomeruli in the first olfactory processing region of the brain. Each glomerulus in the MOB receives axons only from cells expressing the same OR/TAAR receptor gene and connects to a dedicated mitral cell. VNO neurons from both families project to multiple glomeruli in the AOB, located in anterior part for V1Rs and posterior part for V2Rs (see Figure 1.1). Second-order neurons pass the information from each receptor cell type on to higher processing centers. Most chemicals are recognized by a combinatorial code of stimulated glomeruli (Dulac and Wagner, 2006).

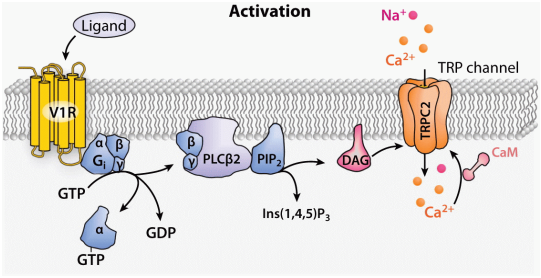


Figure 1.3: Activation of a V1R receptor: G protein–coupled phospholipase C signaling cascade results in opening TRP channel and depolarization of the membrane by Ca^{2+} entry. Adapted from review (Munger et.al., 2009)

1.1.4 Evolution of receptor genes

Available genomic information has allowed analyses of evolutionary relationships between chemosensory receptors. It was confirmed that V1R and V2R class of VNO gene families are surprisingly unrelated to each other, and neither one shares any sequence homology to the OR or TAAR families of genes apart from sharing heptahelical structure (Ryba and Tirindelli, 1997). While V1R genes share some sequence motifs with the T2R bitter taste receptors and with opsin genes (Adler et al., 2000), the V2R genes are closely related to glutamate and $GABA_B$ neurotransmitter receptors, as well as to the T1R sweet and umami taste receptors (Bjarnadóttir et al., 2005). TAAR receptors have been reported to

belong to the same multigene family as serotonin and dopamine receptors (Hashiguchi and Nishida, 2007). Thus the four main types of vertebrate chemoreceptor genes arose and diverged independently. Evolutionary patterns for all olfactory chemoreceptors shown on Figure 1.4 have been examined in seven tetrapods (mouse, rat, dog, opossum, platypus, chicken, and frog) and results strongly suggest that the functional profile of the VNO chemoreceptor repertoire evolves much faster than that of the MOS chemoreceptor repertoire (Grus and Zhang, 2008). Generally, the importance of the VNO and diversity of its genes is gradually declining among higher mammals (Grus et al., 2005), (Young et al., 2005) and (Young and Trask, 2007).

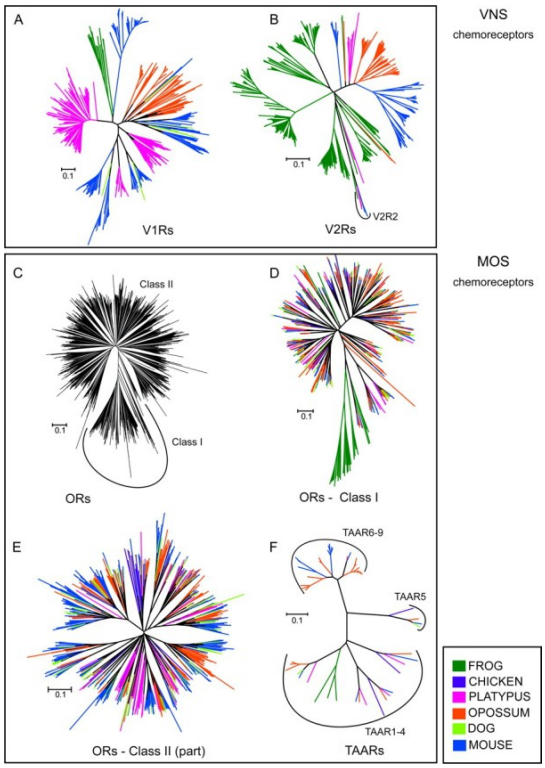


*Figure 1.4: Unrooted phylogenetic trees of all putatively functional frog, chicken, platypus, opossum, dog, and mouse **A:** V1Rs, **B:** V2Rs, **C:** ORs, **D:** Class I ORs, **E:** Class II ORs – from 2 randomly chosen families, and **F:** TAARs. The scale bars show 0.1 amino acid substitutions per site. Adapted from article (Grus and Zhang, 2008).*

1.1.5 Mammalian pheromones

Mammalian pheromones are species-specific organic compounds differing in size, shape, composition, and polarity, which induce innate physiological, social and reproductive behaviors like acceleration of puberty, pregnancy block, dominance/aggressive patterning, mating, mothering and lactation. These actions are usually started by variate patterns of compounds detected by pheromone receptors. TAAR receptors respond selectively to biogenic amines that can be found in various concentrations in urine and are related to a function of sex, status, and stress. V1R receptors respond specifically to small volatile water-soluble pheromones in urine such as sulfated steroids (Nodari et al., 2008), V2R receptors bind nonvolatile peptide and protein pheromone molecules in urine and tears (Mombaerts, 2004).

Chemical	Origin	Possible signal in female	Possible signal in male
2,5-dimethyl-pyrazine	female urine in, mice	suppression of estrous cycle	unknown
2-sec-butyl-4,5-dihydrothiazole	male bladder urine, mice	estrus synchrony, puberty acceleration	male agression, female attraction
2,3-dehydro-exo-brevicomin	male bladder urine, mice	estrus synchrony, puberty acceleration	male agression, female attraction
α- and β-farnesenes	male preputial gland, mice	puberty acceleration	male teritorial status, female attraction
2-heptanone	female or male urine, mice	estrus extension	unknown
6-hydroxy-6-methyl-3-heptanone	male bladder urine, mice	puberty acceleration	unknown
n-pentyl acetate	female or male urine, mice	suppression of estrous cycle	unknown
isobutylamine	male urine, mice	unknown	unknown
methylthio-methanethiol	male urine, mice	unknown	female attraction
major urinary proteins	female or male urine, mice	puberty acceleration, individual recognition	individual recognition, heterozygosity
MHC class I peptides	female or male urine, mice	unknown	attractiveness to different strains
Methyl p-hydroxybenzoate	female dogs in estrus	unknown	female attraction
dog appeasing pheromone	nursing mothers, canine	promoting calm, secure behaviour, establishing a bond with the mother	promoting calm, secure behaviour, establishing a bond with the mother

Sources: (Bradbury and Vehrencamp, 2011), (Goodwin et al., 1979), (Kim et al., 2010)

1.2 Vertebrate models for pheromone related research

1.2.1 Common model organisms

Fish

Fish serve as a models of ancestral state of pheromone sensing, before the diversification associated with the new medium (air) occurred. Even though fish do not possess a vomeronasal organ, sensory cells with receptors of all gene families types are present and form layers within the olfactory epithelium (Liberles and Buck, 2006). V1R and V2R cells are located in middle or surface epithelial layers of *Danio rerio*, *Takifugu rubripes* and *Arothron hispidus* (Shi and Zhang, 2007).

Amphibians

The move onto land by the amphibians is associated with a morphological subdivision between the VR and OR families of cells into separate but interconnected chambers of the nasal organ. Access of odorants occurs through grooves on the sides of the external nares, even when the main air-breathing chamber is closed while underwater (Petti et al., 1999).

Rodents

Most of the genes and principles associated with research pheromones were discovered in rodents, namely mice. Mice are a traditional laboratory animal, they are easily accessible, and also well known for pheromone-related behaviors. Sequencing of mouse and rat genomes and experimental accessibility contributed to numerous discoveries of pheromone receptor families and their orthologs (Herrada and Dulac, 1997), (Ryba and Tirindelli, 1997), (Matsunami and Buck, 1997) and (Liberles and Buck, 2006).

Apes

Monkeys, especially primates are an attractive animal model due to their evolutionary proximity to humans. Their potential for research of pheromones is limited, since many studies reported their repertoire of intact receptor genes significantly reduced. For example, the chimpanzee (*Pan troglodytes*) has only 3 functional TAAR genes and no intact V1R or V2R genes (Grus et al., 2005).

Old World monkeys similarly to marine mammals, birds, some bats and reptiles have completely lost the VNO and the associated genetic components (Young et al., 2005).

1.2.2 The dog as a model organism

Since the first database of sequenced genome of dog (*Canis familliaris*) was made publicly available (Lindblad-Toh et al., 2005) dog has been included in several evolutionary studies of pheromone receptor genes. Although the dog has never been the primary subject of this research, it was found that the dog pheromone receptor repertoire is somewhere between widely diversified rodent repertoires and highly reduced repertoires of primates (Grus and Zhang, 2008). Despite the relatively small number of receptor genes, the dog has pronounced pheromone-affected behaviors. Some commercial preparations (see 1.1.5) are marketed as a means of calming stressed animals or for inducing reproduction (Kim et al., 2010).

1.3 Cloning methods

1.3.1 Gateway cloning system

The Gateway system is a cloning system allowing efficient transfer of DNA-fragments between various plasmids. It takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) for directional site-specific recombinations between vectors and DNA segments containing recombination sites while maintaining the reading frame. This method employs two reactions, utilizing distinct combinations of enzymes and recombination sites:

BP recombination

- recombination of a DNA segment containing a pair of attB sites (attB1, attB2) with attP recombination sites (attP1, attP2) in a Gateway Donor vector
- results in creation of attL and attR sites (attB1 × attP1 → attL1 + attR1, attB2 × attP2 → attL2 + attR2)
- mediated by BP Clonase II
- can be used for creating Gateway Entry clones containing the gene of interest flanked by attL sites

LR recombination

- recombination of a DNA segment containing a pair of attL sites (attL1, attL2) with attR recombination sites (attR1, attR2), such as between a Gateway Entry clone (attL) and Gateway Destination plasmids (attR)
- results in creation of attB and attP sites (attL1 × attR1 → attB1 + attP1, attL2 × attR2 → attB2 + attP2)
- mediated by LR Clonase II
- typically used for moving genes from Gateway Entry clone vectors to destination plasmids, where they are flanked by attB sites

To facilitate effective selection between products of recombination, the ccdB gene inhibiting growth of *E. coli* (Bernard et al., 1993) is present between attP sites of Gateway

Donor vectors, such as pDONR-201. Only recombined constructs (lacking ccdB) can propagate in commonly used strains of *E. coli*. Gateway cloning system vectors and reagents are commercially available from Life Technologies, Inc. (Hartley et al., 2000).

1.3.2 Blunt-end cloning into *Pme*I restriction site

The principle of this method involves creating conditions for efficient cloning of blunt-ended polymerase chain reaction (PCR) products by combining in one reaction mixture two opposing processes: ligation and restriction digestion (by a rare-cutting restriction endonuclease, such as *Pme*I). The ligation of an insert to a vector destroys a unique restriction enzyme (*Pme*I) recognition sequence. Presence of *Pme*I in the ligation mixture limits self-ligation of the vector by restoring the linearized state of the cloning site (Liu and Schwartz, 1992). This principle is used in a commercial cloning system (PCR-Script, by Stratagene/Agilent Technologies), which relies on a proprietary (and correspondingly expensive) *Srf*I restriction endonuclease (Costa et al., 1994). Our method of cloning is similar to the commercial procedure, with adaptations for the *Pme*I restriction site and enzyme. Both *Pme*I and *Srf*I have octanucleotide recognition sequences (*Pme*I: GTTT| AAAC; *Srf*I: GCCC|GGGC) that are rare and occur on average once in 65000 bp, preventing digestion of the DNA being cloned. Ligation into the digested restriction site is accomplished by T4 DNA ligase.

1.3.3 TOPO TA cloning

This method exploits the ability of a single enzyme, topoisomerase I from the Vaccinia virus, to both cleave and rejoin DNA strands with high specificity and efficiency. Type I topoisomerases are known to catalyze site-specific recombinations in prokaryotes and fungi. Topoisomerase I from Vaccinia virus binds to duplex DNA at a specific motif (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a phospho-tyrosyl covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. This protein-DNA complex can subsequently be attacked with acceptor DNAs containing complementary single-strand 3′ overhangs which leads to ligation of the original and acceptors strands and releasing topoisomerase (Shuman, 1994).

This method is especially well suited for the TA cloning of DNAs amplified in Taq

polymerase mediated PCRs. The procedure exploits the non-template-dependent terminal transferase activity which preferentially adds a single deoxyadenosine to the 3′ ends of a double stranded PCR products creating single 3′ A-overhangs (Zhou and Gomez-Sanchez, 2000).

2 Goals of Work

According to recently published articles the mammalian pheromone sensing depends largely on three specialized receptor superfamilies: V1Rs, V2Rs and TAARs. Published results, as well as our own preliminary data indicated that the dog, despite its well known pheromone induced behaviors, possesses only a limited number of putative pheromone receptor genes. This makes the dog an attractive species for studies of mammalian pheromone recognition, potentially allowing complete characterization of the pheromone detection and recognition physiology, and understanding of the logic of the pheromone recognition systems. Thus, the main goals of the project were:

1. To identify all genes likely to encode functional pheromone receptors in the genome of the dog.
2. To design primers for amplification of the identified receptor coding sequences.
3. To isolate genomic DNA from a dog (the Boxer breed).
4. To amplify the DNA of the V1R family of receptors.
5. To clone the receptor sequences into plasmid vectors suitable for subsequent research by the novel method of two-photon polarization microscopy.
6. To implement and test new cloning methods.

3 Materials and Methods

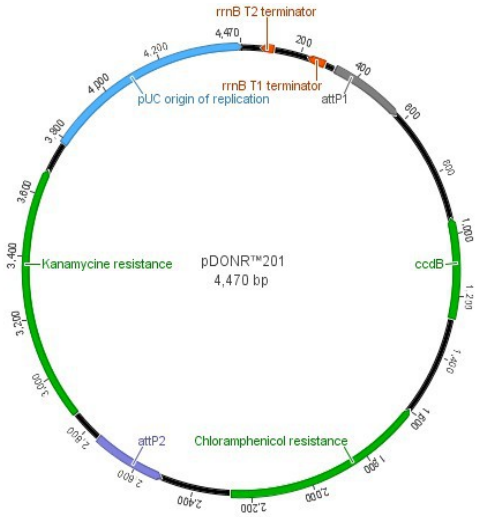
Laboratory equipment

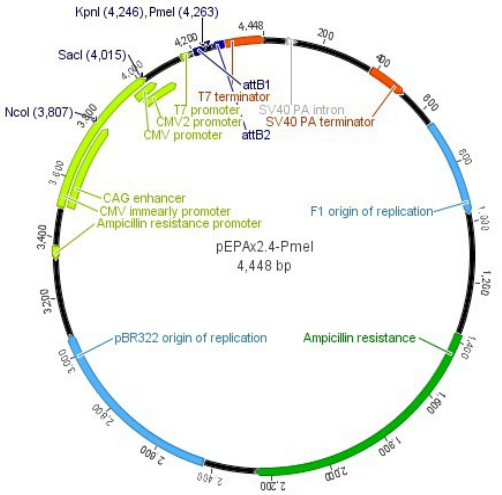
- Adjustable pipettes PIPETMAN Classic (Gilson) and Research vari (Eppendorf)
- Vortex mixer Classic (Velp Scientifica)
- Centrifuge Hettich MIKRO 200 (Hettich)
- Laminar flow box H.18 (Jouan)
- Incubator shaker Innova 44R (New Brunswick Scientific)
- Biological thermostat BT120 (Laboratorní přístroje Praha)
- Fridge RK 6331 W (Gorenje)
- Ultra-Low temperature freezer MDF-792 (Sanyo)
- Microwave oven MWMG 820E (Hyundai)
- Spectrophotometer Pearl (Implen)
- SpeedVac IR Micro-Cenvac NB503CIR (N-Biotek)

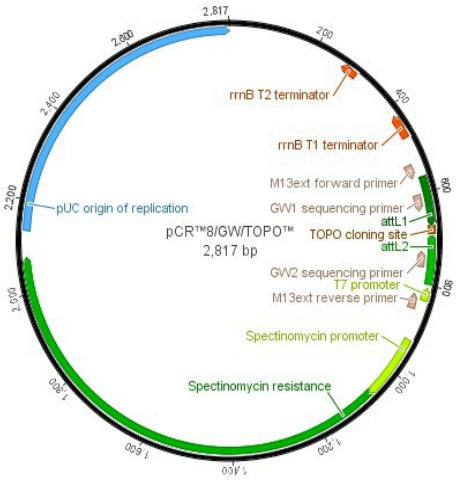
Software

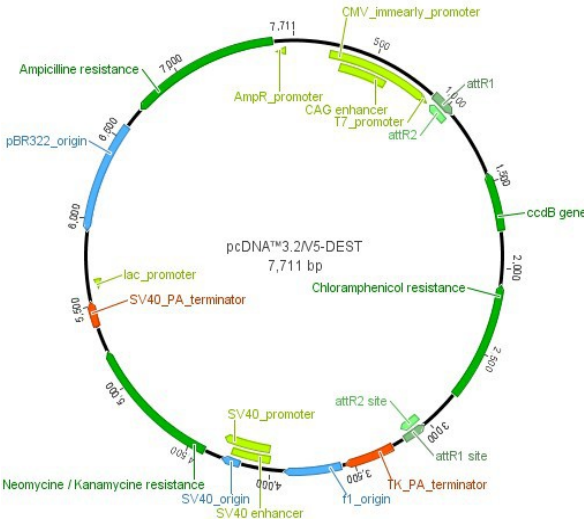
- Laboratory diary, notes – KeyNote NF (Marek Jedlinski, Daniel Prado Velasco)
- Image processing – IrfanView (Irfan Skiljan)
- Data processing – LibreOffice (the Document Foundation)
- DNA analysis, alignments, restriction mapping, chromatograms, phylogenetic trees – GeneTool Lite (BioTools Inc.), CLC Sequence Viewer (CLC bio A/S), Geneious (Drummond et al, 2011), MEGA 5 (Tamura et al., 2011)
- BioMath Calculators (Promega, http://www.promega.com/resources/tools/biomath-calculators/)

Vectors

pDONR201 – commercial vector (Invitrogen/Life Technologies)	
Main features:	
Gateway system entry vector with attP sites	
Recombination region containing a ccdB gene and chloramphenicol resistance gene	
Kanamycin resistance	
Primers for sequencing: seqF, seqR	

pEPAx2.4-PmeI – based on commercial vector pCMV-Sport6 (Invitrogen/Life Tech.)	
Main features:	
Gateway system destination vector with attB sites	
Capable for blunt-end cloning into a PmeI site	
Ampicillin resistance	
Dual expression (bacterial, mammalian)	
Primers for sequencing: T7prom, T7term	
Primers for analytical PCR: attB1, attB2	

pCR8/GW/TOPO – commercial vector (Invitrogen/Life Technologies)	
Main features:	
Gateway system entry vector with attL sites	
Capable for TOPO TA cloning – linearized vector with single 3'-T overhangs and a topoisomerase I attached	
Spectinomycin resistance	
Primers for sequencing: GW1, GW2	
Primers for analytical PCR were designed according to 3.1.2: M13ext-F: GTAAAACGACGGCCAGTCTTA M13ext-R: CAGGAAACAGCTATGACCATGT	

pcDNA3.2/V5-DEST – commercial vector (Invitrogen/Life Technologies)	
Main features:	
Gateway system destination vector with attR sites	
Recombination region containing a ccdB gene and chloramphenicol resistance gene	
Ampicillin and neomycin/kanamycin resistance	
Expression in mammalian cells	
Primers for sequencing: T7prom, V5reverse	

3.1 Identifying target DNA sequences and primer design

Materials

- BLAT search (Kent, 2002)
- BLAST (Altschul et al., 1990)
- Tmpred – transmembrane region prediction tool using a database of naturally occurring transmembrane regions (Hofmann and Stoffel, 1993)
- TMHMM – transmembrane region prediction tool based on hidden Markov model (Krogh et al., 2001)
- SVMtm prediction tool – a transmembrane region prediction tool based on support vector machines (Yuan et al., 2004)
- Primer3Plus – a primer design tool (Untergasser et al., 2007)

3.1.1 Searching dog genome for DNA sequences of pheromone receptors

From published articles and publicly available genetic databases we collected gene sequences coding known vertebrate pheromone receptors of the V1R class and the TAAR family. The V2R class of receptors was excluded from searching, because of published evidence (Young and Trask, 2007), (Shi and Zhang, 2007) that no intact V1R2 genes are present in genome of domestic dog. The V1R and TAAR DNA sequences from a wide range of vertebrates were translated into amino acid sequences and organized into FASTA files suitable for batch submission into sequence search engines (BLAT, BLAST). The FASTA files were used to search the genome of domestic dog (Lindblad-Toh et al., 2005). The identified dog genomic DNA sequences matching the V1R and TAAR sequences used for the search were collected and examined further. The genomic matches and their surroundings were analyzed for presence of open reading frames (ORFs), and the identified ORFs were investigated for presence of transmembrane domain coding regions by a combination of 3 different prediction tools (TMpred, TMHMM, SVMtm) each relying on different principles of transmembrane domain detection.

To determine the complete repertoire of putative pheromone receptor genes present in dog genome, we used the published V1R and TAAR DNA sequences from the following

vertebrates: *Bos taurus, Callithrix jacchus, Canis familiaris, Capra hircus, Danio sp., Equus caballus, Gasterosteus aculeatus, Gorilla gorilla, Homo sapiens, Macaca mulatta, Monodelphis domestica, Mus musculus, Ornithorhynchus anatinus, Ovis aries, Pan troglodytes, Peromyscus maniculatus, Pongo pygmaeus*, *Rattus norvegicus, Saimiri sciureus, Xenopus laevis* (Hashiguchi and Nishida, 2007), (Lindemann et al., 2005), (Rodriguez et al. 2002), (Strausberg et al. 2002), (Young et al., 2005) and more.

3.1.2 Design of primers for receptor encoding DNA sequences

Primers were designed in Primer3Plus from dog genomic sequence containing the target gene, along with 200 bp overhangs on each side. The suggested primer pair alternatives were subsequently analyzed by *in-silico* PCR (computer simulated PCR) to prove their specifity to the target gene, and to reduce the possibility of cross-amplification of unwanted genomic sequences.

3.2 Obtaining genomic DNA of a dog (Boxer breed)

Materials

- a dog puppy (6 weeks old female, Boxer breed, died from a kidney infection)
- DNeasy Blood & Tissue kit (Qiagen – Cat. No. 69504)

3.2.1 Selection of a suitable source of genomic DNA

Since the dog genome sequencing had been carried out on the Boxer breed, carrying out of the experimental work on the same breed was desirable. A dead female puppy of the Boxer breed was generously provided by the MVDr. Jan Dubský Veterinary Clinic in Prague.

3.2.2 Isolation of genomic DNA

The tissue used for genomic DNA extraction was from an ear tip, after hair removal. The DNeasy Blood & Tissue kit (Qiagen) and the manufacturer-provided Spin-Column protocol for animal tissues were used. DNA elution from the spin column was accomplished by washing the column twice with 200 μl of AE buffer.

3.3 PCR methods and DNA isolation

Materials

- Thermal cyclers – T3 Thermocycler (Biometra), TC-XP cycler (Bioer Technology), Cyclogene FPHC3CD (Techne)
- Phusion High-Fidelity PCR kit (FinnZymes – Cat. No. F-553S)
- Taq DNA Polymerase with Standard Taq Buffer (NEB – Cat. No. M0273S)
- dNTPs solution 10mM each (FinnZymes – Cat. No. F-560S)
- Primers specific for target genes or vector regions
- Millipore Milli-Q water
- Horizontal electrophoresis Model 710 (CLP) with PowerPac Basic (Bio-Rad)
- UV transilluminator TCP-20.MC (Vilber Lourmat) with Darkroom CN-TFX (Vilber Lourmat) and camera IP-115-SD (Vilber Lourmat)
- sharp scalpel
- 1× TAE buffer – 40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA
- Agrose for routine use (Sigma – Cat. No. A9539-50G)
- Ethidium bromide – solution 10 mg/ml
- 6× LoadingBuffer (NEB – Cat. No. B7021S)
- 100 bp or 1kb ladder (NEB – Cat. No. N3231S, N3232S)
- ImageJ(Abramoff et al., 2004)
- QIAquick gel extraction kit (Qiagen – Cat. No. 28704)

3.3.1 Amplification of V1R genes from genomic DNA

For amplification of sequences encoding pheromone receptors from the genomic DNA, a set of PCR mixtures containing primers specific for V1R genes (4.2) and high-fidelity Phusion polymerase (error rate 4.4×10^{-7}) was prepared:

Contents	**Final concentration in mixture**
Autoclaved Milli-Q water	up to final volume
HF PCR Buffer containing $MgCl_2$	1×
dNTPs	0.2 mM
Forward primer	0.5 µM
Reverse primer	0.5 µM
Template – Genomic DNA	50-250 ng
Phusion DNA polymerase	0.02 U/µl

The reactions were prepared on ice, in volume of 50 µl, and briefly centrifuged at 6000 rpm. In cases where occurred problems with amplification such as low yield or non-specific products were also dimethyl sulfoxide (DMSO) added to final concentration of 2-6%. The PCR conditions were as follows:

1. Initial denaturation at 98 °C for 60 seconds
2. 36 cycles of denaturation at 98 °C for 10 seconds, primer annealing at 61 °C for 30 seconds, extension at 72 °C for 30 seconds
3. Final extension at 72 °C for 10 minutes

The amplified PCR products were kept on ice until separated on 1% or 2% agarose gel by electrophoresis.

3.3.2 Touchdown PCR

For increasing amplification specificity, touchdown PCR was used. The PCR mixture was prepared as for standard PCR (described above) but higher annealing temperature was set and decreased by 0.25 °C at each cycle to permit the most specific primer binding to occur in early phase of the reaction and allow an efficient product amplification in later phase of the reaction. By using this method we achieved adequate yields even when the specific products were minor in the standard PCR.

3.3.3 Analytical PCR

Colony PCR was used as a cheap and fast way of analyzing bacterial transformants without isolating plasmid DNA. An inexpensive Taq polymerase was used, in small reaction volumes of 20 µl.

First, a bacterial suspension was prepared by dissolving the smallest visible amount of bacteria from a single discrete colony in 5 µl of Milli-Q water. Then, PCR mixtures were prepared on ice, as in section 3.3.1, but using Taq polymerase and buffer, and 2.5 µl of bacterial solution as a template. The reactions were briefly centrifuged at 6000 rpm. The PCR conditions were as follows:

1. Initial denaturation at 94 °C for 180 seconds
2. 36 cycles of denaturation at 94 °C for 45 seconds, primer annealing at 60 °C for 30 seconds, extension at 72 °C for 90 seconds
3. Final extension at 72 °C for 10 minutes

Amplified PCR products were kept on ice until analysis on 1% agarose gel.

3.3.4 Analysis of DNA by agarose electrophoresis

Preparation of gel

To prepare 1% agarose gels, 0.5 g of agarose was dissolved in 50 ml of 1× TAE buffer and brought to a boil in a microwave oven. After complete dissolution of the agarose the solution was cooled to approximately 60 °C and 2.5 µl of ethidium bromide stock solution was added. Then was the solution gently poured into the prepared gel tray with 10 or 20 slots and let solidify about 30 minutes. For better resolution of DNA fractions smaller than 1000 bp, 2% agarose gels were sometimes used, prepared by dissolving 1g of agarose in 50 ml of 1× TAE buffer. After complete solidification the gel was transferred to the electrophoretic bath filled to the operating volume with 1× TAE buffer and used for separation of DNA in an electric field.

Separation of DNA molecules by electrophoresis

DNA samples were mixed with loading buffer in ratio 5:1 and loaded into slots in gel, in volumes not exceeding 20 µl. Also, a DNA ladder was loaded into one slot, in volume appropriate to 0.5 µg per lane. Separation was run with constant voltage of 100 V, and

position of blue component of loading buffer was observed to estimate the position of 500 bp DNA fraction. In case of 2% gels the voltage was set to 50 V to avoid deformation of gel due to higher resistance and temperature. Gels were visualized on transluminator by UV light with wavelenght 312 nm and photographed in darkroom. Images were analysed in ImageJ.

Extraction of DNA from agarose gels

DNA fractions corresponding to the desired size were cut from gel by sharp scalpel and extracted and purified by QIAquick gel extraction kit. The instructions from the protocol using a microcentrifuge were followed, with the following exceptions:

- addition of isopropanol (step 5) was skipped for products smaller than 4000 bp
- binding DNA to QIAquick column (step 7) was carried at 10,000 rpm and the sample was applied twice by pipeting flow-though from first spin back to same column
- recommended washing with QG buffer (step 9) was performed
- to elute DNA from QIAquick column (step 13) we added 30 µl of elution buffer to the center of the QIAquick membrane and let stand for 5 minutes. To maximize yield this step was sometimes repeated.
- after elution, the concentration of products was measured with a spectrophotometer

3.4 Cloning methods

Materials

- Millipore Milli-Q water
- sterile TE Buffer – 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
- BP and LR Clonase II enzyme mixes (Invitrogen, Cat. No 11789-020, 11791-020)
- Gateway donor with attP – pDONR201 vector (Invitrogen – Cat. No. 11798-014)
- vectors with attB bordered *Pme*I cloning site – pEPAx1.4, pEPAx2.4
- Gateway destination with attR – pcDNA3.2/V5-DEST vector (Invitrogen – Cat. No. 12489-019)
- restriction endonucleases, buffers and bovine serum albumin (BSA) – *Pme*I (NEB, Cat. No. R0560S), *Nco*I (NEB, Cat. No. R0193S)
- T4 DNA ligase and buffer (NEB, Cat. No. M0202S)
- pCR8/GW/TOPO TA cloning kit (Invitrogen, Cat. No. K2520-20)
- QIAquick PCR purification kit (Qiagen – Cat. No. 28104)

3.4.1 Gateway system recombinations

BP recombination

The pDONR201-*Pme*I vector was prepared by BP recombination between the pDONR201 Gateway Donor vector and the pEPAx2.4-*Pme*I vector as the source of *Pme*I region. Before recombination, the pEPAx2.4-*Pme*I vector was linearized by *Nco*I digestion (outside the attB bordered region) in accordance with the enzyme manual.

To perform a BP recombination reaction the following mixture was prepared at room temperature, in volume of 5 µl, and incubated at 25 °C for 16 hours:

Contents	Final concentration in mixture
TE buffer, pH 8.0	up to final volume
Linearized attB vector	5 fmol/µl
Gateway attP donor vector	5 fmol/µl
BP Clonase II	1×

Enzymes were inactivated by Proteinase K added to concentration 0.2 µg/µl and a 10 minute incubation at 37 °C. Then mixture was used for transformation according to methods in section 3.5.

LR recombination

The reaction was used to transfer V1Rx DNA fragments from pCR8/GW/TOPO Gateway Entry clone into pcDNA3.2/V5-DEST expression vector. The procedure was as in BP recombination (see above), with the exception of vectors and LR Clonase II.

3.4.2 Blunt-end cloning into vectors adapted with *Pme*I site

Before cloning, the blunt-end cloning vectors were pre-digested by *Pme*I restriction according to conditions recommended by the manufacturer, in 1× NEBuffer 4 with supplement of 100 ng/µl BSA at 37 °C. To perform a cloning reaction, the following mixture was prepared at room temperature in volume of 20 µl and incubated at 20 °C for 16 hours:

Contents	Final concentration in mixture
Autoclaved Milli-Q water	up to final volume
NEBuffer 4 / T4 DNA ligase buffer	1× (see section 4.5.1)
Linearized vector for blunt-cloning	0.5 fmol/µl
PCR product	20-50 fmol/µl
rATP	0.5 mM
*Pme*I restriction endonuclease	0.5 U/µl
T4 DNA ligase	0.4 U/µl

In later experiments, additional post-cloning incubation for 1 hour in 37 °C with addition of half-standard concentration NEBuffer 4 was included to reduce background of

the self-ligated vectors by improving the efficiency of *Pme*I digestion. Enzymes were inactivated by heating the ligation reaction for 10 minutes at 65 °C, and transformation was performed according to methods in section 3.5.

3.4.3 TOPO TA cloning into pCR8/GW/TOPO vector

TOPO TA cloning requires inserts with 3' A-overhangs. For this purpose, Taq DNA polymerase is commonly used.

Addition of 3' A-overhangs to PCR products post-amplification

The reaction mixture was prepared on ice in volume of 50 µl:

Contents	**Final concentration in mixture**
Autoclaved Milli-Q water	up to final volume
Taq PCR Buffer containing $MgCl_2$	1×
dATP	0.2 mM
PCR product	7.2 fmol/µl
Taq polymerase	0,02 U/µl

Reaction was incubated for 20 minutes at 72 °C and proceed to purification.

Purifying inserts after addition of 3' A-overhangs

To purify PCR products after addition of 3' A-overhangs, QIAquick PCR purification kit was used, and instructions from the protocol using a microcentrifuge were followed, with following exceptions:

- binding DNA to QIAquick column (step 4) was carried out at 10,000 rpm and sample was applied twice by pipeting flow-though from first spin back to same column
- to elute DNA from QIAquick column (step 7) 30 µl of elution buffer was added to the center of the QIAquick membrane and let stand for 5 minutes
- after elution concentration of HF PCR products was measured with spectrophotometer
- for optimal efficiency, the subsequent TOPO reaction was performed as soon as possible

Performing the TOPO cloning reaction

The reaction mixture was prepared on ice, according to protocol by Invitrogen, and carried out in a volume of 3 µl. When subsequent transformation using electroporation was planned, concentration of salts in the mixture was reduced to prevent arcing.

Contents	**Final concentration in mixture**
Autoclaved Milli-Q water	up to final volume
HF PCR product with 3' A-overhangs	2.0 fmol/µl
Salt solution*	200 mM NaCl, 10 mM $MgCl_2$
TOPO vector	0.67 fmol/µl

* For electroporation mediated transformation concentration of salts was reduced to 50 mM NaCl and 2.5 mM $MgCl_2$.

3.5 Transformation of *E. coli*

Materials

- ligation mixture or TOPO cloning reaction (with low salts in case of electroporation)
- deep-frozen DH5 alpha, XL1-Blue, Mach1-T1R or TOP10 chemically competent *E. coli* cells, or XL1-Blue *E. coli* electrocompetent cells, in volume of 50 µl per 1.5 ml Eppendorf tube
- water bath or heatblock equilibrated to 42 °C
- Electroporator 2510 (Eppendorf) with cuvette holder chilled to 0 °C
- S.O.C. medium or LB medium prewarmed to room temperature
- Ampicillin to 50 mg/l, Kanamycin to 50 mg/l or Spectinomycin to 100 mg/l
- shaking incubator set to 37 °C and 150 rpm (225 rpm for Mach1-T1R or TOP10 cells)
- selective LB plates containing antibiotics of vector resistance
- QIAprep spin miniprep kit (Qiagen – Cat. No. 27104)
- restriction endonucleases and buffers – *Kpn*I (TaKaRa, Cat. No. 1068S), EcoRI (TaKaRa, Cat. No. 1040S)

3.5.1 Heat-shock mediated transformation

Chemically competent cells were removed from deep freezer and thawed on ice. 3 µl of ligation mixture or TOPO cloning reaction were added, mixed gently by vortexing, and incubated on ice for 30 minutes. Cells were subjected to heat-shock for 30 seconds at 42 °C without shaking and then placed back to ice for 2 minutes. To each tube 1 ml of LB medium or 250 µl of S.O.C. medium (for Mach1-T1R or TOP10 cells) were added and tubes were transferred to shaking incubator and shaken horizontally at 37 °C for 1 hour to allow expression of the Spectinomycin resistance gene. Transformation mix was spread on two prewarmed selective plates, in volumes of 20 µl and 280 µl. The inverted plates were incubated at 37 °C for 16 hours (12 hours for Mach1-T1R cells). Grown colonies were analyzed by detection methods described in section 3.5.4.

3.5.2 Transformation using electroporation

Electrocompetent cells were removed from deep freezer and thawed on ice. 3 µl of ligation or TOPO cloning reaction were added, mixed gently by vortexing and transfered to a 0.2 cm cuvette. Samples were electroporated by pulse of 2500 V, and 250 µl of room temperature S.O.C. medium was immediately added. The solution was transferred to a 1.5 ml Eppendorf tube and shaken horizontally at 37 °C for 1 hour to allow expression of the Spectinomycin resistance gene. The bacteria were plated on two prewarmed selective plates in volumes of 20 µl and 280 µl. The inverted plates were incubated at 37 °C for 16 hours. Grown colonies were analyzed by detection methods described in section 3.5.4.

3.5.3 Plasmid isolation

Single, overnight-grown colony was inoculated into 3 ml of prewarmed selective media in 15 ml Falcon tube and incubated at 37 °C for 12 hours (4 hours in case of Mach1-T1R cells) while shaking horizontally at 200 rpm. The bacterial suspension was used for isolation of plasmids with QIAprep spin miniprep kit. Instructions from the manufacturer's protocol using a microcentrifuge were followed, with the following exceptions:

- bacterial cells were harvested directly into 1.5 ml Eppendorf tubes by centrifugation of 1.5 ml of suspension at 8000 rpm and discarding flow-though followed by another spin with rest of suspension in same tube
- LyseBlue has been added to Buffer P1 and the cell suspension was in all steps inverted until the color changes indicating succesfull step completion occured
- binding DNA to QIAquick column (step 6) was carried at 10,000 rpm and sample was applied twice by pipeting flow-though from first spin back to same column
- the recommended washing with QG buffer (step 7) was performed
- to elute DNA from QIAquick column (step 10), 50 µl of elution buffer was added to the center of the QIAquick membrane and let stand for 5 minutes.
- after elution concentration of products was measured with a spectrophotometer

3.5.4 Analyses of transformants

Colonies growing on selective LB plates were analyzed for presence of the gene of

interest and its orientation in the vector.

Restriction digestion

After plasmid isolation, described in 3.5.3, restriction digestion was performed to determine the size of the isolated plasmid. Clones based on pEPAx2.4-*Pme*I vector were analyzed by digestion with *Kpn*I, using a single *Kpn*I site located 12 bp from the *Pme*I cloning. For clones based on pCR8/GW/TOPO vector, an EcoRI restriction site bordering the TA cloning site was used. Reactions were carried out at supplier recommended conditions. The products were analyzed by agarose gel electrophoresis.

Analytical PCR

In analytical PCR (see 3.3.3) we amplified the att-flanked region of vector. The size of the products allowed identification of clones in large numbers of samples. Colonies carrying a vector with the appropriate sized insert were further analyzed by PCR to determine the insert-to-vector orientation. This was done by a set of two PCR reactions, combining a vector-specific primer (see Vectors) with an insert-specific primers (see 4.1). Colonies with correct insert orientation were used for isolation of plasmids 3.5.3 and long term storage.

Long term storage of bacterial cultures

Selected colonies were inoculated and cultivated as described for plasmid isolation in section 3.5.3. Bacterial suspension was mixed in ratio 9:1 with DMSO, placed on ice and stored in -80 °C freezer for later use.

3.6 DNA Sequencing and sequence analyses

Materials

- DNA from PCR amplification or plasmid miniprep
- LGC Genomics account (http://www.lgc.co.uk/)
- UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/)
- software for chromatogram analysis, alignments and phylogenetic analysis

3.6.1 Preparation of samples

Plasmid DNA was prepared by minipreps (section 3.5.3), and PCR products by isolation of discrete bands from a gel (section 3.3.4). Primers used for sequencing of plasmids are listed in Vectors. PCR products were sequenced using their specific primers listed in section 4.2. DNA solutions (20 µl) of concentration at least 100 ng/µl for plasmid and 20 ng/µl for PCR products were sent to LGC Genomics company and sequenced by 3730xl DNA Analyzer (Applied Biosystems). To obtain reliable results, every V1R gene was sequenced in both directions, from at least two independent clones.

3.6.2 Analyses of sequencing results

DNA sequences obtained from sequencing were compared to dog genomic sequences. The sequencing chromatograms were analyzed if discrepancies were found. Confirmed differences between the sequencing results and genomic sequences were compared with a database of known SNPs (UCSC Genome7). Furthermore, the genomic sequence was verified by sequencing of PCR products. Sequencing results were used for creation of phylogenetic tree describing relative distance of identified V1R genes.

4 Results

4.1 Identified sequences of pheromone receptor genes

Out of 967 sequences submissed into sequence search engines (see 3.1.1) we retrieved 47 BLAT hits with highest similarity. Of those, 17 candidate pheromone receptor gene sequences were analyzed for presence of transmembrane domains. Prediction results varied among different prediction methods. Thus the decision for each gene was therefore established as a consensus among prediction results obtained using different methods.

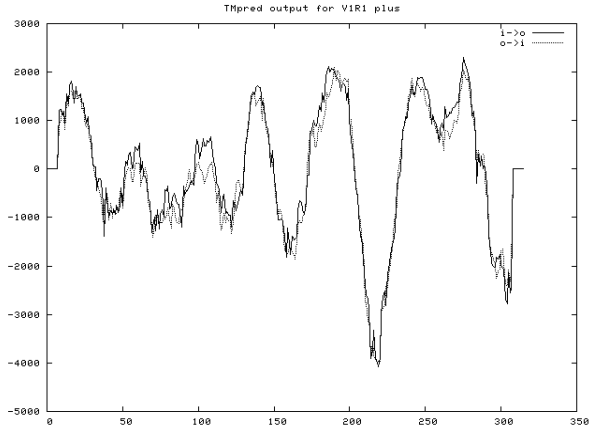


Figure 4.1: TMpred output for V1R1 gene: Transmembrane domains are shown as peaks >0

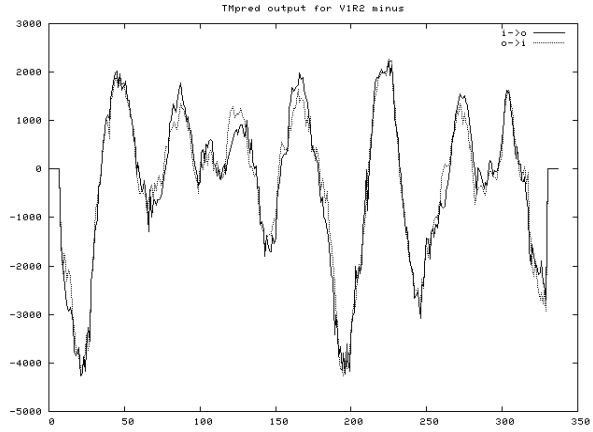


Figure 4.2: TMpred output for V1R2 gene: Transmembrane domains are shown as peaks >0

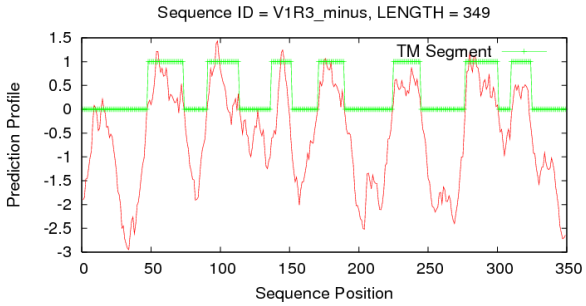


Figure 4.3: SVMtm output for V1R3 gene: Transmembrane domains are shown as peaks >0

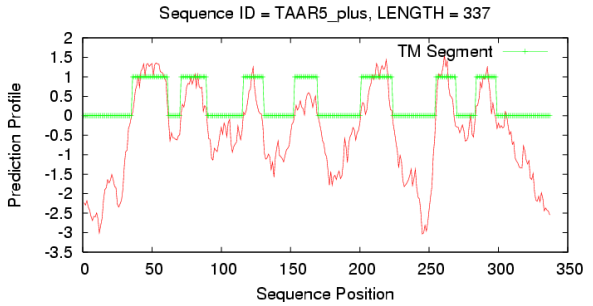


Figure 4.4: SVMtm output for TAAR5 gene: Transmembrane domains are shown as peaks >0

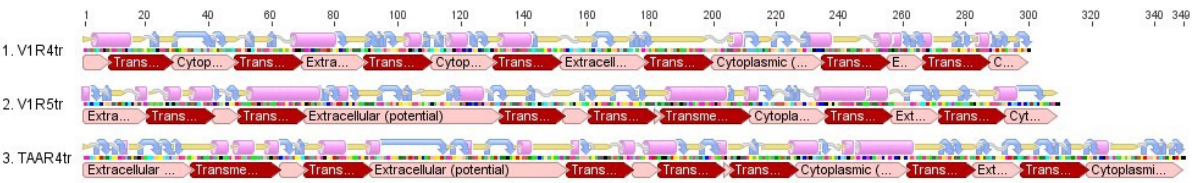


Figure 4.5: Geneious output (analogue of TMHMM – hidden Markov model) for genes V1R4, V1R5 and TAAR4: Predicted protein secondary structure and transmembrane domains (marked as Trans.)

This way we identified eight genes encoding V1R-like proteins with seven-transmembrane domains and many V1R pseudogenes. We also found 2 putative TAAR family genes and 2 pseudogenes (TAAR1 and TAAR2, data not shown):

Name	**Size**	**Chromosome**	**Strand**	**Span**
V1R1	951 bp	chromosome 1	positive	103754428-103755378
V1R2	1017 bp	chromosome 1	negative	105025886-105024870
V1R3	1011 bp	chromosome 1	negative	105062990-105061980
V1R4	900 bp	chromosome 1	positive	113492937-113493836
V1R5	927 bp	chromosome 20	negative	4168232-4167306
V1R6	1044 bp	chromosome 31	positive	39882639-39883682
V1R7	858 bp	chromosome 35	positive	24499396-24500253
V1R8	924 bp	chromosome 6	positive	16711025-16711948
TAAR4	1047 bp	unknown	positive	34175-35221
TAAR5	1014 bp	unknown	positive	41207-42220

Pheromone sensing of dog therefore depends only on 10 receptor genes, unless a new class of pheromone receptor is identified.

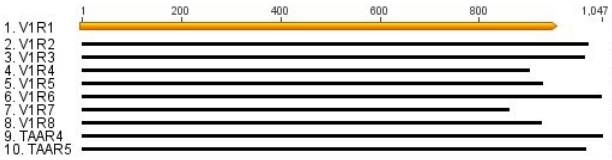


Figure 4.6: DNA sequences of identified pheromone receptors of dog: Comparison of length.

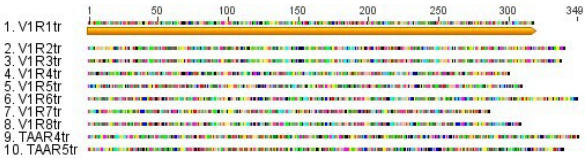


Figure 4.7: Protein sequences of identified pheromone rec. of dog: Comparison of length.

4.2 Primers for amplification of pheromone genes

Primers were designed in Primer3Plus and subsequently analyzed by *in-silico* PCR on dog genome. Best matched primer pairs with their detected weaknesses:

Gene	Primers	Tm
V1R1	F: ATGGCCACCGGGGATC	62.8 °C
	R: TCAGACTGAGCGACCCAG	58.5 °C
V1R2	F: ATGATTCGTGTGGCCCC	60.3 °C
	R: CTATTCATAGGATGGAGGTACTTTATG	57.9 °C
V1R3	F: ATGATTCATGTTGCCCCTTC	59.8 °C
	R: TTAACATGAGGGGATTTTATGTTC	58.4 °C
V1R4	F: ATGATTCTTGTTAACTTGGCATTTG	60.6 °C
	R: TCAGGAATGGCATAGCCTAGA	59.8 °C
V1R5	F: ATGAATAAAAATAACAAACCTTCCA	57.3 °C
	R: TCACAGAAAAATTACACATTTATTGTC	57.8 °C
V1R6	F: ATGATTTCCAGTGACATAATTTGG	59.2 °C
	R: TTATTTATTTATGATAGTCACACAGGGA	58.5 °C
V1R7	F: ATGGTTTCAAACAATATCAAGGG	59.2 °C
	R: TTAATGAATGTGCCAACATTTAGC	60.3 °C
V1R8	F: ATGATTTCCAGTGACATAATTTTTG	58.4 °C
	R: TTAAAAGAGAAGCCTTTTTCTTGC	59.6 °C
TAAR4	F: ATGATGAATTCACCTGACATTTACAACCCTCC	59.1 °C
	R: CTAAGTATCTGCAGGATATAGGTTTAGGGTAGAAG	57.4 °C
TAAR5	F: ATGGACACTGTCCTCAGCCAAGGT	59.6 °C
	R: TCATTCTTGGTACAAATCAATAGTGGGTGTCC	58.9 °C

It was detected that primers for V1R7 gene amplify also its known pseudogene and primers for V1R1, V1R3, and V1R6 could non-specificly amplify other pseudogenes.

4.3 Genomic DNA from a Boxer breed dog

Genomic DNA was extracted from 17 mg of ear tip tissue, and analyzed by agarose electrophoresis. DNA solution (400 µl) with concentration about 300 ng/µl was obtained.

4.4 Amplification of V1R class genes

PCR reactions were initially performed according to the procedure in section 3.3.1 and analyzed by gel electrophoresis (Figure 4.8). Products of reactions providing sufficient amount of DNA were isolated and used for cloning. This procedure was repeated several times for each gene to provide enough DNA for our cloning attempts. The typical yield after separation and extraction from gel (section 3.3.4) was 25-50 ng/µl. PCR reactions to amplify the V1R1 and V1R6 genes required additional optimization steps described below.

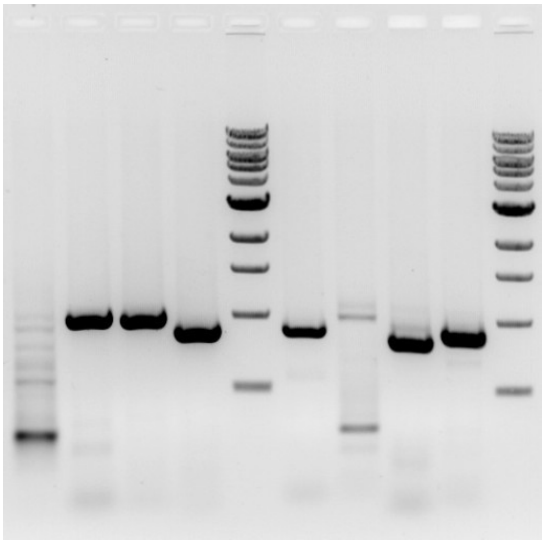


Figure 4.8: PCR with primers to V1Rx genes and genomic DNA as a template:
Slots are in following order: V1R1 | V1R2 | V1R3 | V1R4 | 1 kb ladder | V1R5 | V1R6 | V1R7 | V1R8 | 1 kb ladder

4.4.1 Optimization of amplification of V1R1 and V1R6 genes

Optimization of DMSO and $MgCl_2$ concentrations and annealing temperature allowed amplification of the V1R6 gene. Addition of 4% DMSO in combination with 62 °C annealing temperature provided 50 µl of DNA solution with a concentration 26 ng/µl after extraction. Touchdown PCR method 3.3.2 with annealing temperature 68 °C and 0.25 °C increments combined with 6% DMSO allowed amplification of the V1R1 gene, although as a minor product. After separation and extraction from gel we obtained 300 µl of DNA solution with concentration 14.3 ng/µl.

4.5 Cloning

The following cloning strategies were implemented in order to clone all eight dog V1R genes: *Pme*I cloning into an expression Gateway vector, *Pme*I cloning into a cloning Gateway vector, and TOPO TA cloning into pCR8/GW/TOPO vector.

4.5.1 *Pme*I cloning into an expression Gateway vector

Cloning of PCR products directly into an expression vector by blunt-end ligation into the *Pme*I restriction site seemed to be a fast way to create expression constructs by simplifying the cloning process in a single step. For this purpose a versatile vector pEPAx2.4-*Pme*I was chosen, combining blunt-end cloning capability with the gateway system and the expression capability in both bacterial and mammalian cells.

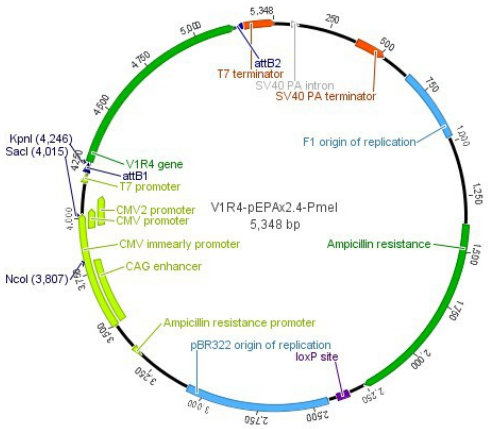


Figure 4.9: V1R4-pEPAx2.4-PmeI – Expression of V1R4 gene in bacterial and mammalian cells

However, despite previous successful use by the supervisor, even after extensive optimizations (different temperatures, reaction times, DNA concentrations, buffer compositions, and enzyme concentrations) and many cloning attempts (>20), the method allowed cloning only of the V1R4 gene (plasmid with gene Figure 4.9).

4.5.2 Creation and *Pme*I cloning into a Gateway cloning vector

Difficulties with direct cloning into an expression vector led us to create a simplified cloning vector for more efficient blunt-end *Pme*I cloning which can be used to transfer of genes by Gateway system (3.4.1). To introduce a *Pme*I restriction site into a cloning vector, we performed a BP recombination between pDONR201 Gateway donor and pEPAx2.4-*Pme*I vector. The resulting vector (pDONR201-*Pme*I) differs from pDONR201 in substitution of the chloramphenicol resistance and ccdB genes by 80 bp sequence with *Pme*I and *Kpn*I restriction sites.

The pDONR201-*Pme*I vector was used for blunt-end cloning, but with little to no improvement in cloning efficiency (7 cloning experiments, no positive clones). The work was further complicated by spontaneous mutation and instability of the pDONR201-*Pme*I construct, demonstrated by restriction analysis (Figure 4.10) and DNA sequencing. Repeated pDONR201-*Pme*I preparations did not alleviate the problems. This led us to abandon the method of *Pme*I cloning and to switch to TOPO TA cloning for cloning the dog V1R genes.

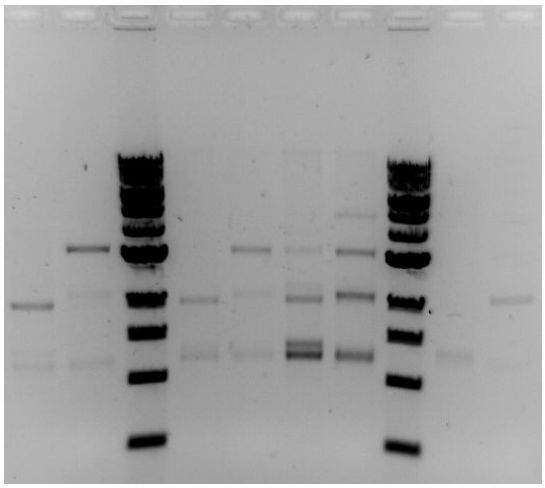


Figure 4.10: Restriction analysis of pDONR201-PmeI plasmids, isolated from E. coli cultures derived from single ancestral colony (shown as a control in slots 9-10), by digestion in PmeI site with 1 kb ladder.
Plasmids from colonies after transformation (1-2), after re-plating on LB selective plates (4-5), after 3 days cultivation on selective plates (6-7)
First from every pair is plasmid before digestion, second after digestion by PmeI enzyme.

4.5.3 TOPO TA cloning into pCR8/GW/TOPO vector

TOPO TA cloning allowed rapid cloning of V1R2, V1R3 and V1R5 genes, although the transformation efficiency was lower than expected (see example Figure 4.11). Cloning of V1R1, V1R6, V1R7 and V1R8 genes required optimization of the cloning procedure (additional DNA concentration and purification steps prior to and after addition of 3'A-overhangs), reaction conditions, and DNA concentrations. Positive clones, bearing inserts in the desired orientation, were identified by analytical PCR, as described in 3.1.2.

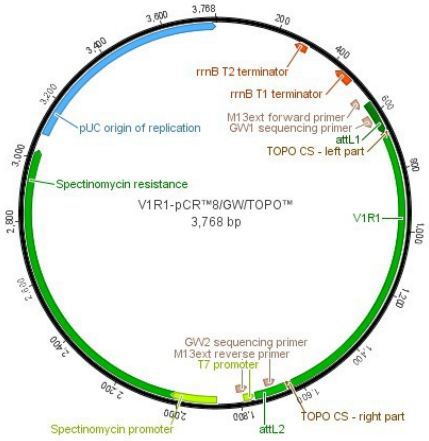


Figure 4.11: Example of construct obtained by TOPO TA cloning: V1R1-pCR8/GW/TOPO

4.6 Analyses of sequencing results

All V1R genes amplified from genomic DNA were sequenced after cloning and compared with the dog database genomic sequences. The V1R2, V1R3, V1R4, V1R5, V1R6 and V1R8 genes were 100% identical to database. These comparisons identified three single nucleotide polymorphisms (SNPs) in V1R1 and V1R7 genes not listed in the dog genome

database. The V1R1 and V1R7 SNPs are described below.

V1R1

- found SNP on position 509/103754936, present in heterozygous state (only the variant allele cloned Figure 4.12)
- SNP-509 in V1R1: Ser170 to Leu170 (Figure 4.13)

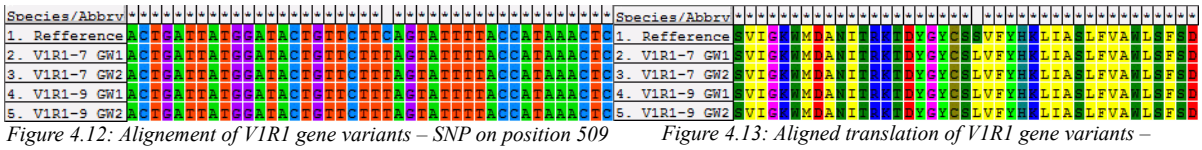


Figure 4.12: Alignement of V1R1 gene variants – SNP on position 509

Figure 4.13: Aligned translation of V1R1 gene variants – SNP-509 shifts serine and leucine

V1R7

- found SNPs on positions 83/24499478, 242/24499637, both present in heterozygous state Figure 4.16 (both variants cloned Figure 4.14)
- SNP-83 in V1R7: Thr28 to Asn28 (Figure 4.15)
- SNP-242 in V1R7: Ser81 to Trp81 (Figure 4.17)

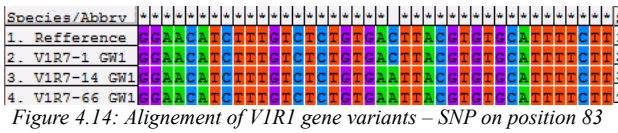


Figure 4.14: Alignement of V1R1 gene variants – SNP on position 83

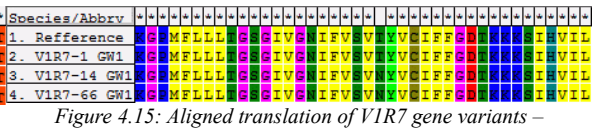


Figure 4.15: Aligned translation of V1R7 gene variants – SNP-83 shifts threonine and asaparagine

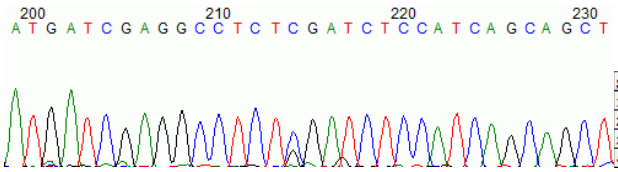


Figure 4.16: V1R7 – SNP on position 242

Figure 4.17: Aligned translation of V1R7 gene variants – SNP-242 shifts serine and tryptopham

Also 5 pseudogenes related to the V1R7 and not identified in the genomic database gene were cloned (Figure 4.18).

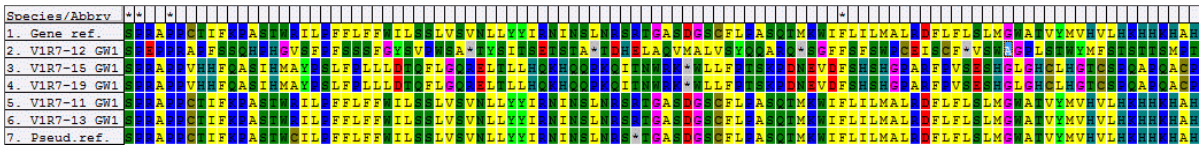


Figure 4.18: Five new variants of V1R7 pseudogenes translated to proteins with reference sequences of V1R7 gene and known pseudogene

Alignment of protein sequences of V1R genes reveals homology in regions which correspond to transmembrane domains Figure 4.19.

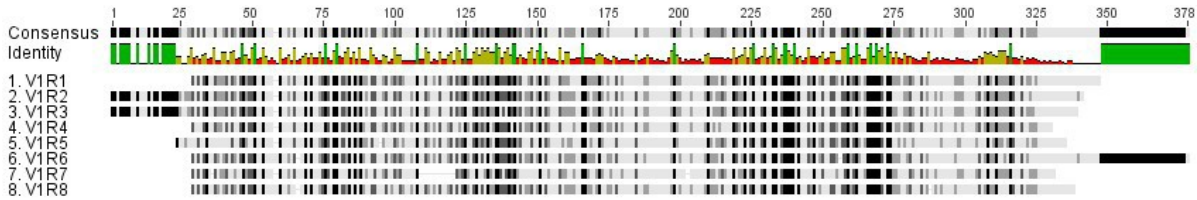


Figure 4.19: All sequences of V1R genes translated to proteins: Homologous regions are marked dark grey

5 Discussion

The dog is well known for its pheromone induced marking and sexual behaviors. Despite the importance of these behaviors, we have identified only a small number of intact putative pheromone receptor genes in the dog genome, in accordance with published information on V1R (Grus et al., 2005) (Young et al., 2005) and TAAR receptors (Stäubert et al., 2010). The small number of pheromone receptor genes in the dog, in comparison, for example, with the mouse (~200 receptor genes) makes the dog an attractive model organism for studying mammalian pheromone reception.

Furthermore, recent results from the Lazar laboratory show that the G-protein signaling pathway (Gαi2) activated by the V1R receptors can be sensitively monitored by a novel microscopy technique, two-photon polarization microscopy, developed in the laboratory (Lazar et al., 2011). Two-photon polarization microscopy should allow identifying agonists of the V1R receptors, and deciphering the logic of pheromone recognition by the V1R receptor repertoire. Therefore, cloning of the dog V1R genes is an important step towards understanding the pheromone recognition in mammals.

We have verified that the dog genome contains only eight intact V1R and two TAAR genes, although it contains significant number of pseudogenes. Primers were designed to amplify coding sequences only and, if possible, of only the desired member of the receptor family. Despite that, we had problems with by-products in amplification of V1R1 and V1R6 genes, which complicated obtaining of sufficient quantities of pure DNA. Minor products could also be found in amplification of V1R2, V1R5 and V1R7 genes. Problems with obtaining sufficient quantity of DNA were overcome by optimizing PCR conditions, such as by addition of DMSO and use of touchdown PCR.

The cloning phase has proven to be considerably more difficult than we expected. Implementation of the method of cloning blunt-ended PCR products into a *Pme*I restriction site was largely unsuccessful (only one successful clone – V1R4-pEPAx2.4). Even after numerous optimization attempts, the method was not effective enough to be routinely usable. The main cause of problems could be the different demands of enzymes to reaction conditions. Another important factor was probably a need for concentrated PCR products, which became available only later, after substantial optimization of the PCR conditions.

Therefore we decided to replace the blunt-end cloning to *Pme*I by another method

using commercial TOPO TA cloning system. Topoisomerase I mediated TA cloning is widely used and a clear protocol same as additional information is available. An important advantage is the absence of ligation step, often problematic in cloning. A disadvantage is a requirement for adding 3' A-overhangs to blunt-ended PCR products. This caused problems mainly in the earlier cloning experiments and was solved by creating a protocol that ensures maximum purity of inserts at each stage of the reaction in combination with a suitable ratio of insert to vector concentrations. The optimized protocol allowed efficient cloning of blunt-ended PCR products produced by proofreading polymerases. TOPO TA cloning allowed cloning of the seven remaining dog V1R genes.

Sequencing of the cloned constructs confirmed that we cloned all eight dog V1R genes. Apart from intact genes, five previously unknown V1R7-related pseudogene sequences were also cloned. We have also cloned three previously unknown SNPs of the intact V1R genes. These SNPs affect the amino acid sequences. Whether, or to what extent, these SNPs affect the function of the respective receptor proteins will revealed in subsequent research.

The cloned V1R genes will be used for further research. After cloning into expression vector (currently in progress), the constructs will be transfected into mammalian cells for research of their function. Experiments using two-photon polarization microscopy will allow monitoring activity of $G\alpha_{i2}$ subunit of G protein coupled with V1R receptors. This can lead to information on how pheromones in the dog are recognized. Information about the dog will be relevant for understanding pheromone systems of other mammals. Apart from purely scientific benefits, our research may lead to practical applications, such as in the control of population of economically important pests (mice, rats, etc.), or conversely, to increase populations of protected species.

6 Conclusion

We have verified that among the known chemoreceptor superfamilies, only eight V1R genes and two TAAR genes are intact and probably functional in dog genome. All the potentially functional dog V1R genes were amplified from genomic DNA of the Boxer breed dog and cloned into plasmid vectors suitable for further research. Sequencing results also revealed polymorphism in V1R1 and V1R7 genes, which may be of functional significance. Along the way, we tested and optimized several cloning strategies. Our results will be used in functional studies of V1R receptors, which will illuminate the mechanisms of pheromone recognition in mammals.

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